Edwin L. Cooper *Editor*

Advances in Comparative Immunology

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Preface

Punch's almanac for 1882: 'Man is but a worm', published in Punch Magazine on 6 December 1881. The satirical cartoon shows how Darwin has evolved from chaos, over earthworms to respectable gentleman.

The first version of *Comparative Immunology* (1976) was an integral part of the early full summation of the field. We included for the first time 16 chapters which treated the immune systems of various phyla in depth: (1) the immune system, (2) phylogeny of the immune response, (3) nature of antigens, (4) phagocytosis, (5) quasi-immune recognition and primordial cell-mediated immunity, (6) primordial cell-mediated immunity, (7) the machinery of the immune system, (8) development of transplantation immunity, (9) characteristics of transplantation immunity, (10) genetic control and transplantation immunobiology, (11) invertebrate humoral immunity, (12) antibody synthesis, (13) the immunoglobulins, (14) activities of immune cells, (15) immunosuppression, and (16) epilogue.

That was the leading edge of our knowledge in 1976. There is a different approach in this book, some 42 years later. This assumes that readers are now educated enough to obviate the need to explicate intricacies of the immune response in great detail. That detail was an essential approach for the first edition – not so for the first revision. After all, many mechanisms are now sufficiently understood. Instead this revision has another approach: a focus on unique models, groups, exotic species – no need to spend time *ad nauseam* explaining antigen-antibody reactions in mice! The successors are animal species perhaps more interesting and exotic. Rather more exciting – the response in bats! After all, bats are carriers of various infections, so an analysis of their immune reactions would seem to be more novel. The study of their other physiological adaptations such as echolocation would be more exciting and new! And what about bigger, non-flying mammals – the elephant, promising clues to the scourge of cancer and dampening, we hope significantly, the urge to extinguish them permanently in their native habitats!

The 1976 edition dealt with humoral immunity in 4 phyla within a single 29-page chapter: echinoderms, mollusks, annelids, and arthropods. The book you hold now disperses these phyla into separate chapters that focus on different functions. Therefore, readers of this new edition can expect to find numerous characteristics of immune phenomena in separate animal taxa being presented in depth. This is evidence that the field has advanced immeasurably since the first edition.

The emphasis now is on function, a veritable *jardin zoologique*, not technique. Moreover, methods and materials must often be modified for phyla that have distinct characteristics. For example, to perform a skin allograft on a fish, we transplant scales and watch the degeneration of melanophores as an indication that the graft is destroyed by the host immune system. In contrast, the equivalent skin graft in a mouse would require suturing or more drastic methods, then watching the healing process including signs of cicatrix formation, hemostasis, and ultimately rejection. Independently of technique, the principle is rejection of *non-self*, and the acceptance of *self*, whether fish scales or mouse skin. Another example of the widening scope of comparative immunology is single cell organisms. In 1976, knowledge of the prokaryotic immune response was nascent, as studies of restriction enzymes to cut viral DNA were just ramping up. Thanks to the discovery/addition of CRISPR (very simply, editing of disorganized nucleic acid) to the toolkits of prokaryotes and immunologists, the explication of *self/not self* and immune reactions at the molecular level should make significant strides forward.

Part 1 of this book looks at the immune system in taxa from prokaryotes to urochordates, all of them invertebrates. The overarching first chapter summarizes the evolution of immunity. This segues into discussions of amoebae, corals, flatworms, and roundworms. Annelids, arthropods, and mollusks are covered in three, two, and two chapters respectively. (To spread these three phyla into seven chapters would have been unthinkable in the first edition; too little was known!) Echinoderm immunology introduces us to the deuterostomes. Urochordates, which include tunicates, are a suitable bookend; they are among the most "primitive" of the chordates.

Part 2 covers cephalochordates up to and including mammals. The cephalochordates include branchiostomes (lancelets or amphioxus), fishes (cartilaginous and bony), reptiles, and birds (with focus on ostriches). This section ends with chapters that consider immunity in bats and elephants, followed by the phylogeny of nasopharynx lymphoid tissue.

Part 3 considers certain broader implications and vulnerabilities due to worldwide climate change, cancer, therapy, and the quest for more diverse food sources. Immune responses in poikilotherms and ectotherms are vulnerable to temperature change making them sources of information that senior comparative immunologists always knew – internal temperature will affect the outcome and reproducibility of trials – a factor of less concern in experimental homeothermic species. The continued search for food sources may turn us to additional edible invertebrates, perhaps more plentiful and less polluting. Variations in immunity within a species and between species are the topic of the chapter on ecoimmunology. Toxicity and disease are explored in earthworms, bivalves, and frogs. The clinical use of maggots in biotherapy is described. The last chapter links cancer and evolution, connecting to evidence for neoplasia in bivalve mollusks seen before in this part.

With such impressive advances in comparative immunology since the first edition of this book, who can predict what the third edition will cover? Surely the maturation of this field within the umbrella of immunology, combined with bright researchers adapting sound techniques, will lead to further basic and applied knowledge.

Acknowledgments and Reminiscences

Rita Beck
It is a pleasure to acknowledge the collaboration of Rita Beck, Publishing Editor at Springer. Rita found an earlier attempt to revise *Comparative Immunology* and realized that no further writing had been initiated. Prompted by a friendly suggestion—why not continue revision?—a productive, cordial, and extremely helpful collaboration was launched, bringing the revision to fruition. I thank Rita for her inestimable help during the writing of this edition and her patience during its revision. We established a realistic due date to coincide with the next International Congress of International Society of Developmental & Comparative Immunology (ISDCI) in June 2018 in Santa Fe, New Mexico. One of our authors, Irene Salinas, is the organizer.

Michael Suzuki
Another person—friend and former PhD student at UCLA—has played an extremely vital role in bringing my revised comparative immunology book to completion. Michael Suzuki knows the field and did much to move forward early experiments of leukocyte biology in earthworms, especially their role in killing foreign cells. Long ago, I had been impressed by Michael's intellect, sense of humor, and taste. Relatively free from teaching undergraduate biology at community colleges in the Los Angeles area, Michael became available part time just when I was desperately in need of a computer expert, unlike myself, a Luddite! In addition to his computer skills, Michael is well read and cultivated and possesses a refined taste in wine, is a capable editor, and always ready to laugh at the vagaries of whatever! Well read and not just religiously devoted to the *New York Times*, Michael moves fluidly between baroque and classical!

Donald Buth
I thank Professor Donald Buth, Department of Ecology and Evolutionary Biology, University of California, Los Angeles (UCLA), for checking the classifications, especially of fishes. Professor Buth is interested in phylogenetic systematics, ecology and evolution in extreme environments, and the evolutionary history of fish; he was recently accorded recognition by his academic senate.

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I would like to acknowledge the work of Deepak Devakumar, Production Editor, Springer, for facilitating the production process. He helped coordinate the transformation of the manuscript into a printed book ready for sale.

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Helene Cooper
Helene, my wife of nearly half a century, has been incredibly effective and efficient at managing our domestic and cultural life in such a way that I never experience any negative surprises with respect to work or creative activity. She is an axle rod on which our familial wheel turns. And if that weren't enough, Helene is and has been supremely supportive, always available, helpful, and dependable. She was the first and only editorial assistant for the journal *Developmental and Comparative Immunology* (DCI). DCI, founded in 1970, was a unique journal devoted to the development and evolution of the immune system. Helene and I were equally excited during four sabbatical leaves in four different countries: Sweden, Switzerland, Egypt, and Italy (generously supported by the Guggenheim, Eleanor Roosevelt, Fulbright, and Humboldt foundations and of course UCLA) or as volunteers on Earthwatch teams aiding other investigators or digging around in archeological pits searching for shards and clues to ancient civilizations or inviting friends and colleagues for long weekends to our farm that has been in the family since 1800 in the Auvergne region of France. Well-being has been and will always be essential manna for a life of creativity. Our children, Astrid and Amaury, and now grandsons Theodore and Adrien Cooper, are thriving and already questioning, alongside their father/grandfather, the differences between frogs and earthworms. Hope lives on!

Career Path
During a long and productive career that has been most enjoyable and exciting, I have had the chance to travel, always searching for ways to explain comparative immunology to those who attended my lectures. Some of my colleagues who remain friends opened their homes and universities to me. Travels have included at least twenty-five countries, four year-long sabbaticals in four different countries supported partially by prestigious foundations, Guggenheim, Eleanor Roosevelt, and Fulbright, as well as the Bologna Institute for Advanced Studies. In addition, the Agency for International Development opened my eyes in 1966 to the culture of Mexico and the Spanish language and gave me the chance to contribute to the basic blueprint for the now ten University of California campus programs that are part of the collaboration between the university and Mexican institutions known as UC MEXUS. After receiving a diploma in Spanish, I became more Mexican, publishing one of my first papers on graft rejection responses in a South American legless amphibian, a relative of frogs and salamanders. I continued my study of French by marrying Helene and in 1976 was invited to present a lecture at the Collège de

France, In addition, I accepted an invitation from Professor Jean Dausset to publish a major review in French in the popular science magazine *La Recherche*.

It is impossible to mention everyone who has earned my appreciation in the writing of this book, for which I would run the risk of omission. I have now joined, to a limited extent, the digital age and suggest that interested colleagues search Google or PubMed to find a variety of publications consisting most recently of reviews and, occasionally, experimental papers. Generous research funds were provided by National Science Foundation, National Institutes of Health, and the American Cancer Society in support of my research in comparative immunology. As my publications increased, there was a corresponding decrease in national funding, which did not dampen my enthusiasm or productivity. As the old adage has it, "I will either find a way, or make one" (supposedly said by Hannibal in 218 BC when his plan to cross the Alps via elephants was challenged).

It was not always easy to win the intense competition for funds since comparative immunology was not mainstream in the late 1960s; still, it was important enough to maintain funding for some of us still embued with questions that could be answered and useful after experimenting with plants and animals (leading to the production of pharmaceuticals). I interpreted this as an obscure hint that a new tactic that could provide support was needed! Why not increase the importance of comparative immunology by making it better understood, thus augmenting its visibility? Aha! Start a much needed journal! Founding a journal, like any project, whether a book or invention was feasible if a unique proposal could be presented to a publisher. Several disappointments greeted my proposal until finally, and seemingly spontaneously, came a positive response in 1976: Pergamon Press, under the guidance of Robert Miranda, offered a contract to publish a journal, which I called *Developmental and Comparative Immunology* to be inclusive. I nervously signed the contract at the Federation meetings in nearby Anaheim, appointing my wife Helene the first and only administrative assistant for 18 years to assist in this international effort. With some minimal fanfare but discreet determination, Volume 1 was published in 1977 with two hastily formed but enthusiastic advisory and editorial boards, lending a measure of prestige!

Some aspects of the journal in the midst of substantial visibility were not usual. DCI was at first a "cut-and-paste" journal, produced like an international quilt (all sorts of fonts!)—but it was published nonetheless, extending the reach of DCI. DCI opened the giant doors of the publishing empire of Robert Maxwell, a CEO with a keen eye for up-and-coming publications. On his 65th birthday and the 10th anniversary of DCI, we, meaning all of his several hundred editors, gathered for a celebration in Oxford, England. Sessions during the day were devoted to the realities of publishing, the advent of computers, and sneak peeks at what publishing was about, including scientific journals. Evenings featured elegant black-tie-and-tail events! Bubbles and exquisite cuisine rounded things off.

DCI matured, opened many more doors, and provided the necessary and continuous visibility so essential for any idea as it might relate to biomedicine. But alas, it matured up to a point, when it came time to make some changes after more than 15 years, when I passed the torch and other chief editors took up the reins. Having published my own papers and organized and published symposia, I gladly stepped

down and gave impetus for national societies under the aegis of DCI, Japan's strong and influential JADCI, for example, is now a flourishing model.

Moreover, certain findings by studies of comparative immunology gave birth to other disciplines and journals, for example, the immune systems of animals and plants, harnessing the resources of the ocean. The idea was to promote the renaissance of ancient medical remedies, paying special attention to the beneficial substances derived from the sea that could be developed into medicinal products! What about bee venom acupuncture, born from the observation of humoral immune system products? And bees are just one of many examples. Finally, tribute was paid to the earthworm, a source of food in other cultures whose humoral immune system had progressed to the point where an agent was isolated from its serum like fluid to prevent blood clots. The innovations kept coming!

One of the important spinoffs from my duties as DCI editor emerged as an incredible windfall while I was lecturing in Japan. Professor Nobuo Yamaguchi was a long-time friend in comparative immunology who was interested in launching, under Oxford University Press, a journal devoted to complementary medicine to fit this growing need to expand aspects of medical practice. For centuries, humans subsisted on natural products derived from plants and animals, before the advent of pharmaceutical houses, and the rapid advance of medical science. To my surprise, I was offered the editor-in-chief position. After a few hours of personal deliberation, I accepted the offer, which provided manna to fuel the emerging discipline of complementary and alternative medicine (Cooper EL, Yamaguchi N (Eds). Complementary and alternative approaches to biomedicine. Advances in experimental medicine and biology. vol. 546. New York: Kluwer Academic/Plenum Pubs 2004). The journal *Evidence-Based Complementary and Alternative Medicine* (eCAM) is published by Hindawi, is open access, and, like DCI, earlier afforded me new opportunities to engage in an activity that gives me great pleasure—writing.

The spectre of starting a new organization provided a windfall of enthusiasm so essential during the birth of a cohesive, dedicated group. eCAM, like DCI and the aforementioned book, began with a bang. Here is a sampling of scholarly contributions as per Scopus, which are direct evidence of enthusiasm: Gibellini L, Marcello P, Milena N, Montagna JP, De Biasi S, Roat E, Bertoncelli L, Cooper EL, Cossarizza A (2011) Quercetin and cancer chemoprevention. *Evid Based Complement Alternat Med*. 2011:591356. doi: 10.1093/ecam/neq053; Cooper EL, Yao D (2012) Diving for drugs: Tunicate anticancer compounds. *Drug Discov Today* 17:636-648; Cooper EL, Balamurugan M, Huang C-Y, Tsao CR, Heredia J, Tommaseo-Ponzetta M, Paoletti MG (2012) Earthworms dilong: Ancient, inexpensive, noncontroversial models may help clarify approaches to integrated medicine emphasizing neuroimmune systems. *Evid Based Complement Alternat Med*. 2012:164152. doi: 10.1155/2012/164152; Cooper EL, Hirabayashi K (2013) Origin of innate immune responses: revelation of food and medicinal applications. *J Tradit Complement Med* 3:204-212; Mackler AM, Heber D, Cooper EL (2013) Pomegranate: Its health and biomedical potential. *Evid Based Complement Alternat Med*. 2013:903457. doi: 10.1155/2013/903457; Huang C-Y, Cooper EL, Fang-Yeu Poh C, Kuo WW, Chen T-S, Sherman R (2014) Special invertebrate models and integrative medical applications: Regulations, mechanisms, and therapies. *Evid Based Complement Alternat Med*. 2014:843961. doi: 10.1155/2014/843961.

Advances in Comparative Immunology Introduction

Why Study Comparative Immunology?

This is the first revised textbook to offer a comprehensive review of recent and exciting advances in comparative immunology. The book presents an evolutionary approach to cellular and humoral immunity and reveals the immune system as ubiquitous and necessary for all animals to survive. Many textbooks are available on immunology, but often they are oriented solely toward medicine or allied professions. This textbook of comparative immunology is a unique beginning text for those advanced students of biology, zoology, immunology, and other disciplines who are interested in more biological or comparative approaches to the analysis of immune competence.

This book provides us with an overview of immune reactions—a possible stepping stone to graduate study in comparative immunology. Specialists, often with a mammalian orientation, can use the text as an introduction to a wealth of other vertebrates and invertebrates and a source of new and meaningful facts pertinent to immunology. Despite this orientation, the book would even be appropriate for medical, dental, and nursing students. Thus, comparative immunology is important to anyone who understands and appreciates the fundamental aspects of immunology and biology and who can grasp significant breakthroughs in immunology when viewed in phylogenetic perspective. Since the first edition, progress in the field has been outstanding!

Immunologists will quickly recognize many fruitful approaches to understanding immunity. To aid the reader, much illustrative material and many references to original works and reviews are given. This expanding information reveals that analyses need not be restricted to rabbits or guinea pigs, since the invertebrates, fishes, amphibians, reptiles, and certainly birds are excellent species for deciphering the basic mechanisms of immune reactions. It should be remembered that cellular immunity, undergoing rapid refinement and extended breadth, had deep historical roots in observations on invertebrate cellular immunity. For this reason a good deal of attention is devoted to specific cellular immunity in invertebrates. The apparent absence of circulating immunoglobulins, but the presence of a complicated and

efficient humoral immune system, in invertebrates should offer fertile ground for speculating on the nature of those pressures that may have led to the evolution of antibody synthesis in vertebrates.

2018: Evaluating the Impact of Comparative Immunology

Actually, comparative immunology may succeed even further by some productive and essential "mimicry." Comparative neurobiologists are now questioning their approaches in the special issue of *Science* called "Challenges in Neuroscience" (Yartsev 2017). They pose the question of whether a comparative approach is important in exploring the nervous system. In essence, their proposal suggests a recognition of and focus on a handful of animal models to explore particular questions—more narrow analysis. Here are several of their suggestions: Utilize the frog *Rana pipiens* as ideal for a strategy to clarify synaptic transmission, or investigate the squid, horseshoe crab, and sea hare *Aplysia* to understand respectively action potentials, retinal physiology, and learning associated with neural memory. Back to immunology: *R. pipiens* can offer extended clues due to the metamorphosis from tadpole to adult frog, setting in motion organogenesis, especially bone marrow, which is the source of immune stem cells.

I would suggest a similar discussion with respect to both the invertebrates and vertebrates in mind—offering more numerous models that can be whittled down to fewer numbers with a focus on the additional, more ecological problem of food supplies and terrestrial and aquatic species as sources of life-saving drugs. Possible models are plentiful even after judicious and practical selection.

The study of immune systems in disparate phyla is also a way to quantify the biological effects of global climate change. Food webs are among the most significant phenomena affected by planetary warming. A population decline in one member species could be disastrous for the remainder of the web. Likewise, a significant increase in the individuals of another species "invading" an extant web might lead to disruption. It is extremely conceivable that the dysfunction or competent function of a species' immune system could be a factor in population collapse or explosion, respectively. For example, temperature change will likely alter the mix of pathogens present in a particular habitat. The ability of species' immune systems to cope with a disruption in any pathogens surrounding them could in turn impact the species' survival.

And it is not disputable that humans and mice are the organisms in which the immune system has been most deeply investigated. The unquestioning extrapolation of these findings to other animal species, including invertebrates and poikilothermic ones, entails risks. Yet it is these nonmammalian species that dominate among animals in food webs. As Yartsev noted, "In the absence of comparative studies, an entire field may be led astray by observations that are either species specific or misinterpreted in the absence of comparative data." He adds later, "… the comparative approach serves as an extremely powerful tool to assess the validity of universal principles on a case-by-case basis. In the absence of the comparative approach, many discoveries may not have occurred, would have reached the wrong conclusions, or would have taken far longer to be unveiled." Thus the

comprehension of nonmammalian immune systems, which may be a large factor in the survival of food web members, will likely be unachievable by the simple extension of what is known about humans and rodents.

Research into temperature changes and the immune system in poikilotherms began years ago. Cellular immunity in Antarctic sea urchins reflected signs of stress with a 4 °C rise in temperature (Branco et al. 2012). Many marine organisms must adjust to acidification as well as warming. Expression of immunity-related genes was altered by lowering the pH in the mussel *Mytilus chilensis* (Castillo 2017). The effectiveness of the insect immune system may be heightened by heat shock (Wojda 2016), but this is different from the chronic temperature rises that are occurring now. Immune competence declined in two species of freshwater fish of the Iberian Peninsula (Jesus et al. 2017). Hibernation and other survival strategies by poikilothermic animals (Storey and Storey 2017) depend heavily on ambient temperatures, which are likely to increase by global warming.

Distinct Periods in Conceptualizing Comparative Immunology

As suggested by Michael Suzuki, PhD, former graduate student, this book required something more than an ordinary preface of just two pages. After careful examination, the discipline of comparative immunology has in fact a long history, even as far back as early indications and records of concern by observers of disease onset. What is left in Egyptian tombs as evidence of war between humans and infectious disease? Aulus Cornelius Celsus recorded evidence of inflammation in mummies in his encyclopedia compiled in the Roman era. The early twentieth century revealed compelling evidence of inflammation, which we surely know today as the beginning that "jump starts" one component of the innate immune response.

This first revision of *Comparative Immunology* is an exciting contribution. Boyhood observations (e.g., ants on a well-worn trail always attack nonmembers) have been transformed into important real-world concepts (e.g., ants recognize *self*/*nonself*, a cardinal principle of immune competence). Early hunches are now channeled into tangible events. As a discipline, comparative immunology is an offshoot of the parent field, immunology, and is an amalgam of immunology and zoology. Comparative immunology has gained wide acceptance in the biological sciences. All animals from protozoans to humans have solved the threat of extinction by evolving an immune-defense strategy that ensures the capacity to react against foreign, *nonself* microorganisms and cancers that disturb the homeostatic *self*. Invertebrate-type innate immune responses evolved first, and they characterize the metazoans. These rapid natural responses act immediately and are often essential for the occurrence of slower, more specific, adaptive vertebrate-type immune responses. As components of the innate immune system, there is an emphasis on several major steps in the evolutionary process: (1) recognition, (2) the phagocytic cell, and (3) the natural killer cell. We now know that some invertebrate and vertebrate mediators are homologous.

The zoological inheritance received by comparative immunology is evident from the astute recognition by the organization of the American Society of Zoologists (ASZ) (now the Society for Integrative & Comparative Biology) in 1975. The ASZ

always exerted a strong influence, and reinforced the will and ensuing success, which was official and provided essential support for establishing a fledgling group; I experienced this first-hand while creating the first organized group. Innate immune systems have been successfully defending invertebrates and plants against microbial infections since time immemorial. The germline-encoded receptors of innate systems are relatively limited in diversity and able to make only coarse distinctions between closely related structures. Nevertheless, they can recognize certain chemical features shared by groups of microorganisms (e.g., pattern recognition receptors) but not by the host, such as lipopolysaccharide of Gram-negative bacterial cell walls. This capability enables innate immunity to detect the presence of an infection, if not the precise cause—perhaps considered a biological, not a structural, distinction.

Because of its evolutionary success, innate immunity is no longer considered primarily a stopgap measure, a temporary expedient for host defense; it is ubiquitous and omnipresent. There seems to be an absence of genetic-recombination mechanisms to generate specificity or "memory" because first and second exposures to a microbial substance elicit similar responses; yet there are exceptions. Acquired immunity first appeared in vertebrates. When they evolved, beginning with fish, thymus-controlled T cells appeared, as did bone-marrow-derived B cells (first found in anuran amphibians with long bones, as mentioned earlier in connection with metamorphosis). These were the precursors of the plasma cells that synthesize and secrete antibodies. Confirming the concept of *self/nonself*, invertebrates possess *natural, nonadaptive, innate, nonclonal, nonanticipatory* immune responses, whereas vertebrates possess *acquired, adaptive, induced, clonal, and anticipatory* responses. The essence is survival.

Thus, *Comparative Immunology*, one of many contributions to immunology appearing around 1976, provided real evidence, not guesswork, constituting the basis for a legitimate discipline. That survey added strong and palpable support for further advances, reaching out to scattered hints of observations that belonged to the ancestral parentage of animals. Through persistent local initiatives at many levels and in many cultures, there accumulated consistent evidence for the ubiquitous phenomenon of immunity. Immune competence is everywhere, globally, and has thrived, providing and giving back its fruits of understanding. As it happens, a cross-fertilization of many disciplines need not threaten the original views that were chiefly derived from mammals and, therefore, human oriented, essential but not universally pertinent.

Retrospective Look at Comparative Immunology

Egyptians' Discovery of Inflammation in Ancient Humans

The earliest surviving records of immune phenomena describe ailments in humans. For instance, inflammation is noted in an Egyptian papyrus of 3000 BC. (Weissman 1990). Bacterial DNA isolated from an Egyptian infant mummy demonstrates that bacteremia and likely septicemia affected that child (Zink et al. 2000). Parasitic infection by *Ascaris* spp. and related helminths were found in 1200 CE mummies of the Guanche people of the Canary Islands (Jaeger et al. 2016). Egyptian mummies exhibited signs of malaria, and symptoms of this parasitic disease were described by Hippocrates among some of his Greek contemporaries (Nerlich 2016).

Some progress in the war against infectious agents affecting humans was made during later centuries, before these agents were identified and methods invented to assay immune responses. For instance, smallpox inoculation is mentioned in a Chinese text of the sixteenth century, written long before the nature of viruses became known. Benefits from such intellectual "low hanging fruits" were finite, and modern advances in understanding human immunity have required tools to observe and comprehend what people were fighting against. Mass production of microscopes and chemicals, invention of chambers for cell and tissue incubation, centrifugation, and usable radioisotopes are only a few of the influential technical approaches that have allowed the dissection of emerging immune responses. Techniques that could reveal how human immunity fights disease were eventually adapted and elucidated the immune systems of other animals, providing ever greater clarity in lower animals in the early to mid-twentieth century, and these efforts continue today.

Advent of the Modern Era: Élie Metchnikoff

Since the time of Élie Metchnikoff, there has been a steady, but most of the time informal, approach to invertebrate immunity (Cooper et al. 2002). Yet more than 20 years later after the discovery of simple phagocytosis, his views were rewarded and recognized by the Nobel Prize in Physiology or Medicine in 1908. For the cellular arm of the immune system his contribution is far-reaching; over the years the implications have been extensive (Cooper 2008). Rather than dismissing his views entirely, the cellular arm of the immune system continued to enlarge, so overarching that it permeated every facet of the immune response, except for activities specific to B cells. The essence of comparative immunology has been pervasive.

Twice in the history of twentieth- and twenty-first-century immunology, the Nobel Prize in Physiology or Medicine has been based on simple basic aspects of inflammation. (The second was Jules A. Hoffman in 2011 for the intricacies of receptor activity that drive immune responses.) Moreover, the experimental subjects have been animal models other than mice, again invertebrates, specifically insects. This lent credence to the advisable use of all species as sources of interrelated phenomena. Metchnikoff spearheaded this significant approach by identifying phagocytosis in *Daphnia*, the water flea. Peering through the flea's translucent body wall, our eminent Russian zoologist from Odessa, enjoying the beach in Sicily, reasoned that white mobile cells (leukocytes) slowly and deliberately encircled the foreign body, rendering this alien cell inactive and preventing death.

The prescience of this observation did much to link aspects of protection to ubiquitous leukocytes or white cells, sensitive to uninvited intrusion and providing the first line of the body's defense to foreign invasion: the militaristic analogy! Thus was born the essence of innate immunity, later declared constant in all living species. Metchnikoff, somewhat like Darwin, became a ubiquitous name associated with the essence of biology in the nineteenth century. There were glimmers of hard science that attempted to invade or break the intellectually stubborn doors of a naturalist's views. Although some have supported Darwin's influence on the development of comparative immunology, this has become evident only in recent years (Cooper 1982, 2008; Cooper et al. 2002).

Darwin was born in 1809 and died in 1882. The young Darwin was well traveled and the quintessential observer in the wild. Metchnikoff, born in 1845, was less the adventurer, more a microbiologist, who died in 1916. They were a generation apart and apparently never met in person. Yet the aura of what they had discovered was surely "in the air," creating an atmosphere whose influence was most probably felt internationally. For each achievement, in summary, there was an element of simplicity despite the end product/result of shared similarities. Although they overlapped substantially in age, there are no records showing mutual influence on each other nor on the fields to which they contributed so vastly. One was the exploring naturalist who traveled extensively, the other staying close to his laboratory to make his discoveries. The universal act of phagocytosis, its ubiquity, laid the foundation for its acceptance as universal—no complicated reagents necessary to assert its pervasive influence. A similar prescient simplicity characterized them both.

Leo Loeb: *The Biological Basis of Individuality* – Emergence of Self/Nonself

Early in my career, as a junior faculty member, I accepted this Loeb book as a most treasured gift from Prof. Nicholas Cohen, the second postdoctoral fellow of W.H. Hildemann. Nick and I overlapped in many ways—our choice and subsequent abandonment of a first PhD research problem: the development of limb regeneration in urodele amphibians. On a page of this gift Nick wrote, "***Let us never forget individuality***."

The Prussian-born physician Leo Loeb contributed the early appearance of the basis for *self/nonself* concepts as expressed in his 1945 *The Biological Basis of Individuality*. (This represented the beginning of crucial and important tomes and treatises in the field, which focus on *self/nonself* and mutually related variations.) Whether it was absolute prescience on the part of Loeb to anticipate *self/nonself*, or it was so obvious that the duality emerged, after a good deal of thought and publication, that there were inherent differences among all individuals, is unknown. Early on, what was crucial to comparative immunology was a recognition that this individuality was characteristic of all species. To add support to this emerging doctrine, Loeb not only perceived or anticipated the concept of *self/nonself* but recognized early on that any differences or likenesses were clearly demonstrable by simple experiments involving the transplantation of grafts in as many animal species as possible. What was revealed was the essence of *self/nonself*. To support his view that all living creatures possess the capacity to show differences when confronted with unlike tissues, Loeb established the field of transplantation immunity, whose basis was the early recognition of *self/nonself*, only later to be accepted by the founders of transplantation biology including Burnet and Brent.

As a favorite animal model, somehow earthworms became the choice of many colleagues close to Loeb who would become the experts providing the confirmatory evidence when transplantation immunology in invertebrates was first discovered (Cooper et al. 1992). To confirm the universality of this kind of response, the recognition of

individuality differentials (later known as self), Loeb the inquiring scientist hastened to include other creatures where easily transplanted tissues could be made, thereby revealing the existence of individuality differentials. Quoting at length from Loeb (1946): "The lumbricidae differ from the planarians in a considerably greater fixity of their organs and presumably in a correspondingly greater specificity and fixity of the substances on which the differences between organs depend (organ differentials, a predecessor of the *self* / *not self* concept). While the organs have not yet become entirely rigid, still the differentiation between head and tail parts is more fixed than in planarians [see chapter by Oviedo]…

"In accordance with this change in the organs we find a greater differentiation in the organismal differentials, as is indicated in the transplantation experiments on lumbricidae which have been carried out especially by Korschelt and his associates, Joest, Rutloff, Leypoldt, Harms, Rabes, and more recently by Mutscheller. The earlier of these experiments antedated the majority of the investigations on coelenterates and planarians. At that time attention was focused on problems which have since" been couched in different terms. Every effort made by Loeb emphasized the individuality of all beings regardless of level of evolution expressed as distinctions between *self* and *nonself* that characterized that individuality. To lend credence to universality, various models explored the unique character of the experimental species. As will be seen later, many of the models in this period were analyzed later, beginning in the 1970s as the parent field of immunology became better understood. Still we must recognize that even among mammals, like the laboratory mouse, information was woefully inadequate with respect to components of the immune system and what each component did when confronted with the demand of the animal to defend itself against that which was foreign, again expressing individuality differentials, later becoming self/nonself.

For example, several researchers working in the 1920s devoted enormous attention to the fate of transplants between different species of earthworms. Only in the 1970s did investigators see that the observation of earlier scientists was immune capability, expressed as the *biological basis of individuality*. However, the 1970s workers were equipped with functioning stronger evidence that specifically defined differentials, i.e., *self/nonself*. The *self/nonself* model remains solid and was only questioned many years later. In the 1990s the view that danger in an organism could act as a *nonself* stimulus arose, offering more testable opportunities for further research (Matzinger 2002a, b).

1960–1980

First appearance of clonal selection; *self* versus *nonself*; integrity of body; ontogeny and phylogeny of immune system; phylogeny separately; defense reactions in invertebrates; Cushing and Campbell, *Principles of Immunology* (1957), whose chapter 12 has an early mention of comparative immunology).

Immunology was beginning to show some resemblance to what we know today as comparative immunology. As a growing field, it was turning toward analysis and

considerations of mechanisms. However, as we see from today's vantage point, mechanisms were really scant, since technology was undeveloped, and the application of technology to questions rarely yielded meaningful analyses and conclusions. Naturally, as an emerging experimental science, advancement of the parent discipline required animal models. The first two acceptable models were the laboratory mouse and rat. Rabbits were also employed as the discipline grew. Eventually other warm-blooded animals, such as chickens, guinea pigs, and hamsters, were sometimes added to the necessary experimental mix of animal models. Immunology concerned with human problems naturally searched for relevant animal models and posed questions that could be answered, thereby opening windows and doors, perhaps advantageous, as would be seen later, what were somewhat unproductive exercises. These early forays into the science revealed little, yet they stimulated researchers' desire to open yet more doors and windows. Once these were opened, what would become the emphasis?

Enter an emerging group of biologists *qua* zoologists (not yet comparative immunologists!) familiar with other animals as incredible sources of relevant immune characteristics. This was further hampered again by a lack of sufficient immune information to establish universal traits among many animal species. These new fledgling immunologists were often not viewed as such by the existing mammalian immunologists, who were locked in imperceptibly with their furry and homeothermic creatures. Still, their value as former microbiologists, now focused on the immunology of infectious diseases, was a source of consolidation and bridge building, capable of straddling the divide less strenuously than the uncomfortable hard-core microbiologists. After all, they were and are more open to the beauties of the living world, able to escape from a firmly closed mindset focused narrowly but understandably on humans. This essential anthropocentricity offers little in our search for the universality of the basics, which include widespread mechanisms of survival; after all, at best survival meant preserving certain warm-blooded creatures and barnyard birds as sources of food. In this book, it is the immune system that we analyze for common denominators, thus opening doors for our entrance into the wide array of animal species to the exclusion of plants—although equipped to guard against disease and ensure survival, their mechanisms are different.

I worked driven by affection and dedicated to the cause of the first version of *Comparative Immunology*, published in **1976**. This was done through multiple invitations actually, one for *Comparative Immunology* via a comparative endocrinologist, Prof Howard Bern of the University of California, Berkeley. The decade of the **1970s** represented an important watershed. Doors opened and my ideas, whose seeds lay dormant to some extent in the 1960s, suddenly burst forth. This was an important period for laying the groundwork of comparative immunology. For me, it began with the award of a Guggenheim Fellowship for studies on comparative immunology at the Karolinska Institute Sweden in **1970**. This period saw the launch of dedicated efforts to understand the evolution of the immune system. It seemed reasonable to think and to imagine that protection against infectious microbes was a property of all creatures, humans included.

Burnet wrote *Immunological Surveillance* in 1970, where he revealed the possibility that immune systems evolved to protect against mutated cells, including cancer cells; two textbooks on comparative immunology, one by Cooper, another by Marchalonis, appeared; nomenclature that is still in use today was laid down; Volume 1, Number 1 of the journal *Developmental and Comparative Immunology* was published in January 1977; books on animal models of comparative and developmental aspects of immunity and disease appeared; volume 4 of *Contemporary Topics* was commissioned (part of a series). Thus, the 1970s were especially productive—different names, but universality among animal groups and problems of disease were important topics.

This was immunology's turning point, a crucial period devoted to comparative immunology. In **1974** I edited *Contemporary Topics in Immunobiology* (Cooper 1974). The fourth volume in this outstanding series presented modern approaches to cellular and humoral immunity and examined relationships between invertebrate and vertebrate immune capacity. Noted scientists, including Nobel Laureate Macfarlane Burnet, discussed the novel theory of immunologic surveillance—an explanation of how cells distinguish between *self* and *nonself*—and related it to cancer biology. Also included were studies of graft rejection in earthworms, as well as in hydras and other marine invertebrates. The work looked at several insect immune defenses and described leukocytes derived from the octopus white body. (By the way, it would be interesting to see how the immune system of the octopus surpasses that of many other animals, considering the complexity of the octopus nervous system and the fact that octopuses have the capacity to show emotion, as demonstrated in some studies.) Finally, this book describes events in phagocytosis and presents a notable but contentious view—tumors in *Drosophila* ***and, perhaps, in some other invertebrates***.

My **1976** book, *Comparative Immunology*, was part of the *Foundations of Immunology* series by Prentice Hall, which included at least five other volumes. This series of monographs was intended to provide readers of diverse backgrounds with an authoritative and clear statement concerning aspects of immunology. This book contained 16 chapters with such diverse topics that were intentionally basic, seeking clarity for a diverse audience. It covered the immune system, phylogeny of the immune response, nature of antigens, phagocytosis, quasi-immune recognition, primordial cell-mediated immunity, the machinery of the immune system, development of transplantation immunity, characteristics of transplantation immunity, genetic control and transplantation immunobiology, invertebrate humoral immunity, antibody synthesis, the immunoglobulins, activities of immune cells, immunosuppression, an epilogue that treated two pertinent topics for adaptive immunity, and, finally, the impact of immunology.

The year **1976** also saw the publication of another book called *Comparative Immunology*, edited by John J. Marchalonis. This book was similar to the one by E.L. Cooper except for a focus on authors, of whom many agreed on certain characteristics. This contrasted with Cooper's emphasis on animal models. Thus Marchalonis's book was more universal with respect to subjects and experts in the field, which counted up to almost 25 comparative immunologists! The Marchalonis

book was perhaps easier to read and covered the subject of comparative immunology in more depth if for no other reason than its breadth (740 pages long) compared to Cooper's (338 pages). Both were and still are valuable, but a comparison of the two with each other revealed that their style and content were not so even.

Enter a different twist to the topic of comparative immunology, a tertiary book, *Comparative Immunobiology*, by Margaret Manning and Rodney Turner. Compared to the two previously mentioned texts, this book contained approximately 180 pages, small and compact, written by two colleagues, and based upon their experiences teaching the subject to students in the last year of a biology sequence. This book was aimed primarily at students who wanted to understand how the immune system works, especially aspects of comparative and evolutionary biology, but who weren't planning on specializing in immunology. Manning and Turner expressed the hope that their book would introduce the phylogeny of immunity to students already knowledgeable in other aspects of immunology.

The year **1977** was a good one, soon after *Comparative Immunology*, when J.J. Marchalonis published his *Immunity in Evolution*. "Evolution of the vertebrate complex immune system has shown no morphological changes apparent from fossil evidence. But comparative studies of immunity in invertebrate species reveal an extraordinary evolution of cellular and molecular mechanisms capable of differentiating *self* from *non self*. In this thorough review, Marchalonis introduced readers to the evolutionary background of immunity and showed how this approach can illuminate this phenomenon in more familiar eutherian mammals." These, of course, are in contrast to monotreme and marsupial mammals, lesser known exotic species, typically Australian.

Although some invertebrates appear capable of immune-like responses (this was proven in the 2000s), only in vertebrates does highly specific biochemical recognition of foreign substances occur. As we would see some 30 years later, this seeming gap or even pessimism with respect to invertebrate responses was due to a lack of strong evidence. Marchalonis traced the evolution of cellular and humoral immunity from the versatile system of cyclostome fishes through the elaborations introduced by subsequent evolving vertebrate groups. Modification of the ancestral immunoglobulin IgM into a variety of Ig types received detailed attention. The emergence of T and B lymphocytes and cooperative interactions were revealed in detail. Marchalonis provided background on evolution and biochemistry so that readers unfamiliar with one or more aspects of this review could follow along with relative ease. Numerous illustrations summarized data, showed evolutionary development, or explained genetic hypotheses. Immunologists, evolutionary biologists, and readers interested in molecular evolution would and will still find frequent use for this book!

1980–Present

The period opening the **1980**s was ripe with a concern for the concept of *self/nonself* discrimination. *Self/nonself* discrimination; *Contemporary Topics in Immunobiology*, volume 9, 1980; cellular recognition reactions in invertebrates; 1st Congress of Developmental and Comparative Immunology held in Aberdeen, Scotland in 1980;

proceedings of that congress were published as *Aspects of Developmental and Comparative Immunology*, J.B. Solomon (ed); two influential books by Jan Klein; Sigel and Cohen (eds), *The Reticuloendothelial System,* volume 3: *Phylogeny and Ontogeny*, published in 1982.

Already *self/nonself* was riding high on more and more confirming evidence, strengthening this view that had firm roots that had been published as early as the late nineteenth century. J.J. Marchalonis and Nicholas Cohen (with MG Hanna, Jr) edited *Contemporary Topics in Immunobiology*, volume 9, 1980. As a special tribute they dedicated the volume to Sir Frank Macfarlane Burnet, who "first used the phrase '*self/non self* discrimination' in 1940. His concepts have provided a challenge to two generations of immunologists….It is with great pleasure that we dedicate this volume to Sir Mac on the occasion of his 80th birthday" (M.G. Hanna, Jr.). Very briefly, at that time there was demonstrable concern for considering cellular recognition reactions in invertebrates: (1) Do invertebrates show specificity in the recognition of antigen? (2) What is the molecular basis for this interaction? (3) Does a phylogenetic lineage of immunoreactive complexity exist, which eventually leads to the complex vertebrate immune response?

The 1980s were also an especially fertile time for expansion in several directions and were characterized by enormity, as illustrated by the two monumental treatises of Jan Klein, plus the volume edited by Sigel and Cohen, *The Reticuloendothelial System*, volume 3. Jan first published *Immunology: The Science of Self/Non-self Discrimination* in **1982**. The content was provocative, but it is the organization that stands alone in structure and deserves some explanation. Rather than organizing the text into four "nonprovocative" sections, for example, Introduction, and so forth, Jan published an unusual set of sections, each of which described aspects of *self/nonself* recognition. Here is his unique presentation. He opened this composition, his book, as his symphony, by confessing that he knew very little about immunology! In my opinion this is not true. His knowledge was as extensive as that of any of us who dared to approach mid-nineteenth-century immunology since what we know now hardly resembles the early days of *self/nonself*. For each of those designations, there are infinitesimal splinterings into various markers, signals, stimuli and receptors, that it boggles the mind to think how far and fast we went from individuality differentials to our present state, past the reigning dogma in the late nineteenth century. Later in the decade, Jan Klein introduced us to another way of looking at the essence of the immune system. This time, *Natural History of the Major Histocompatibility Complex* was published some six years after the "symphonic" look at the immune system. Actually this second book echoed what was enunciated in the book *The Biological Basis of Individuality* by Leo Loeb.

A third member of the club of large sequels (tomes) to the Loeb book is the book edited by Cohen and Sigel, *The Reticuloendothelial System* (Cohen and Sigel 1982). There are at least ten volumes in this series on the reticuloendothelial system; the Cohen and Sigel book is volume 3, *Phylogeny and Ontogeny*, and was published in 1982. (One partner in this series, the Reticuloendothelial Society, renamed itself the Society for Leukocyte Biology in 1984.) The tome's 21 chapters and 740+ pages included most of the invertebrate and vertebrate phyla covered in the present book, but

no mention was made of nematodes. Perhaps the explosion of research in *C. elegans* over the intervening decades provided a motivation to investigate the immunology of roundworms. The preface by Cohen and Sigel mentions a driver of the diversity of animals studied in the current book: "a wealth of new, often unique, models with which to study immunological problems that are not restricted to mammals."

The 1990s were also a busy and productive period. *Immunologie Animale*, published in 1990 by Flammarion (Paris), includes 15 chapters on comparative immunology, for example, invertebrates, fish, and so forth. The period also saw the resurrection of Metchnikoff. Other figures or topics included AI Tauber, *Metchnikoff, and the origins of immunology: metaphor to theory* (1991); earthworms alone; primordial immunity—foundation for vertebrates; developmental immunology; evolution and phylogenesis of immune reactions; comparative histophysiology; modulators of immune responses; publication of *New Directions in Invertebrate Immunology* in 1996, edited by K. Söderhäll, S. Iwanaga, and G.R. Vasta; this book from SOS Publications has more extension to invertebrates of concepts well studied in mammals, including clotting cascades in horseshoe crabs, lectins in insects, and pheromones in ciliate protozoa.

Leslie Brent, professor emeritus of immunology, St Mary's Hospital Transplant Unit, Paddington, London, published the book *History of Transplantation Immunology* over which there was considerable excitement since the second chapter was devoted to the immunological basis of allograft rejection. He opens that chapter with a statement from Sir Peter Medawar (1957): "… the immunology of transplantation not merely for its bearing on cancer research or the repair of radiation damage, but it offers one of the few negotiable pathways into the central regions of biology, where immunology, genetics, embryology and the rest of them lose their identities in problems that bear upon biology as a whole."

The early years of the field focused on various contributions. Of much interest to comparative immunologists, in the middle of that second chapter, Brent immediately poses the following question: Are invertebrates capable of allograft reactivity? Then comes the explosion: For here enter statements expressing skepticism that they are capable, despite the fact that a variety of invertebrate groups supported the idea, with observations in sponges, coelenterates, sea anemones, coral, and worms. Organisms from echinoderms to insects all possess the following immunologically competent characteristics: (1) selective reactivity, (2) cytotoxic or antagonistic reactions following sensitization, (3) inducible memory, and (4) selective altered reactivity on secondary contact—criteria with which most immunologists would concur. The debate moved to comparing Hildemann and Cooper vis-à-vis the mammalian transplantation contingent. The discussion was devoted then to a defense of graft rejection in earthworms and other invertebrates. Not all immunobiologists agreed with the notion, yet Cooper, Rinkevic, Uhlenbruck, and Valembois published "a stout defense" in 1992 in the *Scandinavian Journal of Immunology*. This lively discussion ended on a somewhat sad note with the untimely death of Hildemann, who had been a staunch promoter and advocate of what his post doc Cooper had been doing, inspiring other people.

Subsequent years saw a continued proliferation of books dealing with the panorama of animal evolution and immunity and the appearance of the journal *Trends in Innate Immunity* (Karger), a blurring of the clear border between innate

and adaptive immunity (Cooper 2012), publication of "Adaptive Immunity from Prokaryotes to Eukaryotes: Broader Inclusions Due to Less Exclusivity?" in *Recent Advances in Immunology to Target Cancer, Inflammation and Infections*, edited by J. Kanwar , the discovery of B and T lymphocytes that have some properties of innate immunity and natural killer cells with antigen-specific immune memory.

Self/nonself is an important hypothesis that has guided research in immunology. It is closely connected to adaptive immunity (restricted to vertebrates) and innate immunity (found in vertebrates and invertebrates). *Self/nonself* is now being challenged, and investigators are turning to the danger hypothesis to guide and open new areas of research. Emerging information suggests that genes involved in the development of cancer are present in *Drosophila* and *C. elegans*. Short lifespan may not rule out the presence of genes that are related to the development of cancer (Cooper 2010).

The self/nonself theory has dominated immunology since the 1950s. In the 1990s, Matzinger and her colleagues suggested a new, competing theory, called the danger theory. This theory has received mixed responses, both enthusiasm and criticism. Here we assess the danger theory vis-à-vis recent experimental data on innate immunity, transplantation, cancers, and tolerance to foreign entities and try to determine more clearly whether danger is well defined (Pradeu and Cooper 2012).

Adaptive immunity is now being deconstructed to encompass less stringent rules, including initiation and actual effector activity. Expanding the repertoire of invertebrate innate immunity has greatly facilitated the search for what actually constitutes ***innate*** and ***adaptive***. Strict definitions become *blurred*, casting a skeptical eye on the use of rigid definitions of *innate* and *adaptive immunity* (Kvell et al. 2007). Immunology has experienced remarkable growth. Immutable tenets deserve a brief mention. First, there must be strict divisions between ***adaptive*** and ***innate*** immunity. *Second*, to raise these two views allows for extended inclusions, reveals the essential merits of innate immunity, and acknowledges inclusive invertebrate characteristics. We can even include features of adaptive responses especially to ***danger*** (Pradeu and Cooper 2012). To facilitate this emerging reality means recognizing hazy characteristics that fade into each other, that ***blur***; they are neither black nor white but a "clear gray"—reminiscent of impressionist paintings (Cooper 2010, 2012).

What Hath Comparative Immunology Wrought?

Knowing how the immune system works is lifesaving, now requiring less analysis. Comparative immunology knocked on the hermetically sealed door and opened it, revealing an incalculable cornucopia of biomedical importance and relevance: it has clarified innate immunity, recognized cancer which still has a limited acceptance in lower animals, identified sources of less polluting food, and called for respecting and humanely treating all species. Thus, comparative immunology is no longer esoteric, no longer buried in the earth, but is now out in the open with its relevance and benefits for all to see and enjoy!

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Part I

From Prokaryotes to Urochordates

Evolution of Immunity

Kurt Buchmann

History

It has been estimated that Earth appeared around 4600 million years ago (MYA), and organic molecules of increasing complexity were subsequently established in the surface layers. Primitive anaerobic life forms may have seen light soon after, around 3700 MYA (Hassenkam et al. 2017), and the advent of photosynthesis (2400 MYA) created the basis for an even more intense expansion of diversity. Ever since this early history of Earth, basic organic molecules and primitive organisms have been exposed to infection and parasitism. From the very start of these interactions mechanisms to protect the integrity of the molecules and organisms must have been present, securing their subsequent survival and development. RNA molecules can be interpreted as the first parasites of DNA, and molecular mechanisms for the release and processing of RNA may have appeared to regulate their binding. These became important tools when the first simple microorganisms appeared and were attacked by plasmids and virus. Protective molecular mechanisms including the use of restriction enzymes and clustered regularly interspaced short palindromic repeats protected bacteria against plasmids and irrelevant or harmful genes (Dunin-Horkawicz et al. 2014). The first unicellular organisms needed a wider array of cellular tools to recognize food and secure its uptake. Active contact with other organisms required methods to resist invasion by foreign pathogens, including viruses, bacteria, or even other protozoans.

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The Pillars of Immunity

In its basic form the term "immunity" means "released from a burden," and it covers well the processes initiated in the first microorganisms that were exposed to and challenged by pathogens. Food uptake by simple amoebae is based on contact, recognition, and subsequent rejection or phagocytosis. If the object initiates hostile attacks towards the amoeba, mechanisms for object elimination must be installed. The subsequent step towards development of a multicellular organism was dependent on the ability of the individual cells to recognize self from non-self. This involves the potential to accept self-elements and reject non-self-molecules, and here signaling between individual cells using ligand/receptor interactions is a prerequisite. The balance between food uptake from the environment and protection against potential pathogens in the same environment is the basis for the main pillars of immunity, comprising at least (1) recognition of self/non-self; (2) immunological memory; (3) cell-to-cell signaling; (4) humoral effector mechanisms; and (5) cellular effector mechanisms. These functions may have been central during the anaerobic era before photosynthesis changed the atmosphere by increasing the oxygen content. Even in the new era, the aerobic world, sediments are still partially anaerobic, wherein the ancient processes are kept intact. In addition, in a special pocket of conservation, the anaerobic intestine of both protostomes and deuterostomes, we still find preserved these central interactions between host and pathogen. The recent focus on the gut microbiota and its association with immune regulation in vertebrates, including humans (Lin and Zhang 2017), illustrates the immunological importance of these ancient interactions between cells.

Processes

Many prototypes of these interactive systems were developed during evolutionary processes starting more than 4000 MYA and leading up to the Cambrian explosion around 500 MYA, but after the explosion a plethora of variations of each theme saw the light. Dramatic geological events throughout the history of Earth are likely to have accelerated these evolutionary processes and diversification. Radiation, high pressures, extreme temperatures, and shifts of other physico-chemical conditions not only induce point mutations but may have contributed to duplications of chromosome sets, gene duplications, deletions, and gene domain shuffling—events that have been central in evolution. A never-ending biological challenge came from the co-evolution of the pathogens infecting the hosts (Schmid-Hempel 2009). Viruses (Fig. 1a), bacteria (Fig. 1b), and parasites were exposed to similar conditions as the hosts and presented new and more sophisticated virulence mechanisms, placing on the hosts additional selective pressure, which was met with new forms of immune molecules resulting from various molecular mechanisms including retrotransposition and alternative splicing (Clark et al. 2013).

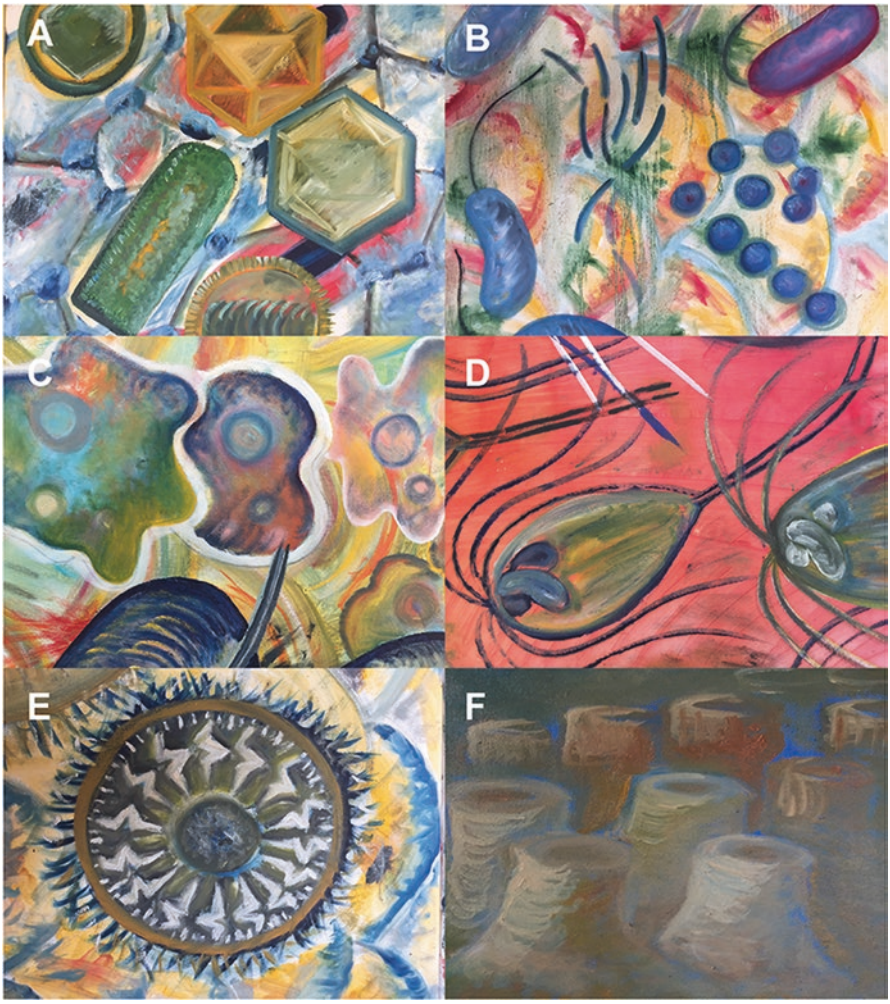

Fig. 1 Early organisms in evolution: (**a**) virus; (**b**) bacteria; (**c**) amoebae; (**d**) flagellates; (**e**) ciliates; and (**f**) animal sponges (Porifera). (Image created by Kurt Buchmann)

Evolutionary Lineages in a Diversity of Functions

Although it is possible to trace a clear line of development in many central functions and protein families, it is also clear that different strategies for reaching immunity have been applied in the different groups (Dzik 2010; Buchmann 2014). Representatives for the different levels of immunological sophistication may give us an understanding of the diverse strategies applied. Immune mechanisms appeared in particular lineages, some were lost, and some were preserved with modifications. Arthropods such as crustaceans, insects, and chelicerates developed a hard

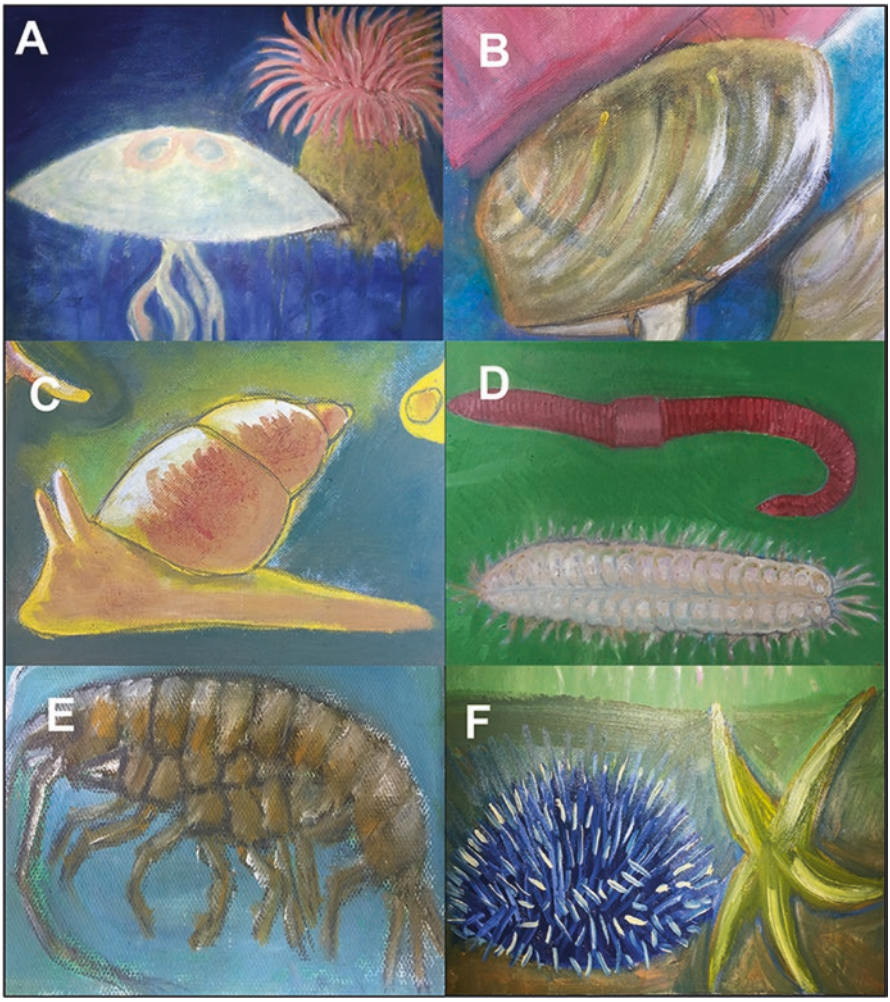

Fig. 2 Early multicellular invertebrates with functional immune systems: (**a**) cnidarians including jellyfish and sea anemones; (**b**) molluscs including bivalves (mussels); (**c**) molluscs including snails; (**d**) annelids including oligochaetes and polychaetes; (**e**) arthropods including crustaceans; and (**f**) echinoderms including sea urchins and sea stars. (Image created by Kurt Buchmann)

exoskeleton which served several anatomical functions as well as gave an efficient first-line defense against pathogens. Thus, descriptions of the immunological armaments in amoebae, flagellates, ciliates, animal sponges (Fig. 1c–f), cnidarians (e.g., jellyfish and sea anemones; Fig. 2a), molluscs (e.g., bivalves and snails; Fig. 2b–c), annelids (e.g., oligochaetes and polychaetes; Fig. 2d), arthropods including crustaceans (Fig. 2e), insects and chelicerates, and echinoderms (Fig. 2f) will indicate how evolved innate responses were before chordates, including branchiostomes (Fig. 3a) and tunicates (Fig. 3b), appeared. The vertebrate lineage involving

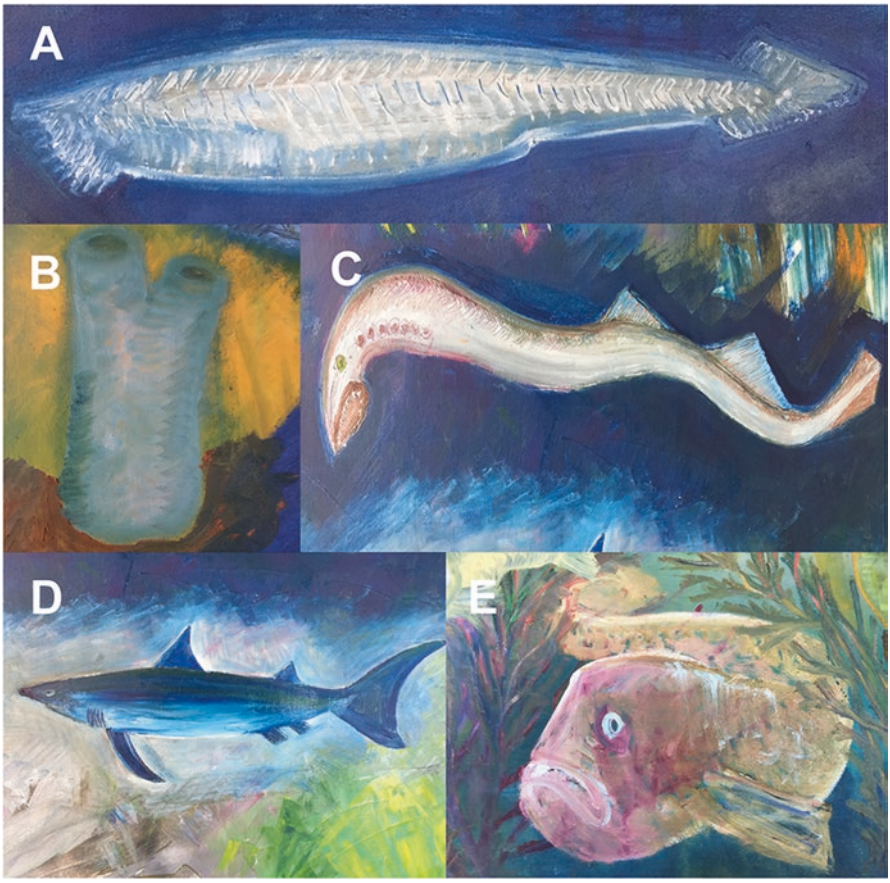

Fig. 3 Chordates: (**a**) cephalochordates (amphioxus); (**b**) urochordates (tunicates); (**c**) early agnathan vertebrates (lampreys); (**d**) gnathostome vertebrates (cartilaginous fish); and (**e**) bony fish). (Image created by Kurt Buchmann)

agnathan fish (such as hagfish and lampreys; Fig. 3c), gnathostomes such as cartilaginous fish (including sharks; Fig. 3d) and bony fish (Fig. 3d), amphibians (Fig. 4a), reptiles (Fig. 4b), birds (Fig. 4c), and mammals (Fig. 4d) started the travel towards sophisticated adaptive responses. Both in more primitive organisms and the highly developed, the basic phagocyte function, with dual functions for repair and for killing (Mills 2012), has kept its central position throughout evolution (Mills et al. 2015), although a richness of terms has been used for their characterization. The macrophage-associated lymphocyte pathways (T-helper [Th] 1 and Th2) became clearly established in bony fish where skewing of responses may be directed by the pathogen nature (Chettri et al. 2014). It is clear that many of the basic innate responses found in invertebrates are still crucial for survival of even the most developed mammal and it is evident that some invertebrate groups without any molecular trace of major histocompatibility complex (MHC) still have the ability to

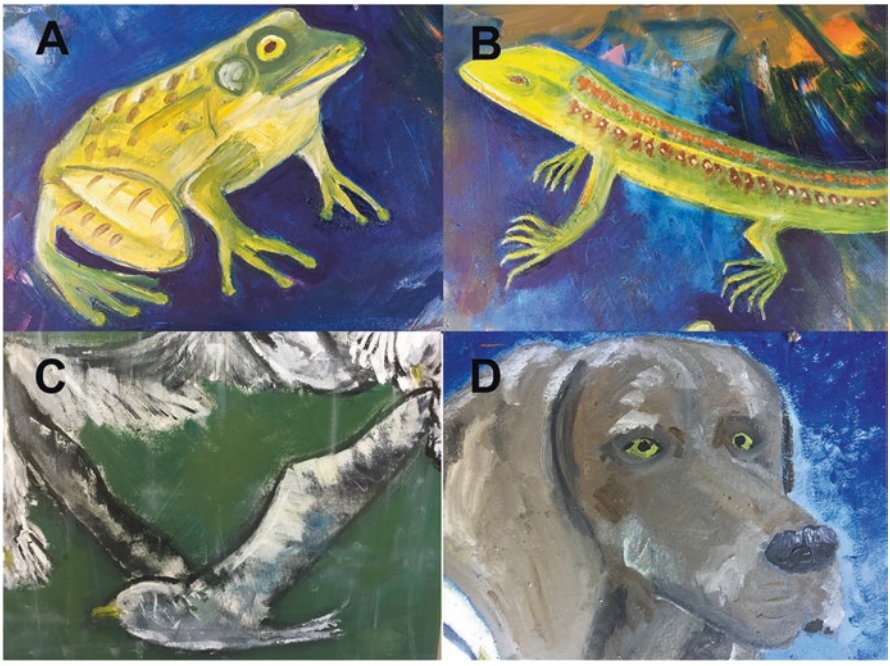

Fig. 4 Higher vertebrates after fish. (**a**) Amphibians (frogs); (**b**) reptiles (lizards); (**c**) birds; and (**d**) mammals. (Image created by Kurt Buchmann)

differentiate self from non-self by the use of other types of recognition molecules. Invertebrates without T and B lymphocytes and MHC still have the ability to exhibit a form of immunological memory by applying alternative memory pathways.

Recognition of Self/Non-self

The adaptive immune system in vertebrates ranging from fish, via amphibians, reptiles and birds, to mammals is based on the MHC, which is the basis for self/non-self recognition in vertebrates (Danchin et al. 2003). The close interaction between MHC and lymphocytes (B and T cells) secures a precise and effective acceptance or rejection of transplants. Proto-MHC genes are found in the chordate amphioxus (*Branchiostoma floridae*) and in insects (*Drosophila melanogaster*) (Danchin et al. 2003) but their occurrence in lower animals was less well-documented before genes encoding MHC-related molecules were detected in the placozoan *Trichoplax adhaerens*, a primitive marine organism consisting of merely two epithelial layers enclosing a number of multinucleate fiber cells (Suurväli et al. 2014). This suggests that the gene complex may have had a basic evolutionary role in immunity, including recognition of self/non-self. Ancestral MHC (proto-MHC) is recognized in urochordates (tunicates) and cephalochordates (amphioxus) (Danchin et al. 2003;

Kasahara et al. 2004) but a composition of the MHC system, which in its basic prototypic form corresponds to the complex in higher vertebrates, first appeared in the cartilaginous fish (Kulski et al. 2002) and finds its full development in mammals. However, even organisms which have never had or secondarily may have lost these genes still need systems for interaction and coordination. The quorum-sensing phenomenon in bacteria also reflects the ability to differentiate between self and non-self and illustrates the universal need for even primitive organisms to sense population densities and coordinate various functions, including colonization (Zhou et al. 2017). Similarly, ciliates have the same social needs and apply pheromones to differentiate genetically distinct cell classes (Vallesi et al. 2016). Many of these ciliate molecules are glycoproteins that occur in numerous forms, allowing a degree of specificity as some are membrane bound while others are exported. The basic experiment to elucidate self/non-self recognition in various animal groups is performance of transplantation experiments using grafts from other species (xenografts) or from individuals of the same species (allografts). Classical xenograft and allograft work in oligochaetes (earthworms) was conducted five decades ago (Cooper 1968, 1969), leaving no doubt that these recognition systems exist. The molecular interactions explaining the reactions have been elucidated in a series of other animal groups. The invertebrate *Hydractinia*, a member of Cnidaria (comprising jellyfish and their relatives), applies two innate histocompatibility loci named alr1 and alr2 (Dishaw and Litman 2009). Highly developed invertebrates such as the echinoderms (sea stars, sea urchins, sea cucumbers) display the ability to reject allografts and accept autografts, which is a classical picture of self/non-self recognition (Arizza and Schillaci 2016). The rich variation and diversity of Toll-like receptor (TLR), scavenger receptor, and NACHT genes in this group, represented by the sea urchin *Strongylocentrotus purpuratus*, with 253 TLRs, 218 scavenger receptor genes and 200 NACHT genes, suggests that these receptor families play a role in the self/non-self recognition process. In more developed animals, such as the urochordates, represented by the tunicate *Botryllus schlosseri*, additional genes involved in fusion (Fu) and histocompatibility (HC) are recognized and termed the FuHC complex. The cell-to-cell interactions in these chordates involve receptors named fester and uncle fester (Gosh et al. 2011). Presence of another self/non-self recognition system in the solitary tunicate *Ciona intestinalis* exists in the interface between sperm and egg. The vitelline coat of the egg may reject sperm and prevent self-fertilization. This recognition process is based on three polymorphic loci termed Themis-A, Themis-B, and vCRL1 (Nicolo et al. 2016).

Immunological Memory

Memory B and T cells associated with MHC in higher vertebrates carry the potential to make a stronger immune response when re-stimulated by a specific antigen and these reactions have been the basis for the original description of memory in adaptive immunity (Bartl and Nonaka 2015). Isotype switching and affinity maturation characterize the mammalian response. Other types of immunological

memory termed innate natural killer (NK) cell memory has been described in mammals including humans (Berrien-Elliott et al. 2015). Whether this follows a direct line via cytotoxic morula cells in early chordates (Franchi and Ballarin 2016) to the innate immune memory, which has been studied in insects and crustaceans, is as yet unknown but phagocytes and phagocytosis play a role in innate immune memory of these arthropods (Cytrynska et al. 2016; Schonhofer et al. 2016). Invertebrates have the ability to use immunological memory based on innate mechanisms and independent of MHC and T cells. The crustacean wood louse *Porcellio scaber* and the mosquito *Anopheles gambiae* exhibit increased occurrence of granulocytes/hemocytes in their hemocoels following re-exposure to bacteria (Roth and Kurtz 2009; Rodrigues et al. 2010). Besides cells, humoral effector molecules may be involved in memory. Insects in general carry memory functions as judged from their ability to respond more strongly to a specific antigen if previously primed. Experimental evidence suggests that immune memory exists in cockroaches, bumble bees, and fruit flies as they all show increased and sustained immunity to a second exposure to certain bacterial isolates following immunization (Cytrynska et al. 2016). The mechanisms have been partly described to include phagocytes, the Toll pathway, and selective usage of a wide array of DSCAM (Down syndrome cell adhesion molecule) receptors. Molluscs, such as the snail *Biomphalaria glabrata*, involve phagocytes in the primary response to a pathogen but produce a soluble factor to sustain lifelong immunity. It is noteworthy that lifelong protection of an invertebrate against the parasite *Schistosoma mansoni* suggests that a basic cellular response against an invading pathogen can be shifted to a humoral long-lasting protective response through production of biomphalysin (Pinaud et al. 2016).

Cell-to-Cell Signaling

Communication between different species of lower and higher organisms is well-studied and many signaling systems may predate the origin of multicellular organisms. Chemoattractants and repellants are known among bacteria, protists, and metazoans. Bacteria are able to communicate and coordinate common functions (colonization) in a way termed quorum sensing (Zhou et al. 2017) and ciliates communicate vividly by a series of pheromones (Vallesi et al. 2016). Amoebae are often found in closed societies (social amoebae) based on close cell-to-cell interactions and ciliates produce a range of specific glycoproteins which make them able to recognize other individual cells of the same species (Zhang et al. 2015). Molecules securing coordination of a colony of individual cells or organisms have therefore been an integral part of communication since the earliest life stages appeared but have reached a high level of sophistication in vertebrates. Intricate processes securing homeostasis of a host organism and coordination of immune responses in vertebrates have developed into extensive networks of cytokines and chemokines (Wang and Secombes 2013; Secombes and Zou 2017). Most cytokine families are found to be well-developed in fish and

in more developed vertebrates but their evolutionary origin can in a few cases be traced back to even lower animals. A tumor necrosis factor (TNF)-α-like cytokine gene (*LITAF*) has been found in the cephalochordate *Branchiostoma* (Jin et al. 2012). Sequences related to transforming growth factor (TGF)-β have been located in the genomes of primitive invertebrates including molluscs, nematodes, insects, and cnidarians (Detournay et al. 2012). A family of astakine cytokines shows regulatory effects on hematopoiesis and leukocyte functions in crustaceans (Lin et al. 2010; Lin and Söderhäll 2011; Watthanasororut et al. 2011). Interferon (IFN) genes have not yet been traced to invertebrates but were first found in sharks, rays, and bony fish, although they may have originated from a cytokine ancestor along with the interleukin (IL)-10 cytokine family (Secombes and Zou 2017). The molluscs *Mytilus galloprovincialis* and *Crassostrea gigas* carry a series of IL-17-related genes, suggesting an early evolutionary appearance of the inflammatory pathway involving IL-17 (Roberts et al. 2008; Venier et al. 2016). Future studies may record ancestral genes of other cytokine families also in invertebrates and it has been documented that mammalian recombinant cytokines TNF-α, IFN-γ, IL-8, and IL-12 bind to receptors in invertebrates and initiate a range of responses (Betti et al. 2006; Fuller-Espie 2010; Malagoli et al. 2008) but evidence that these molecules are encoded in the genome of invertebrates is yet to be found. However, it is noteworthy that earthworm phagocytes (coelomocytes) increase their endocytosis activity when stimulated by recombinant human IL-12 and IFN-γ (Fuller-Espie 2010) and that blue mussels decrease their phagocytic activity when stimulated by recombinant TNF-α (Betti et al. 2006). Macrophage inhibitory factor (MIF) is a central cytokine in responses by snails and crustaceans during various infections ranging from virus to helminths (Garcia et al. 2010; Zeng et al. 2013). *SOCS2* (suppressor of cytokine signaling) genes have been described from the crustacean Chinese mitten crab and may regulate reactions during pathogen invasion (Zhang et al. 2010a, b). *AIF-1* (allograft inflammatory factor-1) genes occur in animal sponges and the pacific oyster, and the cytokine seems to regulate phagocytosis (Zhang et al. 2013). The origin and development of ancient cytokines in invertebrates is well-established but the expanded networks found in vertebrates first arise with fish. Echinoderms (Meng et al. 2009) and early chordates such as tunicates and amphioxus (Zhang et al. 2010a, b) are therefore of special interest. Fish, including both cartilaginous and bony fishes, are the most basic vertebrates to display extended networks of entire cytokine families with some modifications (Wang and Secombes 2013; Venkatesh et al. 2014; Secombes and Zou 2017).

Pathogen Recognition Receptors

Vertebrates produce specific immunoglobulins consisting of heavy and light chains allowing binding to antigens with high specificity and, with their use of affinity maturation recognition of foreign molecules, reach high precision. Vertebrates also possess a basic set of innate recognition molecules which can be

traced to the earliest eukaryote life forms. Pathogens carry certain structures on their surfaces termed pathogen-associated molecular patterns (PAMPs) which can be recognized by pathogen recognition receptors (PRRs) on the host cells. In addition, alarm substances are released when host cells are injured leading to release of damage-associated molecular patterns (DAMPs). PAMPs comprise a wide range of different molecules with prominent members such as lipopolysaccharide (LPS), peptidoglycan, flagellin, double-stranded RNA (dsRNA), and structural carbohydrates. A series of receptors, some of which are membrane bound and others soluble or cytosolic, bind these ligands. They are found from early invertebrates, via fish (Chettri et al. 2011) to mammals, and they include scavenger receptors (Peiser et al. 2002) and TLRs (Boudinot et al. 2014). In addition, binding spectra are wide, including peptidoglycan recognition proteins (PRPs), Gram-negative bacteria binding proteins (GNBPs), DSCAMs, cytosolic NOD-like receptors (NLRs), and retinoic acid-inducible gene I (*RIG-I*)-like receptors (RLRs) (Venier et al. 2016). Both membrane-bound and intracellular receptors are found in everything from primitive levels such as animal sponges (Yuen et al. 2013) to mammals. TLRs represent a central role in innate immunity. They were first described from fruit flies but are accepted as crucial elements in most animals, including fish, amphibians, reptiles, birds, and man (Boudinot et al. 2014). Specific studies have also showed their occurrence in cnidarians (e.g., jellyfish) (Franzenburg et al. 2012), oligochaetes (earthworms) (Skanta et al. 2013), molluscs (e.g., snails) (Elvitigala et al. 2013), crustaceans (Hauton 2012), and echinoderms (sea stars, brittle stars, sea urchins, sea cucumbers) (Meng et al. 2009).

Immunoglobulins as Humoral Effector Mechanisms

Adaptive humoral responses are based on production of immunoglobulins by lymphocytes in vertebrates ranging from gnathostomes (sharks, rays, and bony fish) to amphibians, reptilians, birds, and mammals (Coscia et al. 2016). Antigen binding by classical antibodies is not found in earlier groups which have apply other antigen-binding systems. Recombination activating genes (*RAGs*) are central players in rearrangement of immunoglobulin V, D, and J domains in the process and it is noteworthy that *RAG*-related gene sequences have been detected in the chordate Amphioxus (Zhang et al. 2014), suggesting that these genes may have had other functions in these animals lacking functional antibodies. Where cartilaginous fish carry pentamer immunoglobulins (IgM), bony fish (teleosts) display several immunoglobulin classes (IgM, IgT, IgD, IgZ) (Zhang et al. 2010a, b; Coscia et al. 2016), amphibians exhibit IgM, IgX, IgY, IgA, and IgD, reptiles and birds may carry IgM, IgY, IgA, and IgD (Kaiser 2010; Zimmerman et al. 2010), and mammals are known to have IgM, IgG, IgA, IgD, and IgE (Kaiser 2010). Agnathan fish (hagfish and lampreys) followed another path during evolution as they carry antigen-binding proteins based on leucine rich repeats (LRRs) (Litman et al. 2010).

Complement Factors as Humoral Effector Mechanisms

The complement system and its numerous components exert a range of protective functions which are known to interact with immunoglobulins in vertebrates. However, complement factors follow a separate development and were affiliated with the immunoglobulins relatively lately (Nonaka and Yoshizaki 2004; Peng et al. 2016). Direct binding of various complement factors to the target will not only work as opsonization but may also have a direct lethal effect on the invasive object. The origin of complement and its protective functions predate the appearance of antigen-binding immunoglobulins in sharks and bony fish. It has a central and crucial immunological role—involving the classical, alternative, and lectin-binding activation pathways—in vertebrates but complement-related genes have also been recognized in primitive groups such as the cnidarians (Fujito et al. 2010) and molluscs (Peng et al. 2016), which indicates that this protein complex has been protecting invertebrates during evolution. Insects produce similar proteins related to complement and α_2-macroglobulin (thioester-containing protein [TEP] 1) which bind directly to the target. They can have direct lethal effect but will also opsonize the object and attract effector cells (e.g., hemocytes) to the site (Baxter et al. 2017). The classical complement cascade in vertebrates involves binding of immunoglobulin (and its bound antigen) to complement factor C1q whereafter the associated protease C1r, following a conformational change of the molecule, cleaves C1s to reach an active form splitting C4 and C2. The resulting C4b and C2a fragments combine to an active C3 convertase which will eventually activate the lytic complement cascade and formation of the membrane attack complex (MAC) (Héja et al. 2012). The strong resemblance between C1q (with its associated C1r and C1s) and MBL (mannose-binding lectin) with its associated proteases MASP 1 and MASP 2 (MBL-associated serine proteases 1 and 2) has suggested a close evolutionary connection between these molecular complexes. The occurrence of MBL in early chordates (urochordates, tunicates) has suggested that this is an excellent illustration of the evolution of lectins and their associated proteases and how they established close links to immunoglobulins (Nicola and Loriano 2017). However, additional studies on amphioxus (*Branchiostoma*) (cephalochordates) show that the C1q molecule also operates c1r/C1s in this early group. This suggests an earlier origin of the classical pathway (Gao et al. 2014), but both pathways operate side-by-side even in humans (Héja et al. 2012).

Lectins, Fibrinogen-Related Peptides, and Antimicrobial Peptides as Humoral Effector Mechanisms

Although the important role of MBL, which is a collectin composed of a collagen basis and a carbohydrate binding part, has been documented in both invertebrates and vertebrates, a huge array of other lectins have central roles in immunity. They play a part in self and non-self differentiation, organ development, communication, and immune responses. These carbohydrate-binding proteins occur in all animal

groups and find many applications. Lectins are, based on the nature of their carbohydrate-binding domain (CRD), classified into C-type lectins, S-type lectins (galectins), rhamnose binding lectins (RBLs), F-type lectins (FTLs), X-type lectins (XTLs), I-type lectins (ITLs), P-type lectins (PTLs), and pentraxins (Cammarata et al. 2016). Invertebrates, from primitive sponges, also make use of another protein system termed fibrinogen-related peptides (FREPs), which allows agglutination and clotting of invading microorganisms (Perovic-Ottstadt et al. 2004; Cooper 2010). A wide plethora of additional immune molecules occurs in all evolutionary lineages. Among these antimicrobial peptides (AMPs), having the ability to bind and disrupt break bacterial membranes, are produced by many tissues in the organisms. AMPs are produced in all animal groups and occur in an enormously rich variation, providing the group with the potential to kill a wide range of bacterial pathogens (Arizza and Schillaci 2016; Scocchi et al. 2016).

Other Humoral Effector Mechanisms

Oxygen-dependent killing mechanisms are widely used in species from lower vertebrates to man. In molluscs, entrapment of intruders is associated with production and release of reactive oxygen species (ROS) and nitric oxide synthase (NOS) in hemocytes attracted to the site of injury (Hahn et al. 2000, 2001). These effector molecules include hydrogen peroxide, nitric oxide, super oxide anion, hydrochlorous acid, and hydroxyl ions. Melanization of infection sites is due to enzyme cascades involving the prophenoloxidase system. It is part of a pathogen pacifying process, found in cnidarians, annelids, crustaceans, insects, and echinoderms, in which the target becomes isolated, sequestered, and possibly encapsulated in melanin-rich host cell aggregates (Cerenius and Söderhäll 2004). Lysozyme, which is present in tissue fluids and mucus, is an enzyme that breaks bacterial cell walls. Antiviral responses in insects are to a wide extent based on production of small interfering RNAs (siRNAs) (Baxter et al. 2017). RNA interference is an efficient intracellular protective mechanism in which viral RNA is recognized and degraded through use of Dicer proteins and RISCs (RNA-induced silencing complex), which eventually leads to degradation of the viral RNA (Schonhofer et al. 2016).

Cellular Effector Mechanisms

The first unicellular organisms evolved the ability to recognize and phagocytose particles, including other organisms, a function that provides the needed energy for survival and reproduction. This basic function is preserved and particularly important in the host defense in all animals from early primitive stages to humans (Desjardins et al. 2005; Buchmann 2014). Amoebae may eliminate invaders by simple ingestion, but more sophisticated strategies—probably predating the emergence of metazoans—are available. Social amoebae display a strong and

effective way of silencing external pathogens. The method is applied by higher animals including humans when their neutrophilic granulocytes produce extracellular traps (NETs) consisting of DNA nets loaded with antimicrobial molecules allowing these immune cells to bind, trap, and kill extracellular pathogens. The technique is applied by other vertebrates and invertebrates, but even the social amoeba *Dictyostelium discoideum* produce extracellular traps (ETs). Certain sentinel cells of the colony recognize bacteria or LPS and the traps are released through a Toll/IL-1 receptor domain containing protein TirA and activation of NADPH (nicotinamide adenine dinucleotide phosphate) oxidases (Zhang et al. 2015). The phagocytic cell type is found in all groups ranging from animal sponges (Porifera), cnidarians, molluscs (Venier et al. 2016), annelids (Bilej et al. 2000), arthropods (Salazar-Jaramillo et al. 2014; Baxter et al. 2017), echinoderms (Arizza and Schillaci 2016) via early chordates (Rhodes et al. 1982; Franchi and Ballarin 2016), and fish (Chettri et al. 2011) to mammals. Its functions are basically macrophage-like, which leaves this cell type as one of the footsteps in an evolutionary staircase of immunity (Mills et al. 2015).

The phagocyte function can be found as a wide range of different forms in the various animal lineages. As this is the early prototype of the macrophage it is a basic entity in the communities of social amoebae and animal sponges with amoebocytes in their mesoglea. Subpopulations have been defined in more differentiated animal groups. Earthworms apply coelomocytes, carrying names such as eleocytes, hyaline and granular amoebocytes (Bilej et al. 2000; Engelmann et al. 2016), and the phagocytic abilities of microglia cells in leeches have been investigated by Viziolo et al. (Vizioli et al. 2016). In molluscs, granular hemocytes (basophilic and acidophilic) and agranular hemocytes (hyalinocytes, blast cells, basophils) carry out different functions (Canesi and Pruzzo 2016). Echinoderms, representing the checkpoint between invertebrates and chordates, carry coelomocytes in various forms comprising basic phagocytes, spherulocytes, vibratile cells, and red spherule cells (Arizza and Schillaci 2016). In the early chordates, represented by the tunicates (urochordates), lymphocyte-like cells occur, and in addition a series of hemocytes have been characterized including granular, agranular, unilocular refractile cells, compartment cells, signet ring cells, morula, and finally hyaline cells (Nicolo et al. 2016). The vertebrate lineage displays an increasing complexity of immune cell populations and subpopulations. Agnathans (hagfish, lampreys) present different lymphocyte lineages (Kishishita and Nagawa 2014), which differ to some extent from the populations in sharks and rays, bony fish, amphibians, reptiles, birds, and mammals. Thus, subsets of lymphocytes in jawless fish (hagfish, lampreys) do not possess immunoglobulin and T cell receptors as seen in gnathostomes and higher vertebrates. Instead, these fish carry variable lymphocyte receptors (VLRs) composed of LRRs, leaving agnathans in a unique position compared with cartilaginous and bony fish. Teleosts exhibit a series of lymphocyte subpopulations (characterized by B and T cell receptors) which can be considered the ancestors of the numerous lineages found in higher vertebrates (Abelli 2016). These more developed fishes, amphibians, reptiles, birds, and mammals possess B and T cell lineages.

Diversity of Immune Reactions

All organisms have applied all possibly available protective functions throughout evolution. As long as they conferred the host with an ability to survive attacks by viral, bacterial, and parasitic pathogens their conservation has been justified for a period. However, a trade-off between protection and necessary exposure of vulnerable surfaces has allowed development of a diverse array of potential pathogens. Thus, close contact between the host and its environment during basic physiological processes (feeding, gas exchange) has allowed survival of parasites exhibiting various immune evasion strategies. On the other hand, it has also been a basis for development and conservation of particular types of armaments allowing the host to survive until successful reproduction has been achieved. The immunological tools applied throughout evolution support recognition of self/non-self, immunological memory, cell-to-cell signaling, humoral effector mechanisms, and cellular effector mechanisms. An extensive series of functions which still endow unicellular organisms with the ability to communicate and differentiate self from non-self is not conserved throughout all lineages. A dynamic selection of functions imposed by environmental factors and new virulence mechanisms paved the way for appearance of corresponding functions at various levels of evolution. Basic functions comprising phagocytosis, receptor–ligand signaling, lectin–carbohydrate interactions, and basic cytokine signaling can be traced in all lineages, whereas MHC, lymphocytes, and immunoglobulins appeared at a later timepoint supported by extensive cytokine networks. New immunological tools were, on the other hand, able to interact with existing immune factors. Thus, MBL, MASPs, complement, and immunoglobulins illustrate how involvement of existing mechanisms for killing pathogens was applied to improve the protective armament. Likewise, the TLRs which appeared early in evolution are still pivotal elements of immune functions in the most sophisticated vertebrates.

Applied Aspects of Immune System Evolution

Investigations on invertebrate immunity have unraveled fundamental aspects and mechanisms highly relevant for vertebrates also, including mammalian immunology. Discovery of TLRs and their functions is merely one subject which has enriched immunity research in more sophisticated vertebrate species. Another practical focus point is the potential for developing immune-prophylactic strategies for animal groups which do not apply MHC and memory lymphocytes. Vaccination was originally described by Edward Jenner as immunization of humans with antigens from cow pox virus (Vaccinia virus) establishing a protective immune response against small pox virus. In its broad sense, vaccination may now be considered as a primary immunization leading to immune memory and establishment of a protective response against re-exposure to the pathogen. Since Jenner's early vaccination procedures more than 200 years ago, the methodology has been widely adopted and a huge number of vaccines against various viral, bacterial, and parasitic diseases

have been introduced so that we now are able to perform large-scale routine vaccination of fish, reptiles (Zimmermann et al. 2010), birds, and mammals, including humans. A classical example of how the effective immune system of even lower vertebrates (teleosts) allowed mass vaccination strategies to be implemented is found in the Norwegian salmon farming industry. From the middle of the 1980s the industry has reduced usage of antibiotics by more than 99% despite a fish production increase of more than 2000%. Although management improvements and selective breeding have contributed to this success, the application of novel oil-based vaccines from the early 1990s mainly explains the improved immune status and the reduction of antibiotic usage. The efficacy of vaccines developed for teleosts is based on adaptive responses (MHC and memory lymphocytes) in combination with developed innate responses. Invertebrates that merely carry proto-MHC, no T cell receptors, and no B cells must make use of alternative immune priming systems if vaccination-like methodologies are to be implemented. Recent research has, in fact, described that immune-primed invertebrates are able to erect protective responses against re-exposure to the same pathogen. Crustaceans carry an extended immunological toolbox (Hauton 2012), insects, such as dipterans and coleopterans, may obtain immune memory against various pathogens (Rodrigues et al. 2010; Baxter et al. 2017), molluscs, such as snails, can establish a lifelong immunity against parasitic re-invasion (Pinaud et al. 2016), and echinoderms exhibit a range of immunological factors, providing the basis for the belief that immune memory also occurs in this highly developed group of invertebrates (Arriza & Schillaci 2016). Therefore, continued research within evolutionary aspects of immune functions may bring about not only general scientific enlightenment but could also prove to lead to vaccination strategies for invertebrates. This will eventually benefit productivity in industries delivering nutritive animal products, ranging from insects, crustaceans, bivalves and snails to echinoderms, a basic requirement for an increasing world population.

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Allorecognition and Innate Immunity in the Dictyostelid Social Amoebae

Adam Kuspa

Introduction

Fundamental to the immune function of any organism is the ability of cells to recognize self from non-self in order to curtail exploitation by variant cells of the same species or to fend off potentially harmful cells of other species. The responses to these existential challenges likely provided the evolutionary pressure that helped shape such recognition mechanisms. Social amoebae such as *Dictyostelium discoideum* and their predecessors have survived for ~900 million years in complex soil ecosystems. Vegetative amoebae recognize and respond in complex ways to a myriad of bacteria as food, pathogens, or potential symbiotic partners. As social amoebae achieved multicellularity, self-recognition, or allorecognition, is likely to have been selectively advantageous for reinforcing the cooperative behavior of these otherwise solitary cells. Bacterial recognition and killing during *D. discoideum* development is relegated to a specialized population of innate immune cells that ingest and kill bacteria and that produce extracellular traps (ETs). Thus, several immune recognition events in the social amoebae seem to be shared with metazoa, suggesting that the antecedents of animal immune systems existed in colonial microorganisms.

D. discoideum lives primarily in the soil of deciduous forests, where amoebae track, engulf, and digest bacteria and other microbial prey. As would be expected in any inter-species interaction, in amoebae/bacteria interactions the cellular physiology of *D. discoideum* is modulated during the vegetative growth phase by "kill or be killed" dynamics. Amoebae have mechanisms that enable them to discriminate among different bacteria that presumably allow the amoebae to respond to changes in the soil microbiota in order to achieve optimal feeding and avoid exploitation by

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pathogens (Farbrother et al. 2006; Cosson and Soldati 2008; Bozzaro and Eichinger 2011; Nasser et al. 2013; Lima et al. 2014). So, although mammalian immune cells no longer rely on bacteria as a source of nutrition, some of the bacterial killing machinery, such as the phagosome (Boulais et al. 2010), was likely shaped over more than a billion years by the interactions between bacteria and their unicellular progenitors prior to the radiation of multicellular species.

Social amoebae are facultative multicellular organisms. When starved, they communicate with one another by means of extracellular cyclic adenosine monophosphate (cAMP) signaling, aggregate to form a mound of ~10^5 cells, and differentiate into several distinct tissue types to form a migrating, multicellular slug (Kessin 2001). The slug can migrate away from ammonia and towards light, causing it to climb up and out of the soil, at which point the cells further differentiate to form a multicellular fruiting body. During fruiting body formation, approximately 20% of the cells differentiate into stalk cells and die, thereby forming a rigid stalk that holds aloft the remaining 80% of cells, which differentiate into viable spores. The fact that some of the cells die during this process opens *D. discoideum* to the possibility of exploitation by genetic variants or "cheaters" that produce more viable spores per input cell than the cells they co-develop with.

In this chapter I will consider *D. discoideum* allorecognition and innate immunity during development, and the regulation of its microbiome during growth and at the transition to development. One theme is that the similarities in strategies that eukaryotes use to deal with internal and external threats are likely the product of common genetic heritage that provided a protein "toolbox" to respond to the universal selective pressures that these threats imposed. The mechanistic details of these systems reveal significant complexity in the amoebal immune system and are beginning to allow an assessment of their evolutionary relationship to immune functions found in other organisms, including mammals (Boulais et al. 2010; Zhang and Soldati 2016).

Phylogeny of Multicellular Eukaryotes

The three major groups of the Unikont supergroup (Metazoa, Fungi, and Amoebozoa) each achieved multicellularity by different means: the Metazoa through the evolution embryogenesis to produce mobile individuals, the Fungi through filamentous growth into sessile colonies, and the Amoebozoa through the aggregation of amoebae into mobile individuals. Comparative analyses of the complete proteomes of eukaryotes firmly established that Amoebozoa diverged from the line leading to humans about 900 million years ago, soon after the plant/animal split and just prior to the fungal/animal split (Eichinger et al. 2005; Song et al. 2005; Koonin 2010) (Fig. 1). Earlier sequence analyses had demonstrated that Amoebozoa are a monophyletic clade, rather than a loose assemblage of early diverging protists, as had been assumed (Bapteste et al. 2002; Song et al. 2005). The significance of this is that features that are broadly conserved in amoebae such as the dictyostelids are likely to have originated within the last common ancestor of all extant Unikonts and

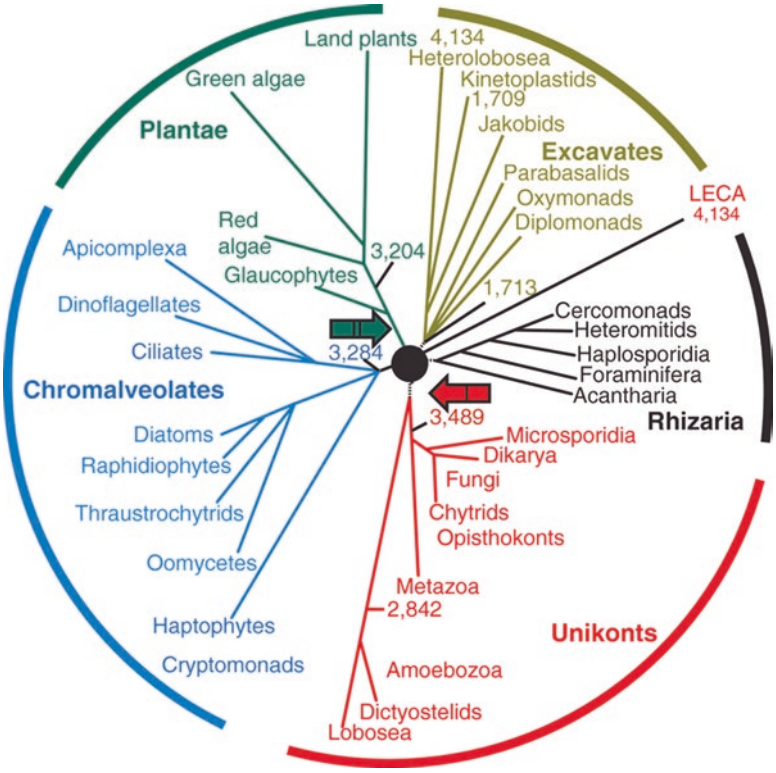

Fig. 1 Evolution of the eukaryotes. A phylogeny of the Excavates, Rhizaria, Unikonts, Chromalveolates, and Plantae, the five eukaryotic supergroups, are shown with the last eukaryotic common ancestor (LECA) at the root (black circle). The numbers indicate the genes that are shared between the LECA and excavate amoeboflagellate *Naegleria gruberi* (4134) and retained in selected lineages from different supergroups (Fritz-Laylin et al. 2010). Branch lengths are arbitrary. Two putative root positions are shown: I, the Unikont-Bikont rooting (Stechmann and Cavalier-Smith 2002, 2003); and II, rooting at the base of Plantae (Rogozin et al. 2009). (Reproduced from Koonin 2010)

so may inform comparisons of immune function across a broad representation of multicellular organisms.

The dictyostelids themselves are comprised of four major groups, each with distinct multicellular forms and physiological characteristics, spanning well over 600 million years of evolution (Schaap et al. 2006; Schilde and Schaap 2013; Tian et al. 2013). Although they all retained the same basic lifestyle of facultative multicellular development and sporulation, the different lineages have undergone dramatic expansions and contractions within common gene families, suggesting that gene function diversification can provide opportunities for specializations without altering the lifecycle (Sucgang et al. 2011; Heidel et al. 2011). For instance, it has been shown that conserved cAMP/phosphodiesterase signaling pathways ramified into intercellular signaling pathways that coordinate multicellular morphogenesis and spore encapsulation (Kawabe et al. 2015; Du et al. 2015; Chen and Schaap 2015).

Decades of developmental genetic studies have uncovered numerous signaling pathways and cellular mechanisms that control growth, differentiation, and morphogenesis of *D. discoideum*, some of which suggest a deep homology between animals and amoebae (Loomis and Smith 1990; Anjard and Loomis 2005, 2006; Robery et al. 2013; Waheed et al. 2014; Ludtmann et al. 2014). Indeed, the genome sequence of *D. discoideum* revealed genes previously thought to be specific to animals (e.g., SH2 [Src Homology 2] domain-based signaling, and several subfamilies of G protein-coupled receptors [GPCRs]) or plants (cellulose degrading enzymes and WRKY transcription factors), indicating that many more cellular systems were available to the last unikont common ancestor than had been assumed prior to the broad availability of genome sequences (Eichinger et al. 2005). Particularly striking examples of this involve the signals that induce sporulation and coordinate it with fruiting body morphogenesis; a cytokinin found mainly in plants and a neuropeptide found in mammals (Anjard and Loomis 2005, 2008). SDF-2 is a peptide that activates cAMP production, and therefore PKA activation and spore encapsulation, by inhibiting signaling through the histidine receptor kinase DhkA (Wang et al. 1999; Anjard and Loomis 2005). Spore differentiation factor (SDF)-2 is processed from the ubiquitous acyl carrier protein (acyl-CoA-binding protein [ACP]) that is produced by prespore cells and secreted through an unconventional secretion system (Anjard and Loomis 2005; Cabral et al. 2010). ACP is processed into SDF-2 by a protease on the surface of prestalk cells and then SDF-2 binds to DhkA on prespore cells (Anjard and Loomis 2005). SDF-2 is identical to the diazepam-binding inhibitor (DBI) that signals through the $GABA_A$ receptors in the mammalian brain and peripheral benzodiazepine receptors, and DBI as well as benzodiazepines such as Valium induce spore encapsulation in *D. discoideum* (Anjard and Loomis 2005). Interestingly, the mammalian peptide signals are similarly produced in cells near those that have the relevant receptors, so although the receptors are not conserved between mammals and *D. discoideum*, the signal processing strategy that allows for coordination between cells may have been conserved (Anjard and Loomis 2005).

Homology or Convergent "Assembly of Parts" From an Ancestral Proteome

A major question for the comparative immunology of metazoa and amoebae concerns the origin of basic defense functions. Which antecedents of extant immunity existed in the last common ancestor of the unikonts and should be considered universal, which were more recent lineage specific inventions, and which resulted from convergent evolution shaped by universal selective pressures? Phylogenomic analyses indicate that the last eukaryotic common ancestor (LECA) was complex, with a diverse proteome and cellular systems found in extant eukaryotes, and likely radiated rapidly into five supergroups (Fig. 1) (Koonin 2010). The question then becomes: which more complex immune functions appeared uniquely in the lineages of these five groups and which share common elements? It is clear that LECA had a more complex gene complement available than had been assumed until recently,

but it is unclear whether the subcellular assemblies and cellular networks that we think of as being fundamental to immunity existed as well. For example, it is clear that phagosomes were used by unikont ancestors to kill bacteria over 1 billion years ago, but were immunoglobulin (Ig) repeat proteins used as the cornerstone recognition systems? Deciphering universal selective pressures that existed at the start of the unikont lineage should deepen our understanding of the biological events that have led to extant immune function in mammals.

An example of billion-year-old proteins that have been used as "raw material" for the evolution of cellular networks useful for immune defense are those used to construct epithelia. Epithelia are sheets of cells found in metazoa that are polarized and held together in specific geometries and used as biological barriers to, for instance, bacterial pathogens. Animals organize cells within epithelial sheets through adherens junctions comprised of α-catenin and β-catenin proteins that connect the actin cytoskeletons of adjacent cells through cadherin proteins (Mege and Ishiyama 2017). Remarkably, *D. discoideum* also forms an epithelium using adherens junctions that are morphologically indistinguishable from those found in metazoa and that are comprised of α- and β-catenin (Grimson et al. 2000; Dickinson et al. 2011). During fruiting body morphogenesis, an epithelium forms around the stalk tube and the barrier it produces prevents stalk-inducing signals from reaching the prespore cells in the nascent sorus (Grimson et al. 2000). Though the strict homology of epithelia may not be provable over such long intervals of time (~900 million years), what is certain is that amoebae and animals use the same proteins to form the same cellular structure, to serve the same biological function in tissue formation. If animal and amoebal epithelia derive from a common ancestral structure, it is worth considering that ancient epithelia served as barriers to pathogen penetration of the "body" of colonial microorganisms that were the last common ancestors of metazoa and amoebae.

Allorecognition and the Stabilization of Cell Cooperation

Social amoebae form cooperative groups of cells when they develop and so can be considered facultative multicellular organisms. Since 20% of the *D. discoideum* cells die in the process of fruiting body formation and become the stalk, the stability of cell cooperation is threatened by variants that can exploit the system by avoiding the stalk fate and making more than their share of spores compared with their prevalence in the starting population (Shaulsky and Kessin 2007; Strassmann and Queller 2011). This is far from a theoretical argument since this kind of "cheating" behavior has been demonstrated in the lab with clonal isolates from wild populations of *D. discoideum* (Strassmann et al. 2000; Fortunato et al. 2003a, b). Moreover, cheater strains have been isolated from mutant screens in the laboratory (Ennis et al. 2000) and their frequency suggests that many loci could potentially produce the cheater phenotype (Santorelli et al. 2008). Evolutionary analyses of the sequence signatures of over 150 "cheater genes" obtained by whole genome sequencing of 20 wild isolates suggests that negative frequency-dependent selection drives a stalemate

between cheater and cooperator genotypes (Ostrowski et al. 2015). This population genomic approach appears to rule out any directional selection of the cheater loci that would tend to polarize phenotypes and suggests a more complex dynamic interaction between loci that stabilize cooperative behaviors.

Since cheating behavior is common among variants in the wild and in syngeneic lab strains, several potential solutions have been tested for the control of cheating that would serve to stabilize cell cooperation over the course of dictyostelid evolution. Perhaps the simplest explanation for why genetically variant cheaters do not overrun populations of cooperating cells is pleiotropy. Foster and colleagues proposed that, since most genes affect many traits, genetic changes that produce the cheater phenotype would also be expected to compromise the overall fitness of the individual and this would limit the spread of cheater variants (Foster et al. 2004). In this regard, it is interesting to note that the first cheater that was characterized, caused by an insertion mutation in the F-box protein FbxA, has severe developmental defects that would almost certainly limit its fitness in the wild (Ennis et al. 2000, 2003).

Kin recognition, or self-recognition (allorecognition) in the case of genetically identical cells, is another plausible mechanism that social amoebae use to direct the benefits of cellular cooperation and such behavior has been demonstrated in *D. discoideum* and in *D. pupureum* (Strassmann et al. 2000; Mehdiabadi et al. 2006). Strassmann and Queller compiled convincing evidence that social amoebae direct the benefits of cellular cooperation toward closely related individuals (Strassmann and Queller 2011). There is significant evidence that the deleterious effects of cheaters within populations of cooperating amoebae are mitigated by the high relatedness of co-occurring isolates in nature (Gilbert et al. 2007; Kuzdzal-Fick et al. 2011). They identified the gp80 protein as the first molecular "greenbeard"—a specific cellular characteristic—which in this case is a cell-surface adhesion protein that directs the benefits of multicellular development to related cells by adhering to other cells that express the protein (Queller et al. 2003). So, the same protein identified individuals as being related by homotypic binding between cells and is also required for the altruistic behavior (development). Interesting complexity of the system was introduced when it was discovered that different isolates of the same species of *D. discoideum* segregated from one another during development and the propensity of two isolates to segregate was inversely proportional to their genetic relatedness (Ostrowski et al. 2008). Therefore, even though all cells of the species will co-aggregate in a process that is directed by chemotaxis to extracellular cAMP, individuals later segregate into less cosmopolitan multicellular organisms comprised of more closely related individuals.

The segregation of wild strains based on genetic relatedness strengthened the case for the kin recognition as a mechanism to preserve cell cooperation as a selective advantage in the social amoeba, but left open the question of mechanism. The genome of *D. discoideum* encodes several distinct membrane protein families that could serve as recognition receptors, or ligands, including dozens of tig/Ig/E-set repeat proteins (later re-named tiger ["Tgr"] proteins) (Eichinger et al. 2005). One tiger protein, TgrC1 (formerly LagC), was a particularly attractive candidate

recognition protein since it had originally been characterized as a cell adhesion protein essential for the formation of the initial multicellular aggregate (Geltosky et al. 1979; Dynes et al. 1994; Wang et al. 2000). Given its structural similarity to immune recognition proteins and its role in integrating cells into a multicellular organism, TgrC1 was considered as a candidate component of the kin recognition apparatus. The *tgrB1* gene was found adjacent to the *tgrC1* gene and the TgrB1 protein is related in its overall structure to TgrC1, and it is also expressed coordinately with TgrC1 through a shared promoter at the time of development when individual cells make adhesive contacts (Benabentos et al. 2009). TgrB1 and TgrC1 are each highly polymorphic in wild populations of *D. discoideum* ranking amongst the top 1% of the most polymorphic proteins encoded by the genome (Benabentos et al. 2009; Ostrowski et al. 2015). The *tgrB1* and *tgrC1* gene deletion mutants each co-aggregate with cells of the parental strain but then segregate into distinct tissues over the course of development (Benabentos et al. 2009). The *tgrC1* mutants are completely left behind in these chimeric mixtures while the parental strain completes development. This indicates that TgrB1 and TgrC1 are required to maintain the integration of individual cells within the multicellular structure, and suggested that they are necessary for kin recognition. Intriguingly, amino acid polymorphism within ~20 amino acid segments of each of the ectodomains of TgrB1 and TgrC1 are highly predictive of the segregation behavior of the wild strains carrying them (Benabentos et al. 2009).

Gene replacement experiments with pairs of *tgrB1*/*tgrC1* genes from wild strains demonstrated definitively that the protein pairs are sufficient to mediate kin recognition (Hirose et al. 2011). When a pair of *tgrB1*/*tgrC1* genes is transplanted into the same chromosomal locus of a laboratory strain, the resulting strain can develop normally on its own. However, such double gene replacement strains fail to recognize their parental strain in mixtures and segregate during the aggregation process to continue development completely independently (Hirose et al. 2017). These results indicate that just two proteins, TgrB1 and TgrC1, determine allotype in *D. discoideum* since they are both necessary and sufficient to mediate allorecognition and they are required for development. The recognition mechanism mediated by TgrB1 and TgrC1 involves self-recognition (allorecognition) rather than active avoidance, or repulsion of non-self. The property can be demonstrated by mixing strains carrying an extra pair of *tgrB1*/*tgrC1* genes with other strains carrying only a native pair of genes. Such a merodiploid interacts well with any other strain that shares one of its *tgrB1*/*tgrC1* pairs (Fig. 2).

One major prediction of allorecognition systems is that they bias the benefits of social interactions to related individuals. This prediction was tested for *D. discoideum* by mixing strains of different allotypes with a strong cheater, a mutant in the F-box protein FbxA (Ennis et al. 2000). In three-way mixtures of an *FbxA* mutant with two strains with different allotypes (distinct *tgrB1*/*tgrC1* gene pairs), the strain that shared the allotype of the *FbxA* mutant suffered a greater cost (produced fewer spores overall) than the strain with a different allotype (Ho et al. 2013). These tests were carried out on an entire petri dish so the fitness was measured over the entire population of hundreds of individual fruiting bodies. Presumably, the strain that did

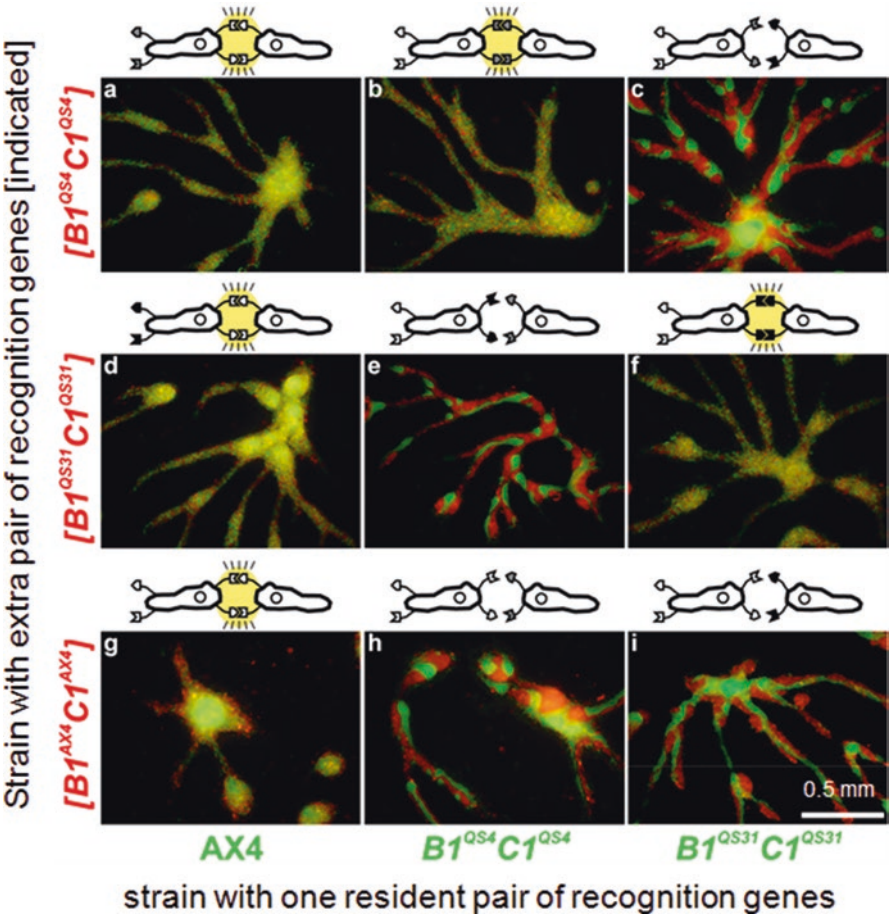

Fig. 2 Allorecognition in social amoebae. Red fluorescent protein (RFP)-expressing merodiploid strains containing an extra pair of *tgrB1–tgrC1* allorecognition genes (red brackets) in the parental AX4 strain are indicated at the left of each row. The *tgrB1–tgrC1* genes were from the wild strains QS4 (**a**–**c**), QS31 (**d**–**f**) and, as a control, from AX4 (**g**–**i**). Each strain was tested for its interaction with green fluorescent protein (GFP)-expressing cells with a single pair of *tgrB1-tgrC1* genes, as indicated below the columns in green: the parental AX4 strain (**a**, **d**, **g**), double gene replacement strain with *tgrB1–tgrC1* alleles from the wild strain QS4 (**b**, **e**, **h**), and double gene replacement strain with *tgrB1–tgrC1* alleles from the wild strain QS31 (**c**, **f**, **i**). The strains were cultivated separately and mixed at equal proportions and allowed to develop as admixtures. Multicellular structures are shown as imaged with fluorescence microscopy at the streaming aggregation stage (8–12 h). Illustrations above the pictures are models of the interactions between the cells, where the cells are shown as elongated amoebae with circular nuclei. TgrB1 and TgrC1 are illustrated as membrane proteins; the shading indicates their origins, and interactions between matching pairs are highlighted. In panels **a**, **d**, and **g** the cells recognize each other as self through the pair of shared *tgrB1–tgrC1* alleles of the parental AX4. In panels **b** and **f** the strains recognize each other as self through the donated *tgrB1–tgrC1* alleles from QS4 and QS31, respectively. In panels **c**, **e**, **h**, and **i** the strains segregate because they do not recognize each other as self due to the absence of any common pair of *tgrB1* and *tgrC1* genes of the same allotype. (Modified from Hirose et al. 2011)

not share the allotype of the *FbxA* mutant cooperated more often with cells sharing its own allotype, thus avoiding exploitation by the cheater. Such experiments provide a simple demonstration that allorecognition mediated by *tgrB1/tgrC1* can suffice to minimize the fitness costs of social cheating.

These laboratory experiments combined with the evolutionary analyses of *tgrB1/tgrC1 gene* sequences in wild populations of *D. discoideum* suggests the TgrB1/TgrC1 allorecognition system is the main mechanism that has served to stabilize cellular cooperation in the social amoebae. It has been proposed that this system cannot fully account for the behavior of wild strains as they appear to produce chimeric slugs and fruiting bodies more often than the laboratory experiments would predict (Strassmann 2016). It is possible that the TgrB1/TgrC1 system provides strong self-recognition discrimination when the cells first integrate into a multicellular organism, and later slug fusion accounts for the prevalence of chimera observed in the wild. In fact, Ho and Shaulsky showed that rare allotypes do in fact join slugs of other allotypes well after the critical time of self/non-self recognition during aggregation, providing a potential explanation for the prevalence of chimeras observed in the wild and also for the maintenance of allotype diversity observed within wild populations (Ho and Shaulsky 2015).

Mechanistic studies have shown that TgrB1/TgrC1 are more than heterophilic cell adhesion proteins and they are beginning to reveal how allorecognition mediates cell cooperation in *D. discoideum.* Examination of the assembly of the allorecognition proteins suggests that TgrC1's recognition of TgrB1 on a closely apposed cell occurs through the interaction of a single Ig repeat on each protein (Chen et al. 2013). This TgrB1/TgrC1 binding between cells stimulates the dimerization of TgrB1 in one cell, followed by the oligomeric assembly of TgrB1 and TgrC1, forming an adhesion complex (Chen et al. 2014). As predicted from the gene replacement experiments, the amino acid sequence polymorphisms in the ectodomains of *TgrB1* and *TgrC1* do in fact predict the pattern of cell cooperation, and the relationship between protein–protein binding strength and recognition specificity between allotype partners has been demonstrated with purified proteins in vitro (Gruenheit et al. 2017). It is curious that the Tgr recognition proteins are type I transmembrane proteins with Ig domain repeats and are structurally and functionally similar to mammalian major histocompatibility complex (MHC) recognition proteins (Hughes and Nei 1988; Boehm 2006) and also structurally similar to Hydractinia allorecognition proteins (Rosengarten et al. 2011). This structural similarity without extensive sequence similarity, across the unikont phylogeny, suggests that these allorecognition proteins were the products of convergent evolution and the selective pressure to present recognition motifs away from the cell surface. Apparently, Ig domain repeats provide a robust protein scaffold that can tolerate the extensive amino acid variation required for effective allorecognition (Barclay 2003).

The function of TgrB1 and TgrC1 as cell surface adhesion proteins required for development has been established over the past 40 years, but the characterization of their role in cellular signaling has only recently begun (Geltosky et al. 1979; Dynes

et al. 1994; Wang et al. 2000; Benabentos et al. 2009; Chen et al. 2013, 2014; Gruenheit et al. 2017). The biological effects of allotype recognition in *D. discoideum* have been elucidated by embedding a small number of cells in a field of developing cells with an incompatible allotype. The behavior of the cells could be used to infer the function of TgrB1–TgrC1 recognition at the cellular level because the missing function of minority singleton cells is restored when they encounter other cells of the same allotype (Hirose et al. 2015). This experimental paradigm was used to demonstrate that allorecognition is required for cell polarization, coordinated cell movement, and tissue integration during developmental aggregation. Minority incompatible cells do not interact with the majority cells move radically while the minority cells meander within the mound, but instead they meander against the radial flow of the majority cells and they do not reorganize their actin cytoskeleton like the polarized cells of the majority. However, when they bump into a compatible minority cell they adhere to one another, reorganize their cytoskeletons within a minute, and begin to move coordinately, but they remain indifferent to the cells of the majority allotype. Eventually, these minority incompatible cells differentiate into prestalk or prespore cells if they can form at least a small assemblage of cells that share the same allotype (Hirose et al. 2015). These findings established that TgrB1–TgrC1 recognition is key to the integration of *D. discoideum* cells into a multicellular organism.

Recent studies indicate that TgrB1–TgrC1 binding initiates a specific signaling pathway that initializes multicellularity. The signaling function of TgrB1 and TgrC1 is beginning to be deciphered. The directionality of signaling initiated by TgrB1–TgrC1 binding was determined by expressing each of them separately in a strain with an incompatible allotype. Strains expressing TgrB1 of a specific allotype could only receive signals from cells of that allotype, and only cells expressing TgrC1 of a specific allotype could provide the signal to cells of that allotype (Hirose et al. 2017). The 77-amino acid cytoplasmic tail of TgrB1 is also specifically phosphorylated during development, but only when TgrC1 of a compatible allotype is present (Hirose et al. 2017). The inference from these experiments is that TgrC1 is the ligand that engages and activates the TgrB1 receptor, which is consistent with TgrC1 inducing TgrB1 dimerization (Chen et al. 2014). Both cooperative aggregation and prespore cell differentiation were tested in these single-gene merodiploids, but it is formally possible that TgrC1 acts as a receptor to regulate other aspects of development that were not specifically tested.

A genetic screen to identify effectors of TgrB1 signaling have so far resulted in the isolation of dominant TgrB1 activation mutants and bypass suppressor mutations in RapA guanosine triphosphatase-activating protein B (RapgapB), a regulator of Rap1 (Parkinson et al. 2009; Li et al. 2016). This first-stage genetic analysis suggests that TgrB1–TgrC1 signaling modulates cytoskeletal dynamics, which is consistent with the rapid (1–2 min) cell polarization response when two cells of like allotype contact each other. Thus, it appears that allorecognition initially modulates the cytoskeleton in a manner that allows adherent cells to coordinate their movements and form a functional tissue.

Amoebae Versus Bacteria

The predation of bacteria by amoebae versus the colonization of amoebae by bacteria is one of the more ancient inter-species competitions, playing out for well over a billion years. *D. discoideum* has been used as a model system to study phagocytosis and the intracellular killing of bacteria and the subversion of those processes by pathogens as it relates to innate immune defense in mammals (Cosson and Soldati 2008; Boulais et al. 2010; Bozzaro 2013; Cosson and Lima 2014). Similarities between amoebae and macrophages have long been recognized—indeed, this insight helped shape Metchnikoff's seminal cellular theory of immunity (Metchnikoff 1905; Hirsch 1959). Amoebae deploy their phagocytic/endocytic system to feed on bacteria, which provides innate defense against potentially pathogenic bacteria in their environment, whereas the primary function of mammalian phagocytes is in immune defense and not in nutrient acquisition. Since amoebae must kill bacteria to satisfy essential nutritional requirements, it is easy to overlook this as a defense function. However, there is a deep homology between amoebal and animal phagocytosis and bacterial killing, requiring the orchestration of dozens of homologs of the unikont proteome, so one might expect other homologous features of the interactions between bacteria and eukaryotic cells have yet to be uncovered (Boulais et al. 2010; Cosson and Lima 2014). Recent examples of this are the unexpected findings that extracellular folate sensing is required for efficient intracellular killing of *Klebsiella pneumonia* by amoebae, connecting the extracellular sensing of bacteria with their intracellular killing by amoebae (Leiba et al. 2017), and the elaboration of DNA-based extracellular bacterial traps described in section "Amoebal Innate Immunity".

Amoebae and macrophages are susceptible to many of the same pathogens and the mechanisms by which these pathogens avoid or subvert the phagocytic process appear to be similar (Cianciotto and Fields 1992; Bandyopadhyay et al. 2004; Danelishvili et al. 2007; Gao et al. 1997; Simon and Hilbi 2015). These findings have generated significant interest in the infection dynamics of amoebae and their use as surrogate systems for the study of macrophage infection, as has been extensively reviewed (Steinert and Heuner 2005; Pozos and Ramakrishan 2004; Dorer and Isberg 2006; Steenbergen et al. 2003; Bozzaro and Eichinger 2011; Lima et al. 2011; Bozzaro et al. 2013; Cosson and Lima 2014; Gerstenmaier et al. 2015; Harrison et al. 2015; Strassmann and Shu 2017). Novel bacterial virulence factors have been identified using *D. discoideum*, highlighting the universality of bacteria–unikont interactions (Pukatzki et al. 2002, 2006; Alibaud et al. 2008; Hasselbring et al. 2011; Tosetti et al. 2014). *D. discoideum* has been used to uncover a type VI secretion system in *Vibrio cholerae* that is relevant to mammalian infection (Pukatzki et al. 2006; MacIntyre et al. 2010; Miyata et al. 2011) and a dozen virulence factors in the human pathogen *Burkholderia pseudomallei* have been delineated by sorting through candidates identified in a genetic screen of *B. thailandensis* infection of *D. discoideum* (Hasselbring et al. 2011). Nramp1 (Natural Resistance-Associated

Macrophage Protein) localizes to the phagosome in both mammalian cells and in amoebae, and this metal ion transporter appears to limit infections of *Mycobacterium* sp. and *Salmonella typhimurium* and *Legionella pneumophila* (Balest et al. 2011; Peracino et al. 2013; Buracco et al. 2015).

D. discoideum and other free-living amoebae have also been found to harbor human pathogens and this has several implications for infection control and health policy. Since human pathogens transit through amoebal populations, there is the intriguing possibility that amoebae provide bacteria the selective pressure to evolve mechanisms to evade predation that are also beneficial for their survival in "accidental hosts" such as humans. In this way amoebae such as *D. discoideum* might serve as evolutionary "training grounds" for bacteria, continually improving the ability of bacteria to cause disease (Fields 1996; Swanson and Hammer 2000; Molmeret et al. 2005; Steenbergen et al. 2003; Casadevall and Pirofski 2007; Taylor-Mulneix et al. 2017). Non-pathogenic amoebal species such as *D. discoideum* are also thought to act as "Trojan horses" that harbor and protect human bacterial pathogens (Molmeret et al. 2005; Thomas and Ashbolt 2011). For example, it has been suggested that amoebae act as reservoirs for *Legionella* or *Chlamydia* and protect them from chlorination and antimicrobial agents (Greub et al. 2004; Thomas and Ashbolt 2011; Thomas et al. 2006), or as amplifying transmission vectors for pathogenetic *Bordetella bronchiseptica* (Taylor-Mulneix et al. 2017).

Amoebal Innate Immunity

Innate immunity in *D. discoideum* begins with the ability of cells to kill bacteria intracellularly and extracellularly during vegetative growth. In the laboratory *D. discoideum* is provided a single species of "food" bacteria such as *K. pneumonia*, but in the wild amoebae must be able to deal with thousands of species of bacteria (Curtis et al. 2002). How the amoebae cope with bacterial diversity may help to inform antibacterial strategies in other eukaryotes, including humans (Bozzaro and Eichinger 2011; Eichinger 2012).

Transcriptional profiling of *D. discoideum* exposed to different species of bacteria suggests that the amoebae mount specific physiological responses to different bacteria (Farbrother et al. 2006; Carilla-Latorre et al. 2008; Sillo et al. 2008, 2011; Nasser et al. 2013). In response to *K. pneumonia* amoebae upregulate the transcription of 780 genes or about 8% of the genome, and the activation of some of those promoters appears to be a highly specific response to a specific bacterium or group of bacteria such as the Enterobacteriaceae (Nasser et al. 2013; Snyder 2013). A number of genes have been identified that are required for survival or growth on one or another specific species of bacteria (Benghezal et al. 2006; Nasser et al. 2013). For example, the adhesion protein gp130 is required for growth on Gram-positive bacteria but not for growth on Gram-negative bacteria, suggesting that it may serve as a receptor for the efficient phagocytosis and killing of Gram-positive bacteria (Chia 1996; Chia et al. 2005; Nasser et al. 2013). The TirA protein is a Toll/interleukin-1 receptor (TIR) domain- and leucine rich repeat (LRR) domain-containing

regulator that is required for robust responses to Gram-negative bacteria (Chen et al. 2007; Walk et al. 2011). Interestingly, loss of TirA also renders amoebae susceptible to infection and killing by a strain of *L. pneumophila* (*icmT*$^{-}$) that is otherwise avirulent (Chen et al. 2007). TirA appears to be part of a cytoplasmic complex that functions to detect the presence of Gram-negative bacteria (Liu 2013). One of the subunits of the TirA complex, EpdR (exoplysaccharide depolymerase-related protein), is a full-length homolog of bacterial exopolysaccharide depolymerases, which are known to bind lipopolysaccharide (LPS) *o*-antigen (Steinbacher et al. 1994; Liu et al. 2018). *EpdR* mutants display compromised growth on Gram-negative bacteria similar to the *TirA* mutant phenotype. Thus, both subunits of the TirA complex identified to date are required for robust growth on Gram-negative bacteria. Interestingly, overexpression of EpdR in a *TirA* mutant results in the partial restoration of growth on Gram-negative bacteria, suggesting that EpdR may act as a downstream effector in the amoebal response to bacteria. EpdR homologs are ubiquitous in bacterial species but are not found in eukaryotes, suggesting that EpdR is a lateral gene transfer from bacteria (Liu et al. 2018). Although the picture is just emerging, current information suggests that *D. discoideum* mounts specific responses to bacteria through specific signaling pathways.

During development, the innate immune function of *D. discoideum* is mediated in part by cells that have functional similarities to mammalian neutrophils. Differentiating amoebae lose the ability to phagocytose bacteria soon after aggregation as they commit to multicellular development (Katoh et al. 2007). As the aggregate forms a migrating slug midway through development, about 1% of the cells differentiate into specialized Sentinel (S) cells (Chen et al. 2007). S cells are able to accumulate xenobiotics within cytoplasmic vacuoles and this has been proposed to provide a detoxifying function for the organism since they are sloughed off as the slugs migrate and are left behind in the slime trail. Perhaps most intriguing is the fact that S cells are highly active phagocytes that are capable of removing bacteria from the interstitial spaces in the slug. Thus, S cells are thought to function as a simple innate immune system to protect the prespore cells that will give rise to spores that will become the next generation of amoebae (Chen et al. 2007; Zhang and Soldati 2016).

S cells form extracellular DNA nets that appear analogous to the ETs produced by neutrophils and eosinophils that are known to trap and kill bacteria (Brinkmann et al. 2004; Goldmann and Medina 2012). S cells purified from slugs elaborate DNA-containing nets upon stimulation with bacterial LPS that are comprised of mitochondrial DNA and protein (Zhang et al. 2016). Observations of populations of purified S cells suggest that each cell can produce one ET without losing viability and without any contact with other cells, indicating that this is a cell-autonomous property. Bacteria bind to the amoebal ETs and appear to be actively killed, similar to what is seen with mammalian ETs. TirA, which is part of an LPS response pathway in vegetative amoebae, is also required for LPS-stimulated ET formation by S cells during development. Reactive oxygen species (ROS) produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase triggers ET production in mammals and this was tested in amoebae to address the issue of homology of ETs

in the unikont lineage (Brinkmann and Zychlinsky 2007; Bianchi et al. 2009; Saitoh et al. 2012). There are three homologs of human NADPH oxidase 2 in *D. discoideum* that produce much of the measurable ROS in developing cells, NoxA, NoxB and NoxC, and S cells from a NoxABC triple knockout strain are severely compromised in ET production (Zhang and Soldati 2013; Zhang et al. 2013, 2016). Both the *TirA* and *NoxABC* mutants fail to clear bacteria from aggregates during development and produce fruiting bodies with live bacteria in their sori (Zhang et al. 2016). These findings suggest that social amoebae and animals share a fundamental cellular function of innate immunity; both S cells and neutrophils produce ETs upon stimulation by LPS that is transduced by TIR domain-based signaling and which is triggered by ROS. The cellular and biochemical similarities between amoeba and animals in the biogenesis of ETs and the mechanism of their exocytosis are yet to be explored in detail, but the documented congruence suggests that the homology of amoeba and animal innate immune cell mechanisms may be more extensive than previously recognized (Zhang and Soldati 2016).

The Microbiome of the Social Amoeba

D. discoideum amoebae lose the ability to feed on bacteria soon after depletion of the food source and well before the amoebae integrate into a multicellular organism. The most obvious indication of this is loss of phagocytic activity as they begin to aggregate into the mound and a "commitment" to development: following 6–8 h of starvation, cells are unable to respond to the addition of a bacterial food source and instead continue to proceed through multicellular development (Katoh et al. 2007). If development is disrupted after this time and the amoebae are disaggregated in the presence of food bacteria, there is a required period of dedifferentiation before the amoebae are capable of returning to vegetative growth (Katoh et al. 2004). The canonical thinking until recently has been that vegetative amoebae consume all bacteria prior to initiating development, that there are no bacteria in the immediate environment when development is initiated, and that a sterile fruiting body is always produced (Raper 1937). Thus, the biphasic life cycle of unicellular growth and multicellular development of the social amoebae appeared to separate the killing of bacteria for nutritional purposes from the killing of bacteria as a defense function. The picture became more complex when it was discovered that some *D. discoideum* strains initiate development before exhausting the bacterial food supply and selectively retain some bacteria throughout the developmental phase. These strains were called "farmers" because the first function to be ascribed to the carriage of bacteria during development was in providing the population a selective advantage in bacteria-poor environments as the bacteria can seed a new food supply for germinating spores as they establish a fresh colony of vegetative amoebae (Brock et al. 2011). As several functions of the bacterial associates, beyond farming, have been uncovered, the mechanistically neutral terms of "carrier" and bacterial carriage has been adopted as an appropriate description of the phenomena.

About one-third of *D. discoideum* strains isolated from the wild have established symbiotic interactions with the bacteria and carry them stably through cycles of growth and development (Brock et al. 2011). Quite complex interactions between social amoebae and bacteria have been documented. For example, carriers can also harbor inedible bacteria that provide a defense function to the amoebae during development (Stallforth et al. 2013; Brock et al. 2013). Closely related species of *Burkholderia* bacteria are commonly isolated from *D. discoideum* and some species can induce the bacterial carriage phenotype in non-carriers (DiSalvo et al. 2015). This "infectious" induction of bacterial carriage also allows additional species of bacteria to be carried within the same organism and within the amoebal population. The induction of bacterial carriage by *Burkholderia* suggests that amoebal physiology is co-opted into an abnormal state, but the process also appears to be regulated by amoebal determinants as described in this section.

Carriers can be distinguished from non-carriers in several ways. Portions of the edges of plaques formed by amoebae as they feed on lawns of *K. pneumoniae* bacteria reveal stark differences in the way carriers and non-carriers feed on bacteria. The extracellular killing of bacteria within plaques can be visualized with fluorescent dyes that distinguish live bacteria from dead bacteria. Amoebal staining is negligible under these conditions due to robust ATP-binding cassette (ABC) transporters in *D. discoideum* that act as efficient export pumps (Anjard and Loomis 2002). *D. discoideum* strains that do not carry bacteria into development (car$^-$), including all laboratory strains, have a mixture of live and dead bacteria at the edge but within the perimeter of the plaques (Dinh et al. 2018). This is easily confirmed by fluorescence microscopy of samples taken from the plaques and can be seen as orange staining, which is a mixture of the red and green fluorescent signal in the image overlay (Fig. 3, upper panels). Plaques made by carrier amoebae feature patches of mostly live or mostly dead bacteria at the edges (Fig. 3, lower panels). This is likely to be an important difference between carriers and non-carriers since carriers spare bacteria as they transition from growth to development and the mechanism of how this is accomplished is not clear. The plaque structure of carriers strongly suggests that populations of carrier amoebae are able to regulate how they kill and feed on bacteria. How the microenvironments are established that allow live bacteria to survive within the borders of carrier plaques while the amoeba initiate developmental aggregation is an area of active investigation. During development, carrier slugs slough off viable bacteria into the slime trail and they form colonies visible to the naked eye (Fig. 4a). When sori of non-carrier fruiting bodies are cultured on standard bacteriological media at 37 °C viable bacteria are only rarely apparent and the slugs are essentially sterile (Fig. 4b).

Carrier strains can have thousands of interstitial bacteria within migrating slugs and fruiting body sori, as well as bacteria that survive as endosymbionts within developing amoebae and spores (Brock et al. 2011; DiSalvo et al. 2015). The ability to carry bacteria while maintaining an innate immune system suggests that social amoeba regulate their microbiome, as observed in other multicellular organisms. Is there differential response, or tolerance, for some species of bacteria? Do some bacteria suppress the innate immune response of the amoebae, as observed in

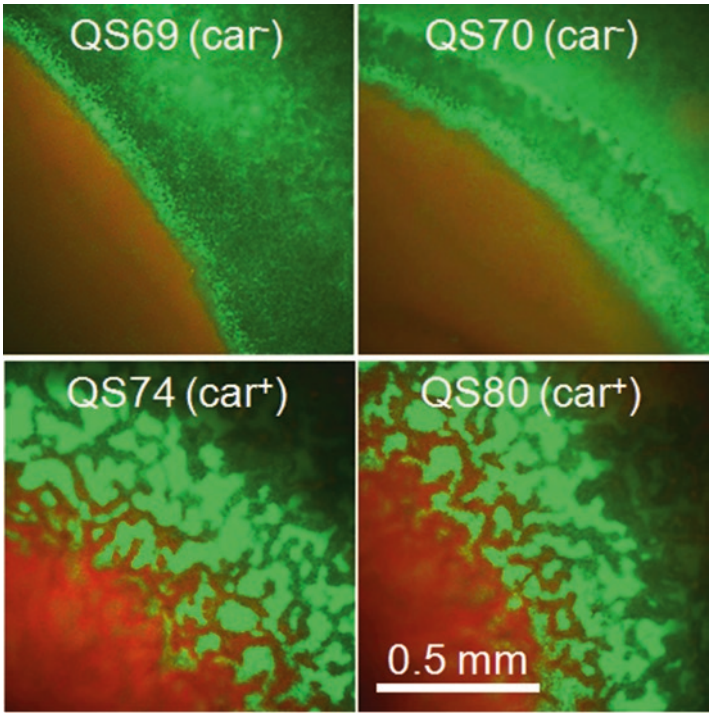

Fig. 3 Extracellular killing of bacteria by social amoebae. Portions of the edges of plaques formed by amoebae feeding on lawns of *Klebsiella pneumoniae* bacteria are shown. The extracellular killing of bacteria within the plaques is made visible with fluorescent dyes that distinguish live (green) from dead (red) bacteria and do not stain amoebae. Plaques of two *Dictyostelium discoideum* strains that do not carry bacteria into development (car^-) and two strains that do carry bacteria (car^+) are shown. A mixture of live and dead bacteria are found at the edge of the plaques of non-carrier amoebae (top panels), as indicated by the orange (red and green) fluorescence, while patches of mostly live or mostly dead bacteria are seen at the edges of plaques from carrier amoebae (lower panels). How the microenvironments are established that allow live bacteria to survive within the borders of carrier plaques while the amoeba initiate developmental aggregation is not known

mammals? Carrier strains have a lower percentage of S cells in their slugs than non-carriers, so a reduced capacity of the carriers' innate immune system might explain bacterial retention during development (Brock et al. 2016). It is also possible that S cells are only able to remove the interstitial bacteria between the amoebae, while the endosymbiotic bacteria that are within amoebae are inaccessible.

The proteins secreted by *D. discoideum* amoebae during growth and in the transition to development are significant mediators of the kill versus carry dynamics with bacteria. Over 300 proteins are secreted by laboratory strains, but the secreted proteome (secretome) of wild isolates appears to be variable and may be regulated by exposure to different bacteria (Bakthavatsalam and Gomer 2010; Nasser et al. 2013; Dinh et al. 2018). The secretome includes novel antibacterial proteins such as DabA

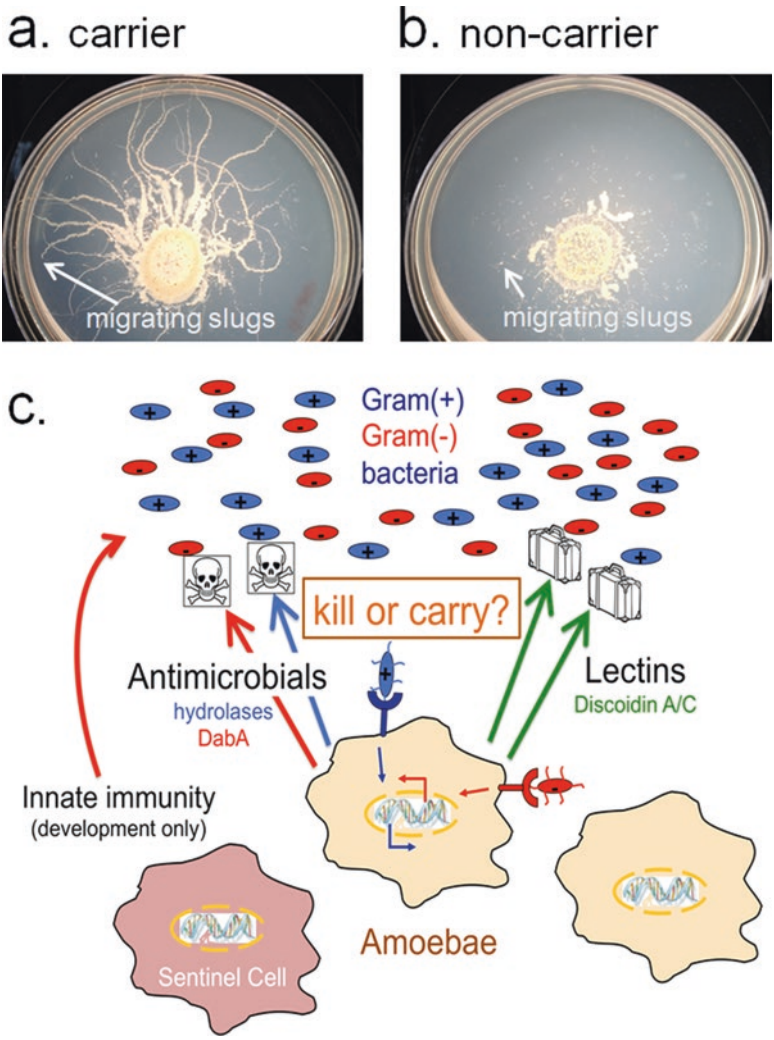

Fig. 4 Microbiome of the social amoebae. Amoebae of a *Dictyostelium discoideum* carrier strain (**a**) and a non-carrier strain (**b**) were spotted in the center of an agar plate and allowed to undergo multicellular development. Bacterial carriage is evident when carrier slugs migrate away from the original spot and slough off bacteria, leaving a trail of bacteria that form colonies over time (**a**), whereas non-carrier slug trails are devoid of bacteria (**b**). (**c**) Carrier strains secrete antimicrobials that kill bacteria and lectins that bind and protect bacteria, allowing some bacteria to survive amoebal feeding by becoming endosymbionts within them and ending up inside spores at the end of development. Sentinel cells differentiate during development and act as innate immune cells that can track and engulf interstitial bacteria in the migrating slug

and three classes of lectins that bind bacteria (Dinh et al. 2018). Carbohydrate-binding lectins are key mediators of the interactions between bacteria and eukaryotic cells. There are many classes of lectins with myriad polysaccharide binding specificities that, upon binding the surface of bacteria, can stimulate phagocytic uptake, modify immune responses, and help to maintain microbiome homeostasis (Ofek and Sharon 1988; Grubhoffer et al. 1997; Gust et al. 2012; Pang et al. 2016). Bacterial carriage by *D. discoideum* appears to be mediated by the lectin discoidin I, which is secreted at 100-fold higher levels by carriers than non-carriers during the growth to development transition (Dinh et al. 2018). About 2.4 million discoidin I trimers bind to the surface of *K. pneumoniae* and protect the bacteria from killing by *D. discoideum* antibacterial proteins such as DabA. A single treatment of *K. pneumoniae* with discoidin is enough to induce bacterial carriage during development. Discoidin-coated bacteria are taken up by phagocytosis but they evade phagolysosomal degradation and become endosymbionts in the developing amoebae (Dinh et al. 2018). Thus, lectin binding alters the cellular response of *D. discoideum* to bacteria in a fundamental way, allowing the establishment of a microbiome during development.

Discoidin I is a homo-trimer with H-type lectin carbohydrate binding domains on the same end of the trimer, which bind galctosamine-containing polysaccharides, and N-terminal discoidin domains on the other side that are structural similar to F-type lectins predicted to bind N- or O-linked glycans found on eukaryotic cell surfaces (Mathieu et al. 2010). It is known that the H-type lectin domains bind bacteria (Cooper et al. 1986) and it is possible that the discoidin domains are free to interact with N- or O-linked glycans on the cell surface of *D. discoideum* amoebae. A receptor has been identified in support of this inference from the discoidin I structure, but specific binding was only experimentally observable by blocking the H-type lectin binding sites on discoidin (Gabius et al. 1985).

Lectinophagocytosis is a common mechanism of bacterial uptake, with the lectins being provided by the bacteria or by the engulfing cell, and it results in efficient uptake through specific receptors (Ofek and Sharon 1988; Le Bouguenec 2005; Athamna et al. 1991). Lectinophagocytosis allows mammals to identify bacteria in the absence of opsinization and results in the killing of the bacteria (Stowell et al. 2014). Though lectins have mainly been characterized for their function in targeting bacteria for killing by immune systems, there have been reports of additional functions in interactions between bacteria and unikonts. For example, lectins in the mosquito gut appear to modulate microbiome homeostasis, but the mechanism has yet to be uncovered (Pang et al. 2016). In *D. discoideum* a broad genetic screen indicated that the *iliE* gene is required for growth on lawns of Gram-positive bacteria but not for growth on Gram-negative bacteria (Nasser et al. 2013). The *iliE* gene is predicted to produce a secreted homolog of the lectin concanavalin A (Nasser et al. 2013). Concanavalin A binds *B. subtilis*, so it is worth considering that *IliE* mediates interactions between *D. discoideum* and Gram-positive bacteria (Doyle and Birdsell 1972). If the biochemical and genetic inferences regarding discoidin and *IliE* are supported by future experiments, it would indicate that secreted lectins are used by *D. discoideum* to establish and regulate its microbiome. The initial studies

of the mosquito microbiome suggest that the use of lectins to modulate resident bacterial populations might be a general strategy.

Summary

The ability of a cell to recognize self from non-self is a universal capacity amongst the Metazoa and the process is mediated by MHC proteins in vertebrates that are highly polymorphic in natural populations (Hughes and Nei 1988). It is believed that MHC polymorphism is selectively advantageous because it imbues heterozygotes with improved resistance to invasion by parasites (Penn et al. 2002). Invertebrates protect themselves from parasite invasion by various mechanisms, some involving molecules that have the properties of MHC proteins—membrane proteins with extracellular Ig-domain repeats that exhibit a high degree of polymorphism (De Tomaso et al. 2005). Studies of TgrB1 and TgrC1 have shown that this class of proteins is present in extant species of early diverging unikont lineages and currently have roles in social amoeba allorecognition. Since two of the three unikont lineages use analogous mechanisms, it is possible that the last universal unikont ancestor employed MHC-like proteins for allorecognition. Whether this ancestor was unicellular, colonial, or conditionally multicellular like Dictyostelium, there were likely parasitic species capable of exploiting any cooperative behaviors of the cells and allorecognition would be a powerful way to thwart the threat.

Figure 4c summarizes the innate immune response of *D. discoideum* in growth and development. During growth amoebae secrete hydrolases and antibacterial proteins that kill bacteria prior to phagocytic engulfment. It is likely that this improves the efficiency of feeding by augmenting intracellular killing capacity of amoebae. During development, specialized S cells patrol the multicellular slug and kill invading bacteria that may be pathogenic and threaten spore production. The existence of S cells indicates that they provide a selective advantage for maintaining the long-term survival of social amoebae in complex soil environments. The overall balance between bacterial killing and bacterial carriage appears to be regulated in part by secreted lectins that allow bacteria to escape both the soluble and cellular innate immune functions. The extensive similarities between mammalian and amoebal innate immunity leads to the intriguing hypothesis that mechanisms in extant immune systems derive from ancient bacterial foraging strategies of the LECA (likely an amoeba!). Some of the evidence for or against this idea will come as we learn more about the mechanism of ET production in animals and amoeba.

Allorecognition and innate immunity in the social amoebae has been studied for a little over a decade and there are many open questions. What downstream signaling events mediate TgrB1/TgrC1 allorecognition? What are the molecular details of how bound lectins protect bacteria from killing by antibacterial proteins or by amoebae? Do lectin coatings render bacteria less pathogenic to amoebae? What regulation governs the balance between bacteria–amoebae mutualism versus bacterial killing by amoebae? Do lectins impose selectivity on the retention of bacteria during formation of the amoebal microbiome? What are the genetic and physiologic

properties of amoebal carriers that promote mutualism with bacteria? The last 10 years of study of the social amoeba have revealed innate immune cells, allorecognition, and a regulated microbiome, and I expect many more surprises to come.

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Cnidaria: Anthozoans in the Hot Seat

Caroline V. Palmer and Nikki G. Traylor-Knowles

Introduction

Cnidaria is a diverse phylum, representing animals of dramatically different morphologies, life histories, and ecological functions but united by the presence of a specialized cell type—the cnidocyte. Cnidocytes secrete organelle-like capsules with eversible microtubules called cnidae (Daly et al. 2007). Anthozoa is the most speciose class of the phylum Cnidaria, with an estimated 7500 extant species (Daly et al. 2007), including the subclasses Hexacorallia and Octocorallia, which comprise stony corals and anemones, and soft corals and gorgonians, respectively (Won et al. 2001) (Fig. 1). Anthozoans are phylogenetically basal, both within the Metazoa as a whole and arguably within Cnidaria (Kayal et al. 2013), with Scleractinia (stony corals) appearing in the mid-Triassic (c. 250 million years ago [MYA]) (Romano and Palumbi 1996), possibly having evolved from anemones.

The first dinoflagellates, single-celled eukaryotes—"protists"—also purportedly appeared during the Triassic (Fensome 1993; Stanley 2006), and eventually, after a series of extinctions, formed an obligate endosymbiosis with a wide range of multicellular organisms, including anthozoans (Stanley 2006; Aranda et al. 2016). The majority of extant anthozoans live in this obligate endosymbiosis with members of the genus *Symbiodinium* (Aranda et al. 2016)—a relationship that underpins the ecological success of the class. In this intimate association, the *Symbiodinium* can provide over 90% of the energy requirements of the anthozoan (Muscatine and Porter 1977) and, in the case of hard coral, facilitate exoskeleton calcification, enabling the formation of tropical reef ecosystems (Fig. 2). In return, and in lieu of

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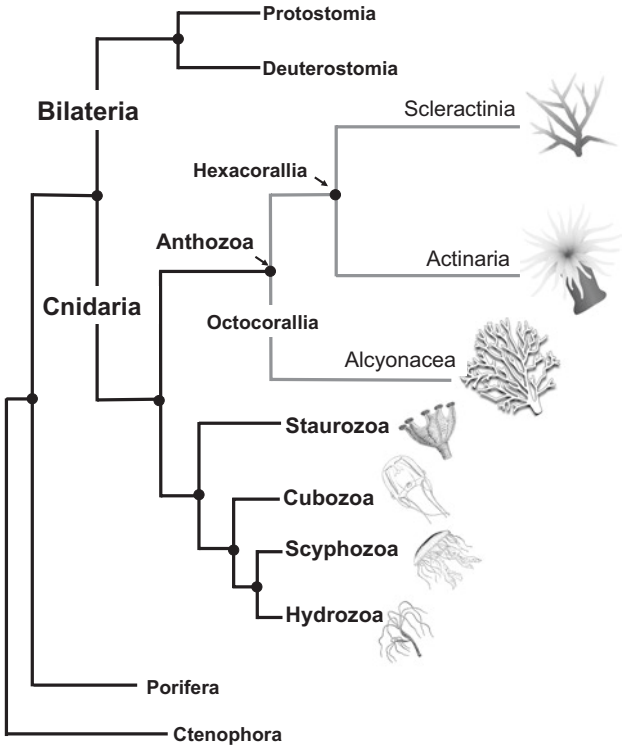

Fig. 1 Phylogenetic tree showing the position of Cnidaria

Fig. 2 A diverse and colorful Indo-Pacific coral reef. (Photo credit: Giles Winstanley)

living in typically nutrient-poor waters, the anthozoan host protects its algal partner and uses it to recycle waste carbon and nitrogen (Jeong et al. 2012). Under stress conditions, such as increased water temperature or infection, this obligate endosymbiosis can break down, turning the coral white as the cHL: Intelectin-1orophyll-pigmented *Symbiodinium* leave or die, revealing the coral skeleton through the translucent host tissue. The "bleached" anthozoan (Fig. 3) host is then susceptible to starvation, disease, and death (Fig. 4). This scleractinian coral–algal association is the best studied of the relationships anthozoans have with microbiota. It is

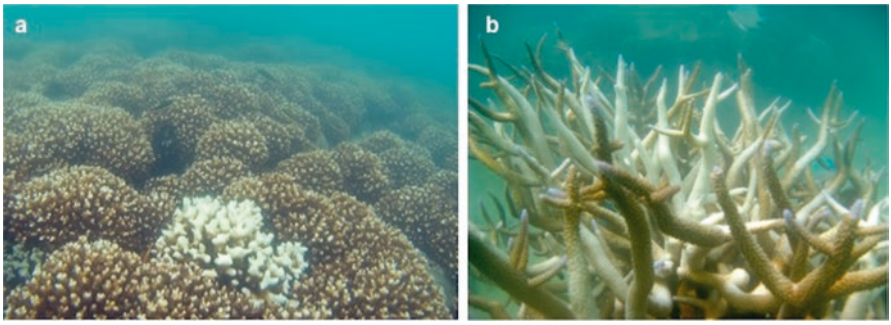

Fig. 3 Bleached scleractinian coral: (**a**) *Pocillopora* sp., Bahia Tomas, Costa Rica; and (**b**) *Acropora* sp., Orpheus Island, Great Barrier Reef, Australia. (Photo credit: C.V. Palmer)

Fig. 4 Bleached and diseased *Acropora millepora* (scleractinian coral), Orpheus Island, Great Barrier Reef, Australia. (Photo credit: C.V. Palmer)

becoming increasingly apparent that the specific, variable, and diverse microbiome associated with anthozoans is crucial to their health and likely modulated, in part, by coral immune mechanisms (Bourne et al. 2016). Deciphering the immunological intricacies of coral–microbe symbioses is an ecologically important field of research and will likely provide insight into the establishment and functioning of symbioses throughout the animal kingdom. This is particularly so as, despite their phylogenetic position (Fig. 1) and apparent morphological simplicity, anthozoans are immunologically complex (Miller et al. 2007; Shinzato et al. 2011b), with large genomes and gene families that are comparable with those of the Bilateria (Augustin and Bosch 2010). Unlike many bilaterians, however, anthozoans have evaded gene loss (Miller et al. 2007), making them an interesting group for studying the evolution of immunity as well as mutualisms.

The immune system is a highly integrated suite of mechanisms and processes that enable organisms to resist infection and maintain tissue integrity to promote survival (Medzhitov 2008; Cooper 2010). Like all organisms, anthozoans possess innate immune mechanisms (Palmer and Traylor-Knowles 2012), but as invertebrates they lack the more complex adaptive arm of immunity. Innate immunity provides a non-specific and immediate response to perceived endogenous and exogenous threats in a bid to re-establish homeostasis (Beutler 2004; Medzhitov 2008). Concomitantly, anthozoans use a diverse repertoire of immune receptors (Miller et al. 2007) (Table 1), signaling pathways (Wolenski et al. 2011), and effector and "stress" responses (Palmer et al. 2008), which eliminate pathogens, seal wounds, mitigate self-harm, and defend self by maintaining homeostasis (Palmer and Traylor-Knowles 2012; Mydlarz et al. 2016).

Shifts in environmental conditions, driven by climate change and local anthropogenic disturbances, are threatening the long-term survival of many species and systems, and coral reefs are among the most threatened (Hughes et al. 2017). Unfavorable environmental conditions are negatively affecting the health of coral reefs; the ancient, co-evolved symbiotic relationship that is so important to coral health is being pushed beyond its limit, resulting in mass bleaching and die-off events (Hughes et al. 2017). Longer-lived organisms, such as scleractinian corals, are particularly vulnerable to the anthropogenically increased rates of climate change, which exceed the time needed for a population to adapt through natural selection (van Oppen et al. 2017). To conserve coral reefs through this high rate of change, the potential of genetically engineered "super corals" that can withstand environmental change, is being explored (van Oppen et al. 2017). It is increasingly apparent that immunity, as the basis of the maintenance and reestablishment of health, needs to be at the forefront of coral reef health and disease research. Anthozoan immunology offers hope that we will better understand the drivers behind coral health in order to more effectively conserve and restore the reefs systems that are of high ecological and societal value.

Table 1 Summary of gene, protein, and signaling pathways involved in cnidarian immunity and stress response

Gene, protein, or pathway	Immune factor	Experiment	Organism	Citation
Toll-like receptor pathway	TLRs	Genome analysis	*Acropora digitifera*	Shinzato et al. (2011)
	TLRs	EST Database	*Acropora millepora*	Miller et al. (2007)
	TLR, TIR domain-only	Phylogenetic analysis	*Acropora digitifera* *Acropora millepora* *Fungia scutaria* *Montastrea cavernosa* *Pocillopora damicornis* *Seriatopora hystrix* *Exaiptasia pallida* *Anthopleura elegantissima*	Poole et al. (2014)
	TLR2, TRAF3	White band disease transcriptome	*Acropora cervicornis*	Libro et al. (2013)
	TLRs	Bleaching and disease transcriptome	*Orbicella faveolata*	Anderson et al. (2016)
	TLRs	Stress transcriptome	*Pocillopora damicornis*	Traylor-Knowles et al. (2011)
	TLRs, TOLLIP	Larvae 454 transcriptome	*Acropora millepora*	Meyer et al. (2009)
	TLRs	Reciprocal transplant + heat stress transcriptome	*Acropora hyacinthus*	Seneca et al. (2015)
	TLR	Bacteria challenge transcriptome	*Pseudodiploria strigosa*	Ocampo et al. (2015)
	TLR2	EST Database	*Acropora millepora* *Acropora palmata*	Voolstra et al. (2011)
	TIR-1	qPCR, physical wounding with infection of bacteria	*Acropora aspera*	van De Water et al. (2015c)
	TLR	Injury-induced head regeneration transcriptome	*Hydra* sp.	Wenger et al. (2014)
	TLR	Transcriptome analysis	*Anthopleura buddemeieri* *Aulactinia veratra* *Calliactis polypus* *Telmatactis* sp. *Nemanthus annamensis*	Van der Burg et al. (2016)

(continued)

Table 1 (continued)

Gene, protein, or pathway	Immune factor	Experiment	Organism	Citation
	TLR	Phylogenetic analysis	*Nematostella vectensis* *Hydra* sp. *Acropora millepora*	Hemmrich et al. (2007)
	TIR domain	Bioinformatic analysis	*Nematostella vectensis*	Reitzel et al. (2008)
	TIR domain only	Genome analysis	*Exaiptasia pallida*	Baumgarten et al. (2015)
Lectin pathway	Millectin	Bacteria exposure; phylogenetics analysis and binding assays	*Acropora millepora*	Kvennefors et al. (2008)
	Millectin	LPS and peptidoglycan immune challenge, measured with qPCR, immunohistochemistry	*Acropora millepora*	Kvennefors et al. (2010)
	Lectins	Algal–coral cell interactions, flow cytometry	*Fungia scutaria*	Wood-Charlson et al. (2006)
	Tachylectin	EST Database	*Acropora palmata* *Montastraea faveolata*	Schwarz et al. (2008)
	Tachylectin-2	Phylogenetic analysis	*Oculina patagonica* *Montastraea faveolata* *Acropora millepora*	Hayes et al. (2010)
	PdC: C-lectin	Suppression subtractive hybridization	*Pocillopora damicornis*	Vidal-Dupiol et al. (2009)
	Rhamnose-binding lectin	Gene expression measured with qPCR, bacteria, and symbiont binding assays	*Pocillopora damicornis*	Zhou et al. (2017)
	C-type mannose receptor 2, C-type lectin domain family 10 member A	White band disease stress transcriptome	*Acropora cervicornis*	Libro et al. (2013)
	C-type lectin	White plague disease transcriptome	*Orbicella faveolata*	Daniels et al. (2015)
	HL: Intelectin-1-1	Anthropogenic stress transcriptome	*Acropora millepora*	van de Water et al. (2015a, b, c)
	C-type lectin	Reciprocal transplant + heat stress transcriptome	*Acropora hyacinthus*	Seneca et al. (2015)

	CEL-III type lectins	qPCR, early larval settlement and development	*Acropora millepora*	Puill-Stephan et al. (2012)
	C-type lectins, fucolectins, D-galactoside/L rhamnose-binding lectins, galectins, tachylectins	Bacteria challenge transcriptome	*Pseudodiploria strigosa*	Ocampo et al. (2015)
	Tachylectin precursor	Thermal stress microarray	*Montastrea faveolata*	DeSalvo et al. (2008)
	PdC: Pocillopora damicornis C-lectin	qPCR, bacteria challenge	*Pocillopora damicornis*	Vidal-Dupiol et al. (2011a)
	Tachylectin-5A	Response to *Aplanochytrium* spp. parasite transcriptome	*Gorgonia ventalina*	Burge et al. (2013)
	C-type lectin	qPCR, bacterial challenge	*Acropora millepora*	Brown et al. (2013)
	Mannose phospholipase lectin receptor related, C-type lectin superfamily	Injury-induced head regeneration transcriptome	*Hydra* sp.	Wenger et al. (2014)
	Calreticulin, C-type lectin domain family 4 member F, Ficolin 1, Ficolin 2, Fucolectin 1, Fucolectin 4	EST Database	*Exaiptasia pallida*	Sunagawa et al. (2009)
	C-type lectin domain	Bioinformatic analysis	*Nematostella vectensis*	Reitzel et al. (2008)
	C-type lectin domains	Bioinformatic analysis	*Nematostella vectensis*	Wood-Charlson and Weis (2009)
	C-type lectin	Heat stress, larvae, microarray	*Acropora millepora*	Rodriguez-Lanetty et al. (2009)
NOD-like receptor pathway	nucleotide-binding domain and leucine-rich repeat containing gene family s	Genome analysis	*Acropora digitifera*	Shinzato et al. (2011a, b)
	nucleotide-binding domain and leucine-rich repeat containing gene family s	Phylogenetic analysis	*Acropora digitifera*	Hamada et al. (2013)
	NLCR: Nod-like receptor C5	White band disease transcriptome	*Acropora cervicornis*	Libro and Vollmer (2016)
	NACHT-domain containing	Bacterial challenge transcriptome	*Pseudodiploria strigosa*	Ocampo et al. (2015)
	nucleotide-binding domain and leucine-rich repeat containing gene family	Transcriptome analysis	*Anthopleura buddemeieri* *Aulactinia veratra* *Calliactis polypus* *Telmatactis* sp. *Nemanthus annamensis*	Van der Burg et al. (2016)
	nucleotide-binding domain and leucine-rich repeat containing gene family, TRIP-6	Genome analysis	*Exaiptasia pallida*	Baumgarten et al. (2015)

(continued)

Table 1 (continued)

Gene, protein, or pathway	Immune factor	Experiment	Organism	Citation
Complement pathway	C3	Genome analysis	*Acropora digitifera*	Shinzato et al. (2011a, b)
	C3	EST Database	*Acropora millepora* *Nematostella vectensis*	Miller et al. (2007)
	Bf, C3	Anthropogenic stress transcriptome	*Acropora millepora*	van de Water et al. (2015a, b, c)
	MASP1	Bleaching and bleaching recovery transcriptome	*Orbicella faveolata*	Pinzon et al. (2015)
	MPEG-1	White band disease transcriptome	*Acropora cervicornis*	Libro et al. (2013)
	C3	qPCR, bacterial challenge	*Acropora millepora*	Kvennefors et al. (2010)
	lncRNA	Bleaching vs. healthy transcriptome	*Protolpalythoa varibilis* *Palythoa caribaeorum*	Huang et al. (2017)
	C3	qPCR, early larval settlement and development	*Acropora millepora*	Puill-Stephan et al. (2012)
	C3, Bf, MASP, A2M, CD109	Bacteria challenge transcriptome	*Pseudodiploria strigosa*	Ocampo et al. (2015)
	C3	Cloning of C3 using RT-PCR	*Swiftia exserta*	Dishaw et al. (2005)
	C3, Bf	qPCR, physical wounding with infection of bacteria	*Acropora aspera*	van De Water et al. (2015a, b, c)
	Complement component-related sushi domain containing, C1q related, complement component 1, MASP	Injury-induced head regeneration transcriptome	*Hydra* sp.	Wenger et al. (2014)
	C2, C3, C5, C8	EST Database	*Exaiptasia pallida*	Sunagawa et al. (2009)
	Bf, MASP, C3	Phylogenetics, qPCR, bacteria challenge	*Exaiptasia pallida*	Poole et al. (2016)
	C3	Microarray analysis	*Fungia scutaria*	Schnitzler and Weis (2010)
	C3	Chronic heat stress study	*Porites astreoides*	Kenkel et al. (2013)
Scavenger receptor pathway	SR-A, SR-E, SRCR only, SR-B	Phylogenetics and functional analysis	*Acropora digiterfera* *Acropora millepora* *Fungia scutaria* *Nematostella vectensis* *Anthopleura elegantissima* *Exaiptasia pallida*	Neubauer et al. (2016)
	SRCR	Bacteria challenge transcriptome	*Pseudodiploria strigosa*	Ocampo et al. (2015)
	SRCR	Longitudinal bisections transcriptome	*Calliactis polypus*	Stewart et al. (2017)

Interleukin receptor pathway	IL-R1-like	Phylogenetic study	*Acropora digitifera* *Acropora millepora* *Fungia scutaria* *Montastrea cavernosa* *Pocillopora damicornis* *Seriatopora hystrix* *Exaiptasia pallida* *Anthopleura elegantissima*	Poole et al. (2014)
	IL-IR1, IL-IR2, IL-IR3	EST Database	*Acropora millepora*	Miller et al. (2007)
	IL-R1	Transcriptome of longitudinal bisections	*Calliactis polypus*	Stewart et al. (2017)
	IL-1R-like	Transcriptome analysis	*Anthopleura buddemeieri* *Aulactinia veratra* *Calliactis polypus* *Telmatactis* sp. *Nemanthus annamensis*	Van der Burg et al. (2016)
	IL-1R	Phylogenetic analysis	*Nematostella* *Hydra* *Acropora millepora*	Hemmrich et al. (2007)
	ILR1, ILR2, ILR3, ILR4	Genome analysis	*Exaiptasia pallida*	Baumgarten et al. (2015)
Tumor necrosis factor receptor pathway	TNFRSF: Tumor Necrosis Factor Receptor Super Family 1A	White band disease transcriptome	*Acropora cervicornis*	Libro et al. (2013)
	TRAF6	EST Database	*Acropora millepora*	Miller et al. (2007, b)
	TNFRSF: Tumor Necrosis Factor Receptor Super Family 16	White band disease transcriptome	*Acropora cervicornis*	Libro and Vollmer (2016)
	TRAF3	Reciprocal transplant + heat stress transcriptome	*Acropora hyacinthus*	Seneca et al. (2015)

(continued)

Table 1 (continued)

Gene, protein, or pathway	Immune factor	Experiment	Organism	Citation
	TRAF3, TNFRSF: Tumor Necrosis Factor Receptor Super Family 27, TNF, TNFRs, LITAF: Lipopolysaccharide-induced tumor necrosis factor-alpha factor	Heat stress transcriptome	*Acropora hyacinthus*	Barshis et al. (2013)
	TRAFs, TNFRs	Reciprocal transplant between different thermal regimens transcriptome	*Acropora hyacinthus*	Palumbi et al. (2014)
	Lymphotoxin-β receptor	LPS-exposed transcriptome	*Orbicella faveolata* *Pseudodiploria strigosa* *Porites* *Porites astreoides*	Fuess et al. (2017)
	TRAF6	qPCR, physical wounding with infection of bacteria	*Acropora aspera*	van De Water et al. (2015a, b, c)
	TNF-related, TNF family member, TNFRSF: Tumor Necrosis Factor Receptor Super Family , TRAFs	Injury-induced head regeneration transcriptome	*Hydra* sp.	Wenger et al. (2014)
	Caspase 3, Caspase 8, Caspase 10	EST Library	*Exaiptasia pallida*	Sunagawa et al. (2009)
	TNF	Bioinformatic analysis	*Nematostella vectensis*	Reitzel et al. (2008)
	Caspase 8, TRAF6	Genome analysis	*Exaiptasia pallida*	Baumgarten et al. (2015)
RIG-like receptor	DDX: DEAD-box helicases 60	White plague disease transcriptome	*Orbicella faveolata*	Daniels et al. (2015)
	Rig-like receptors	Bleaching and Disease transcriptome	*Orbicella faveolata*	Anderson et al. (2016)
	Rig-I/MDA: Melanoma Differentiation-Associated5 gene 1, Rig-I/MDA: Melanoma Differentiation-Associated5 gene 2	Bioinformatic analysis	*Nematostella vectensis*	Zou et al. (2009)

NF-κB pathway	TAK: TGF-beta activated kinase-1, TRAF-6, NF-κB	EST Database	*Acropora millepora*	Miller et al. (2007, b)
	MyD88	Phylogenetic study	*Acropora digitifera* *Acropora millepora* *Fungia scutaria* *Montastrea cavernosa* *Pocillopora damicornis* *Seriatopora hystrix* *Exaiptasia pallida* *Anthopleura elegantissima*	Poole et al. (2014)
	NF-κB	Larvae 454 transcriptome	*Acropora millepora*	Meyer et al. (2009)
	NF-κB pathway	LPS exposed vs. control transcriptome	*Orbicella faveolata* *Pseudodiploria strigosa* *Porites* *Porites astreoides*	Fuess et al. (2017)
	NF-κB pathway	Bacteria challenge transcriptome	*Pseudodiploria strigosa*	Ocampo et al. (2015)
	NF-κB	qPCR, physical wounding with infection of bacteria	*Acropora aspera*	van De Water et al. (2015a, b, c)
	MyD88	Knockdown of MyD88	*Hydra magnipapillata*	Franzenburg et al. (2012)
	NFAT5, NF-κB, I-κB, NF-κB activating protein	Injury-induced head regeneration transcriptome	*Hydra* sp.	Wenger et al. (2014)
	NF-κB	Functional analysis of development	*Nematostella vectensis*	Wolenski et al. (2013)
	NF-κB, IκB, Bcl-3, and IκB kinase	Cloned cDNA and expressed in cell culture	*Nematostella vectensis*	Wolenski et al. (2011)
	NF-κB	Characterization of Cys-Ser polymorphism	*Nematostella vectensis*	Sullivan et al. (2009)
	NF-κB, IκB, Bcl-3, NFAT: Nuclear factor of activated T-cells	Phylogenetic study	*Nematostella vectensis*	Sullivan et al. (2007)
	NF-κB inhibitor α	EST Library	*Exaiptasia pallida*	Sunagawa et al. (2009)
	MyD88, NF-κB	Transcriptome analysis	*Anthopleura buddemeieri* *Aulactinia veratra* *Calliactis polypus* *Telmatactis* sp. *Nemanthus annamensis*	Van der Burg et al. (2016)

(continued)

Table 1 (continued)

Gene, protein, or pathway	Immune factor	Experiment	Organism	Citation
	MyD88, IRAK: Interleukin-1 receptor-associated kinase, TRAF6, TAK: TGF-beta activated kinase1, IKK, NF-κB NF-κB, IKKα, TBK1, TRAF6, TRAF3	Phylogenetic analysis Genome analysis	*Nematostella* *Hydra* sp. *Acropora millepora* *Exaiptasia pallida*	Hemmrich et al. (2007) Baumgarten et al. (2015)
Integrins	Integrin-α	Proteomic and bioinformatics analysis of the skeletal organic matrix	*Stylophora pistillata* *Pocillopora damicornis* *Acropora digitifera* *Favia* sp.	Drake et al. (2013)
	Integrin βcn1, AmIntegrin-α1	Expression analysis of coral fertilization	*Acropora millepora*	Iguchi et al. (2011)
	AmItgα1, AmItgβ2	Phylogenetics and expression analysis of coral gastrulation	*Acropora millepora*	Knack et al. (2008)
	Integrin βcn1	cDNA isolation	*Acropora millepora*	Brower et al. (1997)
	Integrin β, Integrin-α8, Integrin-α4	Bioinformatic analysis	*Nematostella vectensis*	Reitzel et al. (2008)
	α-Integrin	Wound healing and regeneration microarray analysis	*Nematostella vectensis*	Dubuc et al. (2014)
TGF-β signaling	TGF-β	Bioinformatics, cellular analysis of TGF-β	*Exaiptasia pallida*	Detournay et al. (2012)
	TGF-β, TGF-β type I receptor	Bioinformatic analysis	*Nematostella vectensis*	Reitzel et al. (2008)
Heat shock proteins	HSP70	ELISA assay, lab experiment of tidal fluctuations	*Veretillum cynomorium*	Teixeira et al. (2013)
	HSP70	Lab temperature study	*Eunicella singularis*	Pey et al. (2011)
	HSP70	Lab heat shock study	*Corallium rubrum*	Haguenauer et al. (2013)
	HSP70, HSP90	Lab heat stress study	*Montastrea annularis*	Carpenter et al. (2010)
	HSP70	Red soil exposure	*Pocillopora damicornis*	Hashimoto et al. (2004)
	HSP16, HSP60	Lab heat stress study	*Porites astreoides*	Olsen et al. (2013)
	HSPs	Heat stress and embryogenesis, microarray	*Acropora palmata*	Portune et al. (2010)
	HSP70	Heat shock	*Goniopora djiboutiensis*	Sharp et al. (1997)
	HSP60	Heat stress and shock study	*Anemonia viridis*	Choresh et al. (2001)
	HSP60	Salinity stress study	*Seriatopora caliendrum*	Seveso et al. (2013)

	HSP60	Skeleton eroding band disease	*Acropora muricata*	Seveso et al. (2012)
	HSP90	Heat stress study	*Dendronephthya klunzingeri*	Wiens et al. (2000)
	HSPs	Heat and UV stress transcriptome	*Anthopleura elegantissima*	Richier et al. (2008)
	HSP70, HSP90	Heavy metal and oil dispersant exposure study	*Montastraea franksi*	Venn et al. (2009)
	HSP60, small HSPs	Cellular diagnostic for corals in their natural environment	*Montastraea annularis*	Downs et al. (2005)
	HSP70	Characterized protein	*Stylophora pistillata*	Tom et al. (1999)
	HSP70	Heat shock study	*Anemonia viridis*	Sharp et al. (1994)
	HSP90	Heat stress and bleaching microarray	*Montastraea faveolata*	Desalvo et al. (2008)
	HSP 93, HSP83, HSP70, HSP68, HSP45, HSP39	Heat stress study	*Aurelia* sp.	Black and Bloom (1984)
	HSP70	Protein assay of corals from naturally hot environment	*Porites lobata*	Barshis et al. (2010)
	HSP95, HSP90, HSP78, HSP74, HSP33, HSP28, HSP27, HSP82, HSP72, HSP68, HSP48	Heat stress study	*Montastraea faveolata* *Aiptasia pallida*	Black et al. (1995)
	HSP70	Heat stress transcriptome	*Acropora hyacinthus*	Traylor-Knowles et al. (2017b)
	HSP90, HSP97	Heat stress on embryos, microarray	*Montastraea faveolata*	Voolstra et al. (2009)
	HSP90, HSP70	EST Library	*Exaiptasia pallida*	Sunagawa et al. (2009)
	HSP70	Larvae transcriptome using 454 sequencing	*Acropora millepora*	Meyer et al. (2009)
	HSPs	Reciprocal transplant between different thermal regimens	*Acropora hyacinthus*	Palumbi et al. (2014)
	HSP60, HSP70, chIpsHSP: cHL: Intelectin-1oroplast small heat shock protein, sHSPS	Heat stress–no light and heat stress high UV	*Montastraea faveolata*	Downs et al. (2000)
	HSP70, HSP60, HSP35	Heat shock study	*Acropora grandis*	Fang et al. (1997)
	HSP60	Heat shock and light intensity shock	*Stylophora pistillata* *Turbinaria reniformis*	Chow et al. (2009)
	HSP90, HSP70, HSP20	Bioinformatic survey	*Nematostella vectensis*	Goldstone et al. (2008)
	HSP60	Field-sampled animals for different temperature regimens	*Anemonia viridis*	Choresh et al. (2001)

(continued)

Table 1 (continued)

Gene, protein, or pathway	Immune factor	Experiment	Organism	Citation
	HSP60, HSP70	Comparative study of HSP60 and HSP70	*Hydra vulgaris*	Bosch and Praetzel (1991)
	HSP70 sHSP	Petroleum contamination study	*Pocillopora damicornis*	Rougee et al. (2006)
	HSP90	Heat stress study	*Eunicea fusca*	Ross (2014)
	HPS70, HSP90, chaperone GP96: heat shock protein 90 kDa beta member 1	Heat stress, larvae, microarray	*Acropora millepora*	Rodriguez-Lanetty et al. (2009)
	HSPs	Heat stress transcriptome	*Acropora hyacinthus*	Barshis et al. (2013)
	HSP60	Heat stress treatment	*Hydra attenuate* *Hydra oligactis*	Bosch et al. (1988)
	HSP20, HSP70, HSP40	Acidification stress on larvae	*Acropora millepora*	Moya et al. (2015)
	HSF1, HSP70, HSP90	Acidification stress study	*Acropora digitifera*	Nakamura et al. (2012)
	HSP70	Heat and acidification on larvae	*Pocillopora damicornis*	Putnam et al. (2013)
	HSP112, HSP89, HSP74 HSP102, HSP98, HSP56, HSP25, HSP44, HSP38, HSP33	Heat and cold stress study	*Leptogorgia virgulata*	Kingsley et al. (2003)
	HSP90, HSP60, HSP16	Chronic heat stress study	*Porites astreoides*	Kenkel et al. (2013)
	HSP16	Heat light stress	*Porites*	Kenkel et al. (2011)
	HSP70	Bacteria challenge study, qPCR	*Acropora millepora*	Brown et al. (2013)
	HSP90, HSP70	Heat stress study, qPCR	*Acropora aspera*	Legget et al. (2011)
	HSP90	Heat stress study, larvae, microarray	*Montastraea faveolata*	Polato et al. (2010)

Bcl, B-cell lymphoma, Bf Factor B, *cDNA* complementary DNA, *chIpsHSP* chloroplast small heat shock protein, *Cys-Ser* Cysteine-Serine, *DDX* DEAD-box helicases, *ELISA* enzyme-linked immunosorbent assay, *GP96* heat shock protein 90 kDa beta member 1, *HL* Intelectin-1, *HSP* heat shock protein, *IKK* IκB kinase, *IL-R* interleukin receptor, *IRAK* Interleukin-1 receptor-associated kinase, *LITAF* Lipopolysaccharide-induced tumor necrosis factor-alpha factor, *lncRNA* long non-coding RNA, *LPS* lipopolysaccharides, *MASP* mannose-binding lectin-associated serine protease, *MDA* Melanoma Differentiation-Associated, *MyD88* myeloid differentiation primary response gene 88, *NFAT* Nuclear factor of activated T-cells, *NF-κB* nuclear factor-κB, *NLCR* Nod-like receptor C, *NLR* nucleotide-binding domain and leucine-rich repeat containing gene family, *NOD* nucleotide-binding oligomerization domain, *PdC Pocillopora damicornis* C, *qPCR* quantitative polymerase chain reaction, *RT-PCR* reverse transcription polymerase chain reaction, *sHSP* small heat shock protein, *TAK* TGF-beta activated kinase, *TBK* NF-Kappa-B-Activating Kinase, *TGF* transforming growth factor, *TIR* terminal inverted repeat, *TOLLIP* Toll interacting protein, *TLR* Toll-like receptor, *TNFR* tumor necrosis factor receptor, *TRAF* tumor necrosis factor receptor-associated factor, *TRIP* Thyroid Receptor Interacting Protein

Anthozoan Innate Immunity

Cnidarians use a diverse suite of characteristic innate immune mechanisms to maintain and re-establish homeostasis (Palmer and Traylor-Knowles 2012). Unlike the majority of animals, which possess either a protective exoskeleton (e.g., Arthropoda) or a thick epidermal tissue layer (e.g., mammals), the protective physical and biochemical layers of an anthozoan include only a single-cell host epithelium and surface mucus layer (SML). Once these protective barriers have been breached, and in the presence of a threat, an innate immune response occurs in the three broad immunity phases described across phyla: *recognition* of a threat, *signaling pathways* to activate appropriate response, and *effector responses* that eliminate the threat and mitigate self-harm (Hoffmann et al. 1999).

Anthozoans, like all invertebrates and higher organisms, use a suite of pattern recognition receptors (PRRs) and soluble proteins to recognize a broad array of conserved microorganism-associated molecular patterns MAMPs e.g., lipopolysaccharides (Loker et al. 2004; O'Neill et al. 2013) and host-derived damage-associated molecular patterns (Medzhitov and Janeway 2000a; Beutler 2004; Palmer and Traylor-Knowles 2012). In insects and other arthropods, the binding of MAMPs and/or DAMPs to PRRs, such as the Toll-like receptor (TLR) (Medzhitov and Janeway 2000b), activates serine protease cascades (Cerenius et al. 2010) and rapid-acting transcription factors, such as nuclear factor (NF)-κB. This leads to gene transcription and ultimately protein translation, which induces immune signaling pathways and appropriate effector responses (Medzhitov and Janeway 2000a). These receptors, signaling pathways, and downstream responses are being elucidated in anthozoans (see the summary in Table 1). Here we discuss progress in cnidarian immunology, with a focus on anthozoans, in relation to the broader field of invertebrate immunology.

The Mucosal Epithelia

SMLs evolved with the Cnidaria and are present in all multicellular phyla (Bythell and Wild 2011). Similar to the mucosal surfaces of the intestinal cell epithelia of the human gut (Artis 2008), the anthozoan SML overlays single-cell epithelia and is home to an array of commensal bacteria, distinct from the microbiota of the surrounding environment (Sweet et al. 2011). While the methods of many coral mucus studies may have led to variable accounts of the SML-associated microbiota (Sweet et al. 2011), it is evident that the SML represents a physical protective barrier and a niche for many members of the coral microbiome (Kaiko and Stappenbeck 2014).

The coral SML is composed of a mixture of secreted compounds, including large glycoproteins called mucins. Mucins are released from epithelial mucocytes and form gels of varying viscosity (Jatkar et al. 2010) that are ultimately responsible for providing epithelial protection (Bythell and Wild 2011). The coral SML is dynamic, enables the transfer of gases and storage of metabolites (Bythell and Wild 2011), is used to remove sediment (Fig. 5), and also varies with the environment and over

Fig. 5 Mucus sheet sloughing off of massive *Porites* sp., Orpheus Island, Great Barrier Reef, Australia. (Photo credit: C.V. Palmer)

time (Brown and Bythell 2005). Importantly, the SML offers a niche for commensal coral-associated microbes that fulfill important functions including nutrient provision and antimicrobial defense (Ritchie 2006; Krediet et al. 2013). While the ability of host immune systems to regulate populations of commensal bacteria is conserved across phyla, it is unclear how innate immune mechanisms distinguish beneficial and commensal microbes from potential pathogens (Rohwer et al. 2002; Artis 2008; Bourne et al. 2016). In the case of the coral SML, innate immunity of the host epithelium must be *hypo-responsive* to commensal microbes, while remaining *reactive* against pathogens (Rakoff-Nahoum et al. 2004; Artis 2008).

Effector Responses: Activation and Signaling

Effector responses eliminate a recognized threat that may be exogenously derived—like pathogens and toxins—or endogenously derived, such as signals from stressed or malfunctioning cells (Medzhitov 2008). The effector response resulting from endogenous activation of the immune system is sometimes referred to as a "cellular stress response", and may be triggered by changes in environmental conditions (e.g., Kültz 2005). The immediate and typically non-specific nature of innate immunity means that many effector responses are often mediated without gene transcription, and are instead reliant on serine protease cascades and redox signaling (Cerenius et al. 2010). In the following sections we discuss anthozoan effector responses and provide information on the current information on related receptors and signaling pathways (Table 1).

Immune Cells, Phagocytosis, and Wound Healing

Mobile immune cells eliminate pathogens via phagocytosis, seal wounds, and release bioactive compounds at sites of infection. Cell–cell and cell–extracellular matrix communication is key for each of these effector responses, and often involves integrins (Johnson et al. 2009). Integrins are a group of transmembrane α–β heterodimer receptors that are involved in cell migration and differentiation, fibrillar matrix formation, and signal transduction (Takada et al. 2007). Integrins have been identified within many anthozoan genomes, and show a surprising amount of complexity (Table 1) (Knack et al. 2008). For example, there are three α- and four β-integrin subunits identified in *Nematostella vectensis*, the starlet sea anemone (Putnam et al. 2007; Reitzel et al. 2008), and two β-subunits in the hard coral *Acropora millepora* (Brower et al. 1997; Miller et al. 2007). In a study on *N. vectensis* wound healing and regeneration, one of the highest upregulated genes during wound healing was α-integrin (DuBuc et al. 2014). α-Integrin is part of the mitogen-activated protein kinase (MAPK) signaling pathway (Table 1), which is proposed to be one of the primary mechanisms involved in *N. vectensis* wound healing (DuBuc et al. 2014).

The lectin-activated complement pathway is also important for cellular immune responses. This pathway is highly conserved and promotes phagocytosis and pathogen killing by aggregating and opsonizing pathogens (Fujita et al. 2004). Lectins are a diverse family of PRRs that include ficolins and mannose-binding lectins (MBLs), which recognize specific bacterial MAMPs (Fujita et al. 2004). The primary complement pathway components include complement C3, Factor B (Bf), lectins, and MBL-associated serine protease (MASP) (Carroll 1998). A wide diversity of lectins have been found within cnidarians, and are activated, along with other complement pathway components, in response to various stimulants including pathogen challenge, initiation of symbiosis, thermal stress, and wound healing (see Table 1) (Ocampo et al. 2015). For example, in the scleractinian coral, *Pseudodiploria strigose*; the lectins C-type, fucolectins, D-galactoside/L rhamnose-binding lectins, galectins and tachylectins C3, Bf, and MASP; and pathway components A2M and CD109 are all activated in response to a pathogen challenge. (Ocampo et al. 2015). In *Hydra*, components of the lectin-activated complement pathway are upregulated during wound healing and regeneration of the head (Wenger et al. 2014). In particular, MASP is upregulated in bisected animals, and it is proposed that the lectin-activated complement pathway may promote opsonization of invading pathogens (Wenger et al. 2014). The presence and activation of the lectin-activated complement pathways in cnidarians demonstrates its key role in phagocytosis and cellular responses during an immune response to infection.

Congruent with the identification of immune cell receptors and complement pathway components within anthozoan genomes, multiple anthozoan immune cells have been identified (Palmer and Traylor-Knowles 2012). Mobile, phagocytic cells—amoebocytes—were first identified in the sea anemone *Actinia equina*, and were shown to have bioactive capabilities within the mesenterial filaments (Hutton and Smith 1996). Additionally, a population of cells showing phagocytic activity were identified in the sea anemone *Exaiptasia pallida* using fluorescent-activated cell

sorting (Rosental et al. 2017). Within gorgonians, immune cells have been identified in *Swiftia exserta* in response to injury (Olano and Bigger 2000) and unstimulated immune cells have been located using enzymatic histochemistry, suggesting a bioactive role in immunity (Menzel and Bigger 2015). Additional investigations in the gorgonian *Plexaurella fusifera* have provided insights into the processes and cells involved in anthozoan wound healing (Meszaros and Bigger 1999). Amoebocytes have also been observed in response to infection in *Gorgonia ventalina* (Mydlarz et al. 2008; Couch et al. 2013). In this response, melanin-producing amoebocytes of *G. ventalina* encapsulated infected tissue (Mydlarz et al. 2008). Similarly, encapsulation has been observed in a hybrid of *Sinularia maxima* (Slattery et al. 2013). Within scleractinian corals several types of immune cells have also been identified, including granular amoebocytes (Vargas-Angel et al. 2007; Renegar et al. 2008), melanin-containing cells (Palmer et al. 2010), chromophore cells (Domart-Coulon et al. 2006), agranular (hyaline) cells (Palmer et al. 2011b), and fibroblast-like cells in response to injury (Palmer et al. 2011b). Similar characteristics among many scleractinian coral immune cells suggest that they may originate from a common stem cell (Palmer et al. 2011b), consistent with observations of *Hydra* (Bosch et al. 2010).

Tissue damage requiring wound healing is a common occurrence for many organisms. Having an open lesion leaves an organism susceptible to infection, making it imperative that wounds are rapidly and effectively sealed. In scleractinian corals, wounding occurs naturally primarily via predation (fish bites; Fig. 6), boring invertebrates, algal abrasion, fragmentation, and storm damage, and is often associated with distinct changes in tissue coloration (Fig. 7). Wound healing across the Metazoa occurs in four sequential stages using specialized cells (Galko and Krasnow 2004; Martin and Leibovich 2005), and has been described in the scleractinian coral *Porites cylindrica*, based on histological analysis (Palmer et al. 2011b). The wound healing process is characterized by (1) insoluble clot (plug) formation to seal the lesion, prevent fluid loss, and minimize infection (Theopold et al. 2004), via the transglutaminase and melanin synthesis pathways in invertebrates (Palmer et al. 2012); (2) infiltration and phagocytosis of cellular debris and foreign organisms; (3)

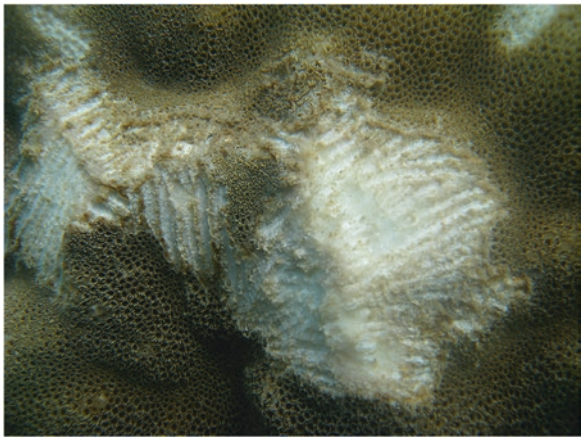

Fig. 6 Very recent predation scars on a massive scleractinian coral of the genus *Porites*. (Photo credit: C.V. Palmer)

Fig. 7 Visibly distinct pigmentation on *Porites* spp (Photo credit: C. V. Palmer)

proliferation and formation of granulation tissue that consists of multiple cell types, collagen, and a basic extracellular matrix, providing a platform for re-epithelialization (Galko and Krasnow 2004; Biressi et al. 2010); and (4) re-epithelialization and wound maturation, often involving apoptosis (Martin and Leibovich 2005; Biressi et al. 2010; Palmer et al. 2011b). Immune cells involved in wound healing of the hard coral *P. cylindrica* include melanin-containing granular cells (Fig. 8), agranular amoebocytes, fibroblast-like cells, and granular amoebocytes (Palmer et al. 2011b).

The Melanin Synthesis Pathways

Melanin synthesis pathway by products (e.g., reactive species and quinones) and deposited melanin pigment are key constituents of the invertebrate immune repertoire (Söderhäll and Cerenius 1998). They are also the first classic invertebrate immune responses to be documented within anthozoans (Couch et al. 2008; Mydlarz et al. 2008; Palmer et al. 2008; Gimenez et al. 2014; Zaragoza et al. 2014). In arthropods, melanin synthesis is initiated by PRRs that trigger the activation of serine protease cascades, leading to the cleavage of the prophenoloxidase (PPO) zymogen and resulting in the formation of active phenoloxidase (PO) enzymes (Cerenius et al. 2010). The POs then initiate rapid proteolytic cascades involved the catalysis of monophenol hydroxylation, diphenol oxidation, and autocatalytic reactions that results in melanization (Cerenius et al. 2010). PPOs and POs exist in various isoforms that represent different components of several melanin synthesis pathway types. These pathway types include tyrosinase (Cerenius et al. 2008) and laccase

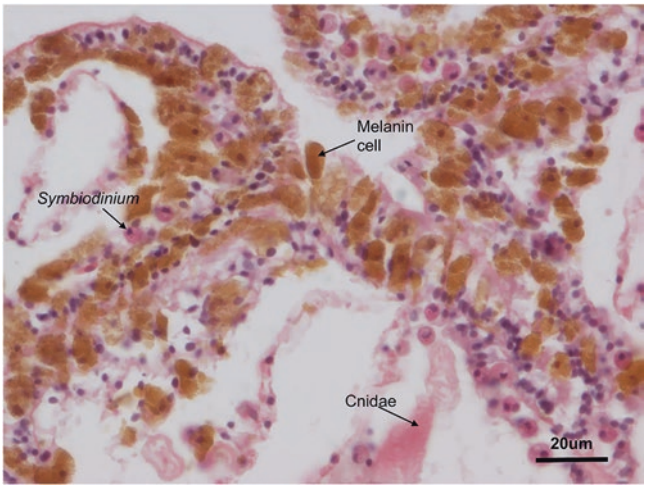

Fig. 8 Histological sections showing an epidermal tissue of scleractinian coral *Porites* sp. with a high concentration of melanin-containing granular cells as well as endosymbionts *Symbiodinium* and cnidae. (Photo credit: C.V. Palmer)

(Luna-Acosta et al. 2010), and likely have different functions (Sugumaran 2002; Cerenius et al. 2008; Palmer et al. 2012). For example, the tyrosinase-type melanin synthesis pathway is highly cytotoxic and therefore ideal for resisting infection (e.g., Bidla et al. 2008; Seppala and Jokela 2011), whereas the laccase-type pathway is less cytotoxic and likely has a role in cuticle formation within many invertebrates (Cerenius et al. 2008; Luna-Acosta et al. 2010).

The receptors and mechanisms involved in melanin synthesis pathway activation have not been elucidated for anthozoans, as for many other invertebrates (Takahashi et al. 2015). However, genes homologous to those associated with melanin synthesis pathways in some arthropods have been identified within the genomes or transcriptomes of several anthozoans, such as C-type lectins (Yu and Kanost 2004) (Table 1). In scleractinian corals many different types of lectins have been discovered, including C-type lectins, rhamnose-binding lectins, tachylectins, fucolectins, and galectins (Table 1). One well-studied example of the C-type lectin is "millectin", discovered in *A. millepora.* Millectin can bind to both pathogens and algal symbionts (e.g., Kvennefors et al. 2008) (Table 1). However, another study found no increase in C-type lectin expression during bacterial challenge (Brown et al. 2013). The conflicting evidence for lectin reactivity suggests that the complete picture on the role of lectins in anthozoan immunity is still unknown (Palmer and Traylor-Knowles 2012).

In addition to the lectin pathway, many other pathways are linked to melanin synthesis. The TLR and Toll pathway are involved in melanin pathway activation in insects (Cerenius et al. 2010), and concomitantly TLR genes are present in anthozoans (Table 1). Similarly, tyrosinase genes, which are involved in melanin pathway activation, have been found within the genomes of the sea anemone *N. vectensis* and

the hydrozoan *Hydra magnipapillata* (Esposito et al. 2012). Expression of trypsin-like serine proteases has also been documented during immune challenge in the scleractinian coral *A. millepora* (Weiss et al. 2013), and a laccase-3 gene and shrimp PPO-activating enzyme (PPAE) homolog found in the scleractinian coral *Pocillopora damicornis* (Vidal-Dupiol et al. 2014). Also, a single contig predicted to encode a PO was found within the transcriptome of the Caribbean reef-building coral *P. strigosa* (Ocampo et al. 2015). Crucially, as rapid proteolytic cascades control melanin synthesis, gene expression studies will likely only loosely represent the immune activity of this effector response (Cerenius et al. 2010). As such, PO activity is frequently measured enzymatically in invertebrate immunology to determine presence and regulation of melanin synthesis pathways (e.g., Cerenius et al. 2008; Haine et al. 2008; Palmer et al. 2010).

Biochemical PO and PPO activities of the tyrosinase-type pathway were first reported in two reef-building coral species, *A. millepora* and *Porites* sp. (Palmer et al. 2008) and the gorgonian sea fan *G. ventalina* (Mydlarz et al. 2008). Tyrosinase-type PO and/or PPO activity has since been enzymatically demonstrated within numerous scleractinian corals from the Caribbean and Indo-Pacific (Palmer et al. 2012), soft corals (Alcyonacea), anemones (Actiniarian), and zoanthids (Zoantharia) (Palmer et al. 2010, 2011c, 2012a; Mydlarz and Palmer 2011; D'Angelo et al. 2012; Anithajothi et al. 2014; Gimenez et al. 2014; Sheridan et al. 2014; Zaragoza et al. 2014; van de Water et al. 2016). Laccase-type PO activity, which potentially has a role in coral photosensing and structural support (Palmer et al. 2012), has also been biochemically demonstrated in multiple scleractinian corals from the Indo-Pacific (Palmer et al. 2012) and Caribbean (Mydlarz and Palmer 2011), and in larvae and juveniles (Palmer et al. 2012). These reports demonstrate the ubiquity, and therefore likely significance, of melanin synthesis across anthozoans.

Melanin-associated encapsulation and structural support involves the degranulation of immune cells within which the melanin synthesis pathway is active (Galko and Krasnow 2004). Examples of these immune cells in other invertebrate organisms include crystal cells of insects (Bidla et al. 2008) and hemocytes of crustaceans (Söderhäll and Smith 1986). Melanization and associated amoebocytes have been shown to form a barrier against fungal infection, and were first documented within the anthozoan sea fan *G. ventalina* (Petes et al. 2003; Mullen et al. 2004; Mydlarz et al. 2008). Melanin-containing cells and/or melanin deposits have also been found within a suite of healthy Indo-Pacific corals (Scleractinia and Alcyonacea) (Palmer et al. 2010) and multiple Caribbean coral species (Mydlarz and Palmer 2011), suggesting such cells may be ubiquitous among anthozoans. The increase in melanin cell density in both compromised and infected coral tissue (Palmer et al. 2008, 2009a) and their degranulation at lesions (Palmer et al. 2011b) indicates their prominent role in coral immunity (Fig. 8).

Though the cytotoxicity of melanin synthesis hasn't been explicitly explored within corals, the upregulation of PO in injured (D'Angelo et al. 2012; Sheridan et al. 2014; van de Water et al. 2015c), pathogen challenged, and infected corals (Palmer et al. 2008, 2011a, c; Zaragoza et al. 2014) suggests pathway activities are

part of an effector response, as well as immune signaling (Mydlarz and Palmer 2011; Palmer et al. 2012). However, it has also been proposed that coral PO is used in growth, rather than immunity, due to the correlation between PO activity, fluorescence, and cell proliferation (D'Angelo et al. 2012). However, given the key role of PO in immunity throughout invertebrate phyla (Cerenius et al. 2010), upregulated PO in fast-growing coral tissue likely provides additional protection to the most at-risk parts of the coral colony. Growing tissues that demonstrate proliferation of melanin-containing granular cells and increased PO are also those most likely to come into contact with potentially harmful competitors, algae, and biofilm-associated microbes, as is the case during larval settlement (Palmer et al. 2012).

Coagulation

Coagulation is the process by which a liquid, such as invertebrate hemolymph, is converted into an insoluble clot—often in the form of a gel (Theopold et al. 2004). In invertebrates, coagulation ensures the rapid re-establishment of tissue integrity upon injury by preventing fluid loss and entrapping pathogens during infection (Cerenius et al. 2010). While there are likely multiple clotting mechanisms within invertebrates, one key pathway involves transglutaminases that form a gel upon interaction with plasma proteins (reviewed by Cerenius et al. 2010). Transglutaminases have previously been identified within several different invertebrates including molluscs (e.g., Nozawa et al. 2001) and many types of arthropods (see Cerenius et al. 2010), and is often followed by melanization for clot hardening (reviewed by Theopold et al. 2004). Transglutaminase activity has only been documented within one anthozoan: the reef-building coral *P. cylindrica* (Palmer et al. 2012). Within *P. cylindrica*, transglutaminase activity increased in response to injury (Palmer 2010), confirming its role within anthozoan wound sealing.

Antimicrobial Activity

Cnidarian antimicrobial peptides (AMPs) are cationic and hydrophobic, targeting the cell walls of microorganisms, and often providing broad-spectrum defense (Destoumieux-Garzón et al. 2016). Across the Metazoa, AMPs are typically located within granular immune cells and in association with epithelial tissue layers (Zasloff 2002). AMP transcription is initiated by the activation of the TLR pathway and subsequently the transcription factor NF-κB complexes with other adaptor proteins (Anderson 2000). AMPs are then used to disrupt microbial cell membranes (Shai 2002) and inhibit bacterial metabolic processes (Brogden 2005; Vidal-Dupiol et al. 2011b). A wide variety of invertebrates have been shown to possess a diverse suite of AMPs (e.g., Lemaitre and Hoffmann 2007; Otero-Gonzalez et al. 2010; Destoumieux-Garzón et al. 2016). Within Cnidaria, AMPs have been identified within Scyphozoa jellyfish, *Aurelia aurita* (aurelin) (Ovchinnikova et al. 2006), the Hydrozoa *Hydra* (arminin) (Miller et al. 2007; Augustin and Bosch 2010; Franzenburg et al. 2013), and Anthozoa, as reviewed by Mydlarz et al. (2016). The AMP, Damicornin, has been isolated from the scleractinian coral *P. damicornis* and demonstrated activity in response to Gram-positive bacteria and fungi (Vidal-Dupiol et al. 2011b). Two other AMPs have been bioinformatically characterized from

P. damicornis—a mytimacin-like protein that binds to lipopolysaccharides and a bactericidal/permeability-increasing protein (LBP-BPI) (Vidal-Dupiol et al. 2014). The antimicrobial compound Homarine has previously been shown to demonstrate antifouling and predator deterrent functions in other invertebrates and was subsequently found to be a critical AMP for gorgonian *Leptogorgia virgulata* (Shapo et al. 2007). In several *Hydra* species the AMP, arminin, has been shown to have a distinct, species-specific function in dictating which bacterial communities can associate with specific polyps of *Hydra* (Franzenburg et al. 2013). This specificity is maintained even when different species of *Hydra* are co-cultured, suggesting that host immunity determines the composition of the bacterial community (Franzenburg et al. 2013). The diversity and the continual discovery of these bioactive compounds has promise for discovering novel AMPs that could have important medical applications.

As members of Cnidaria, anthozoans represent some of the most poisonous known organisms, producing toxic, bioactive compounds for defense and predation (Parisi et al. 2014). These bioactive chemicals are a key area of bioprospecting due to their potential for human medicine for their anti-inflammatory, cytotoxic and antinociception activities (reviewed by Cooper et al. 2014). Production of secondary metabolites enables this taxonomic group to be one of the most effective sessile benthic colonizers (Harvell et al. 1993; Changyun et al. 2008; Kelman et al. 2009). Between 2008 and 2014, 244 diterpenoids, a class of compounds with antimicrobial activity, were isolated from Gorgonian corals (Changyun et al. 2008), and while the biological function has not been assigned to each compound, this provides a glimpse of the potential complexity of immune, defense, and microbial interactions that are continually occurring on a coral reef. Compounds such as diterpenoids, sesquiterpenoids, and sterols are used for chemical defense and allelopathy, the use of chemicals to influence competitors' biology, by soft corals and gorgonians, providing protection against predation (Van Alstyne et al. 1994) (reviewed in Changyun et al. 2008). Similarly, the antimicrobial activity of scleractinian and gorgonian extracts has been widely reported (e.g., Harvell et al. 1993; Kim et al. 2000; Gochfeld and Aeby 2008; Palmer et al. 2011c), though the nature of the chemical compounds and mechanisms employed are not always clear. A homogenate of coral tissues is often used to measure antimicrobial activity, and this contributes to activities being highly variable. This is particularly notable in immune challenge experiments, where some experiments result in increased bacterial growth while others demonstrate effective antimicrobial activity (e.g., Gochfeld and Aeby 2008; Palmer et al. 2011c). However, there are cases where extracted compounds have clear antimicrobial activity, for example diterpenoids extracted from the soft coral *Sinularia flexibilis* (Aceret et al. 1998). Similarly, diterpenoids and sterols are also involved in *S. flexibilis* allelopathy (Fang et al. 2005) (reviewed in Changyun et al. 2008). Scleractinian corals are highly dependent on allelopathy (Koh 1997; Gochfeld and Aeby 2008; Kelman et al. 2009; Slattery and Gochfeld 2012), with high competition for space from other corals, algae and biofilms (Chadwick and Morrow 2011).

Apoptosis

Apoptosis is a tightly regulated form of cell death that occurs during normal development, stress, injury, and infection (Brentnall et al. 2013). It is linked to the endogenous activation of innate immunity in response to signals generated by damaged or malfunctioning cells, and typically occurs when stress and redox imbalance exceeds the tolerance limits of the cell (Medzhitov 2008). The intrinsic apoptosis pathway is activated and regulated by proteolytic enzymes called caspases (Brentnall et al. 2013). Extrinsic apoptosis is triggered by cell surface receptors in the presence of specific ligands, such as those on the membrane of cytotoxic cells, and is primarily mediated by the highly conserved tumor necrosis factor (TNF) receptor (TNFR) ligand superfamily (Quistad et al. 2014; Quistad and Traylor-Knowles 2016).

Within anthozoans, both caspases (Moya et al. 2016) and members of the TNF superfamily have been identified (Quistad et al. 2014; Quistad and Traylor-Knowles 2016) and they display more diversity than within other organisms (Quistad and Traylor-Knowles 2016), demonstrating the functional conservation of apoptotic pathways within the Metazoa. As for all animals (Jacobson et al. 1997), apoptosis plays a role in wound maturation in coral, by eliminating excess cells produced during the proliferation phase of wound healing (Palmer et al. 2011b) and disease mediation (e.g., Ainsworth et al. 2015; Lawrence et al. 2015). However, apoptosis in coral also occurs in response to changing environmental conditions, such as with reduced water pH (Kvitt et al. 2015) and has been most intensively studied in relation to thermal bleaching (e.g. Hawkins et al. 2013). Apoptosis is activated in the host through stimulation by the reactive species nitric oxide during the process of temperature-induced breakdown in anthozoan-algal mutualisms, known as bleaching (Hawkins et al. 2013). Apoptosis enables the release of *Symbiodinium* from the host endodermal cell and interacts with autophagy to expel the redundant symbiont (Dunn et al. 2007; Tchernov et al. 2011). Concomitantly, the TNFR signaling pathway, which can initiate either inflammation via NF-κB or apoptosis (Aggarwal 2003), is activated in response to thermal stress (Barshis et al. 2013; Palumbi et al. 2014; Rose et al. 2015).

Reactive Species

Reactive species are essential molecules derived from oxygen or nitrogen, or other molecules, that are more reactive than the element from which they were derived, which in some cases, such as oxygen, is itself toxic (Halliwell and Gutteridge 2015). Reactive species are involved in cellular reduction–oxidation (redox) reactions that occur under normal processes of metabolism, cell signaling, development, and immunity (Bartosz 2009). Examples of reactive species include superoxide anion radical, hydrogen peroxide, hydroxyl radical, nitric oxide radical, peroxynitrite, and electronically excited states such as singlet molecular oxygen that vary reactivity (Halliwell and Gutteridge 2015; Sies 2015). These cytotoxic and abundant molecules are continually kept in check by suites of antioxidant compounds and enzymes, so as to prevent damage to biomolecules and cells (Halliwell and Gutteridge 2015). Changes in local abiotic conditions, such as chronic or acute changes in temperature, pH, or salinity, induce the production of cellular reactive species (Tomanek

2015), potentially leading to a state of oxidative stress in which antioxidants are no longer able to maintain redox homeostasis. Oxidative stress occurs when the production of toxic reactive species overwhelms a system's ability to eliminate them with antioxidant molecules and enzymes (Halliwell and Gutteridge 2015; Tomanek 2015). This situation can lead to extensive damage; due to their transmissibility across membranes, reactive species have the potential to adversely affect all parts of the cell—from DNA to lipids to membranes—leading to disease and potentially necrosis (Halliwell and Gutteridge 2015). Oxidative stress, as a result of increased reactive species produced by photosystem II of the algal endosymbiont of coral, is a key factor in coral bleaching—the breakdown of symbiosis with *Symbiodinium* spp. (Lesser 1997; Gardner et al. 2017). However, during bleaching the coral host also increases reactive species, creating an unfavorable environment for the symbionts, leading to their death and/or expulsion (Weis 2008). This is one example of how anthozoan stress and immune responses are inextricably linked with the cytotoxicity of reactive species.

Cytotoxic reactive species can also be used to a host's advantage by being produced deliberately during immune responses to kill pathogens. These may be as "by-products" of immune pathways, such as the melanin synthesis pathway (Cerenius and Söderhäll 2004), or by oxidase enzymes during phagocytosis, known as a respiratory burst (Berton et al. 2015). Respiratory bursts have been described within gorgonians (Mydlarz and Jacobs 2006) with the resultant reactive species released into the local environment (Shaked and Armoza-Zvuloni 2013). Additionally, in order to prevent self-harm, the increased production of reactive species and/or a measurable immune response is often coupled with an increase in antioxidants (Bartosz 2009), including within anthozoans (e.g., Mydlarz and Harvell 2007).

Antioxidants

The potential damage caused by oxidative stress means that the stakes are high when increasing reactive species production. In order to mitigate or minimize self-harm, a suite of antioxidants are always present and upregulated with increases in reactive species, such as during abiotic stress events or an immune response. Many compounds have antioxidant capacity, including pigments such as melanin (Meredith et al. 2006) and carotenoids (Cornet et al. 2007), but enzymatic antioxidants, such as superoxide dismutase, catalase and glutathione (peroxidases), and thioredoxin-dependent systems, are crucial in maintaining redox homeostasis (Williams et al. 2013).

Anthozoans possess many different types of enzymatic antioxidants, including peroxidases (Downs et al. 2002; Mydlarz and Harvell 2007), superoxide dismutase (Diaz et al. 2016), and catalase activity (hydrogen peroxide-scavenging) (Hawkridge et al. 2000; Mydlarz and Palmer 2011; Palmer et al. 2012). Consistent with a damage mitigation role during abiotic stress, coral antioxidant activity varies with shifts in environmental conditions and particularly during coral bleaching (e.g., Downs et al. 2002; Merle et al. 2007; Jin et al. 2016). Similarly, corals upregulate antioxidants in response to both injury and infection (Mydlarz et al. 2010; Mydlarz and

Palmer 2011; Palmer et al. 2011c; Palmer 2010), which indicates the necessity of redox stabilization during an immune response.

Heat Shock Proteins

Heat shock proteins (HSPs) are ubiquitous soluble, constitutively expressed proteins responsible for a suite of cellular housekeeping functions that are essential to organism survival. HSPs fall into ten family categories, present in all metazoans. HSPs are molecular chaperones, assisting in protein folding and preventing denaturing and, though first identified in relation to heat shock in *Drosophila*, are not restricted to roles in thermal stress mitigation (Srivastava 2002). HSPs help ensure homeostasis, and are therefore involved in both abiotic stress and immune responses (Srivastava 2002; Tenor and Aballay 2007). HSPs can generate reactive species and activate melanin synthesis (Baruah et al. 2014), and, similar to many immune factors, transcription is related to the Toll family of immune receptors (Tenor and Aballay 2007). Anthozoans possess many types of HSPs (Table 1).

The first anthozoan HSPs were found within the scleractinian *Montastraea faveolata*, the sea anemone *E. pallida* (Black et al. 1995), and the scleractinian *Goniopora djiboutiensis* (Sharp et al. 1997). Subsequently, a HSP found in the scleractinian coral *Stylophora pistillata* (SP HSP70) was used to monitor coral stress responses (Tom et al. 1999). Congruently, coral HSPs are upregulated during environmental change events, including thermal stress and bleaching (Barshis et al. 2013; Ross 2014; Traylor-Knowles et al. 2017b), pH change (Moya et al. 2015), and in response to disease (Seveso et al. 2015). HSPs have also been proposed to confer resistance of corals to heat stress (Palumbi et al. 2014). Additionally, HSPs have also been shown to be involved in the stress response to laboratory bacteria challenge experiments (e.g., Brown and Rodriguez-Lanetty 2015). Specifically, HSP70 was discovered to be involved in promoting a primitive form of the defense response in the sea anemone *E. pallida* (Brown and Rodriguez-Lanetty 2015). Brown and Rodriguez-Lanetty further propose that this short-term priming could confer immune strength during seasonal times of pathogen exposure. While the traditional studies of HSPs have focused on their role in general stress response and thermal stress response, more evidence now suggests that they may have a broader role in the immune response in anthozoans.

Fluorescent Proteins

A striking feature of many anthozoans is their multiple fluorescent proteins (FPs) (Alieva et al. 2008) (Fig. 9). FPs consist of a chromophore that spontaneously forms in the presence of oxygen and which is housed within a hydrophobic core in a robust ß-barrel structure (Sample et al. 2009). The barrel restrains the vibrations of the excited chromophore and prevents radiation-less loss of energy (Sample et al. 2009; Seward and Bagshaw 2009). FPs utilize their protein microenvironments to modulate and refine their photophysical characteristics, enabling them to absorb light at different wavelengths (Sample et al. 2009). FPs function by absorbing a photon of light at a low wavelength, for example within the blue or UV spectra, which then shifts the chromophore to an excited state through pronotation (Sample et al. 2009).

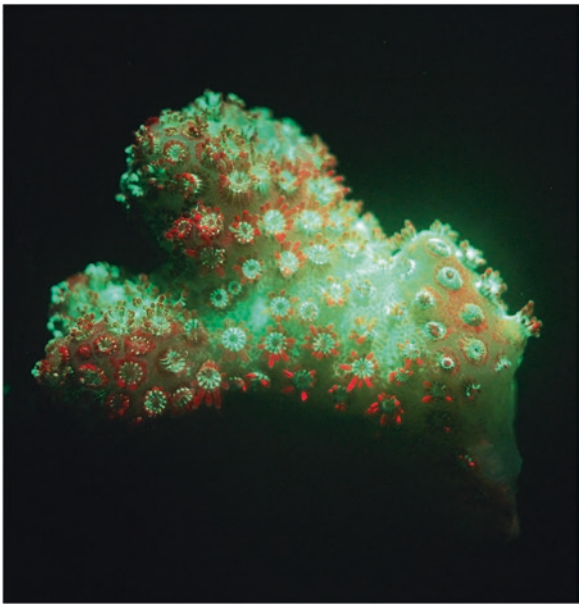

Fig. 9 Reef coral fluorescence. (Photo credit: G. Ampie)

Energy is emitted as light of a longer, red-shifted wavelength as the chromophore returns to its ground state. In the excited state, FPs produce reactive species, which, if not moderated, can lead to photobleaching of FPs and impact the local microenvironment through oxidative stress (Halliwell and Gutteridge 2015). The protein scaffold of the FP is a molecular sink for reactive species, which thus protects the chromophore and surrounding microenvironment (Sample et al. 2009). Across different FPs there are multiple types of chromophore, which contribute to the spectral diversity found in FPs across taxa (Bogdanov et al. 2009). Cnidarian FP diversity is used commercially as markers of gene and protein expression, and has revolutionized modern biomedicine (Sample et al. 2009).

All organisms, from microbes to large mammals, use pigments for signaling, crypsis, or mitigation of oxidative stress. In the animal kingdom, the type and intensity of coloration is often indicative of immune competence (e.g., Nolan et al. 2006). However, while color production in the ocean is common (Widder 2010), the biological roles of the higHL: Intelectin-1y conserved scleractinian FPs remains debated (Takashashi-Kariyazono et al. 2016). The function of FPs in scleractinians is postulated to be primarily for the maintenance of the endosymbiotic relationship with *Symbiodinium* (Gittins et al. 2015). *Symbiodinium* is of great importance to the overall health of scleractinian coral, so it is beneficial for the scleractinian coral host to protect them. FPs alter the local light environment of the scleractinian host, and therefore may facilitate photosynthesis (Smith et al. 2017), provide protection during high light conditions (Salih et al. 1998), and act as a photo-attractant (Hollingsworth et al. 2005). However, the presence of FPs in anthozoans that lack *Symbiodinium* (azooxanthellate) (Wiedenmann et al. 2004) suggests that they have additional or alternative roles within cnidarian biology.

The visible increase of fluorescence in scleractinians with compromised tissue (Fig. 7) (Palmer et al. 2008; Palmer et al. 2009a) and its coincidence with elevated PO activity (Palmer et al. 2008; D'Angelo et al. 2012; Palmer et al. 2012; Palmer 2010) suggests that FPs may have a role in immunity. Concomitantly, and consistent with the general description of the biochemistry of FPs (Sample et al. 2009), scleractinian FPs scavenge hydrogen peroxide (Palmer et al. 2009b), indicating that FPs are potentially very useful for managing oxidative stress potential during immune responses and periods of environmental stress (Seneca et al. 2010; Roth and Deheyn 2013; van de Water et al. 2016).

Ecological Immunity: Focus on Scleractinian Corals

Ecological immunology theory postulates that variations among and within constituent immunity and immune responses are due to energetic trade-offs among costly life history traits like reproduction, growth, and maintenance/immunity (Sheldon and Verhulst 1996; Sadd and Schmid-Hempel 2009). Scleractinian corals, from both the Indo-Pacific (Palmer et al. 2010, 2012) and Caribbean (Mydlarz and Palmer 2011; Palmer et al. 2011c), demonstrate high intra-taxon variation in baseline levels of immunity, which for the Indo-Pacific scleractinians predicts susceptibility to both disease and bleaching at the family level (Palmer et al. 2010). These family-level differences in immunity corresponded to life history strategies; for example, fast-growing scleractinians with high reproductive output (e.g., Acroporiidae) are among those with the lowest constituent immunity (Palmer et al. 2010). Similarly, a short-term temporal study of three Indo-Pacific scleractinians found that constituent PO activity varied among species and with water temperature fluctuations (van de Water et al. 2016), indicating that immune function is influenced by the environment and life history of the scleractinian.

One of the first signs of coral distress is the increased production of mucus (Brown and Bythell 2005) (Fig. 5). The increased production of mucus requires large energetic investments, which leads to the depletion of metabolic reserves, resulting in an immune-compromised state (Sheridan et al. 2014). Similarly, following a thermal bleaching event, involving the loss of energy-providing *Symbiodinium*, disease is often the cause of scleractinian death (Fig. 4) (Brandt and McManus 2009). The breakdown of this mutualism results in a starvation state and an energy deficit, leaving the scleractinian host ill-equipped to resist infection, as for other invertebrates (e.g., Siva-Jothy and Thompson 2002). Similarly, infection is energetically costly as resources are invested in resisting, or tolerating, the disease (Mayack and Naug 2009).

The effects of paying the high energetic costs to promote health have been observed in scleractinian corals when comparing thermally bleached and diseased colonies to healthy ones (Palmer et al. 2011a). Both the thermally bleached and diseased colonies of the scleractinian *A. hyacinthus* had lower levels of PO and peroxidase activity than healthy controls (Palmer et al. 2011a). The exception being at the disease lesion edge, where immune response was equivalent to constituent

levels of immunity in healthy controls (Palmer et al. 2011a). As with other invertebrates, the ability of a coral host to deliver an optimal immune response and maintain healthy constituent immune levels depends on the availability of energy (Sheridan et al. 2014). Energy availability and trade-offs therefore likely contribute to the intra-species variation in immunity observed within scleractinians (Wright et al. 2017; Palmer 2010). It is increasingly apparent that the environmental context needs to be considered with measurements of immunity (van de Water et al. 2015a; Wright et al. 2017). It is also evident that innate immune responses and responses to environmental change ("stress responses") are intertwined. Immunity therefore has the potential to be used as an effective indicator of coral health (Palmer et al. 2010; Traylor-Knowles and Palumbi 2014; Jin et al. 2016). Thus, the expansion of coral ecological immunology is important for analyzing the influence of more frequent climate extremes on scleractinian corals as well as the ecosystems that they support.

Immunity, Climate Change, and Conservation of Scleractinian Corals

In this Anthropocene era, humans have placed themselves in an arms race against an aggravated natural world. Scientists and conservationists are racing to understand complex systems, develop technologies and conserve organisms and ecosystems before human-induced climate change shifts them beyond repair in this sixth mass extinction event. Coral reefs have declined globally and at an accelerating rate since the 1980's e.g. (Bruno and Selig 2007; Hughes et al. 2017). Now, even with dramatic reductions in global carbon production, the persistence of coral reefs as biodiverse, economically significant systems (Graham et al. 2014) rests largely on our ability to conserve and effectively restore them through increasingly severe conditions. Cnidarian immunity will be critical in determining the long-term success of scleractinian species and coral reef communities (Palmer and Traylor-Knowles 2012; Mydlarz et al. 2016).

Anthozoans demonstrate a "stress response" to environmental perturbations, which is often investigated as distinct from immunity. With warming waters, increased hurricane activity, increased pathogen load, and ocean acidification, climate change stands to significantly undermine cnidarian immunity and therefore coral reef health. Scleractinian immune mechanisms have the potential to mitigate many of these assaults; however, studies investigating how these factors influence constituent immunity and immune responses are somewhat limited (e.g., Palmer et al. 2011c; van de Water et al. 2015c; Traylor-Knowles et al. 2017a). As such, cnidarian immunology has not yet been encorporated into the fields of coral conservation and restoration, though they stand to be highly informative.

In the face of climate change, our inefficacy in conserving and protecting wild coral reef systems is concerning. For example, the Great Barrier Reef, one of the world's best-protected marine systems, has undergone catastrophic coral loss in the past 2 years (Hughes et al. 2017). While the term "restoration" refers to the act of

returning something to its original condition, in the context of climate change it is well-acknowledged that reefs of the future will look quite different to the reefs of the past (Graham et al. 2014). With this in mind, starting in the 1990s, coral restoration projects using coral out-planting (coral gardening) have been under taken to promote biodiversity conservation and ecosystem function of scleractinians (Rinkevich 1995). Coral gardens are now abundant globally (Rinkevich 2014) and are a main method for restoring reef habitats. Coral gardening involves propagating small fragments of specific scleractinian genotypes on tree-like structures in mid-water nurseries, monitoring them to understand their disease and stress tolerances, and then out-planting them to prepared reef areas (Rinkevich 2014). Rapid coral colony growth through micro-fragmentation methods are promising; however, the physiological consequences of high fragmentation are as yet unknown (Forsman et al. 2015) and, as growth is high in terms of energetic cost, may come at the cost of impaired immunity (van der Most et al. 2011). However, numerous coral gardening restorations projects have been deemed successful, and arguably offer a viable mechanism for mediating the effects of climate change through ecological engineering (Schopmeyer 2012; Rinkevich 2014).

An alternative method to coral gardening restoration is larval seeding, where scleractinian coral larvae are reared ex situ and introduced in high densities to denuded reefs. However, the long-term benefits are still not well-understood (Edwards et al. 2015). Additionally, assisted evolution, via the use of selective breeding, epigenetics and microbiome manipulation, has recently begun to be used to create corals that are able to tolerate more extreme climate conditions (van Oppen et al. 2017). Assisted evolution has the potential to be integrated into ongoing coral gardening restoration efforts, and it is argued that the rate of coral loss means this is essential. van Oppen et al. (2017) have proposed targeting genes that underpin the "ubiquitous minimal cellular stress response" (Kültz 2005) and highlight coral stress and thermal resilience studies (e.g., Barshis et al. 2013). It is increasingly clear that cellular stress responses cannot be decoupled from immunity (Baruah et al. 2014; Pinsino and Matranga 2015), and therefore including specific immune-related genes as targets for assisted evolution and restoration will likely be of benefit.

Conclusion

In this chapter we have summarized the rapidly expanding field of cnidarian immunology, touching on the identified immune mechanisms, their roles in immune responses, and the relevance of cnidarian immunology to understanding ecological patterns in health and disease, and in improving restoration efforts. There are many gaps in our knowledge, including understanding the gene versus proteolytic regulation of responses to immune challenge and adverse conditions, as well as the identification of additional anthozoan-specific immune mechanisms. Many mechanisms still remain to be more extensively explored within anthozoans, including endocrine-like signaling (Song et al. 2015; Tarrant 2015) as well as locating sites of immune

compound production, storage, and use. Lastly, cnidarian immunology will greatly benefit from more understanding of how the immune pathways fit together and influence each other. Cnidarian immunology is a burgeoning field that stands to inform conservation efforts, and biomedicine, as well as the field of comparative immunology.

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Platyhelminthes: Molecular Dissection of the Planarian Innate Immune System

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Introduction

Vertebrate animals exposed to pathogens frequently activate both their innate and adaptive immunity. Innate immunity or the non-specific immune system has an important role as the first line of defense against pathogens. Because innate and adaptive (i.e., specific) immunity tend to work together in the fight against microorganisms, research often addresses them together. Invertebrate organisms do not rely on adaptive immunity and therefore offer the unique opportunity to address the innate immune system without intervention of adaptive immunity (Abnave et al. 2017; Lai and Aboobaker 2017; Loker et al. 2004; Peiris et al. 2014). Although animals are generally exposed to similar pathogens, invertebrate organisms effectively defend against microbes by using innate immunity (Salzet et al. 2006; Schulenburg et al. 2007). Thus, analysis of the innate immune system in invertebrates not only has potential to inform about the biology of the first line of defense but also to provide meaningful mechanistic alternatives to reinforce and make a more effective innate immune system in vertebrate animals.

The innate immune system capitalizes on the extended presence of invertebrate organisms before the Cambrian explosion, giving them several hundreds of million years to evolve and diverge (Wilson 1987). Additionally, the diverse environments in which invertebrate organisms co-exist may also provide interesting evolutionary insights about alternative mechanisms developed to defend against pathogens. Take as an example the sponges, which were the first to branch out from the common

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ancestor of all metazoans. These organisms rely on a complex network of intercellular mediators for an immune response (Wiens et al. 2007; Wilkinson et al. 1984). Mediators known as chemokines are important for deducing proteins for self and non-self recognition (Degnan 2015; Wiens et al. 2007; Wilkinson et al. 1984). Among those recognition receptors are Toll-like receptors, which are important in recognizing pathogens and activating the innate immune response. Phagocytic cells are their main source of protection against/elimination of microbial and parasitic pathogens. Besides phagocytosis, immune effector cells produce various cytotoxic agents to destroy pathogens. Sponges effectively filter around 2.5×10^8 viral particles/ml and between 75% and 99% of suspended bacteria, while distinguishing between commensal and pathogenic microorganisms (Mukherjee et al. 2015, 2016; Wiens et al. 2005). Thus, millions of years of refined immunity allow sponges to continually eliminate a wide range of pathogens (Mukherjee et al. 2015, 2016; Wilkinson et al. 1984).

The diploblastic Cnidarians, which include the freshwater hydra, also eliminate pathogens in the absence of motile phagocytic cells. Hydra display intriguing general innate immune features, allowing this freshwater organism to identify pathogens and respond with antimicrobial peptides and a signaling cascade related to the nuclear factor (NF)-κB pathway (Miller et al. 2007). Hydra also possess a protective mucous layer covering the ectoderm, which keeps microbes from colonizing them (Nyholm and Graf 2012). Furthermore, recent studies in hydra revealed that beneficial virus–bacterial–host interactions are essential to maintain the microbial ecology and increase the innate protection against invading microbes (Bosch et al. 2015; Fraune and Bosch 2007; Miller et al. 2007; Wenger et al. 2014). Altogether, Cnidarians seem to have a more complex innate immune response than sponges but they both seem to effectively recognize and eliminate pathogens in their respective aquatic environments. On the other hand, invertebrate organisms that live on land, such as the nematode *Caenorhabditis elegans* (ecdysozoan), dwell in the soil and feed on bacteria, and are therefore in constant contact with soil-borne microbes. These protostomes use their cuticle and grinder, creating a sort of physical barrier against pathogens. In addition, this organism mounts effective innate immune responses through evolutionarily conserved signaling pathways including the p38 mitogen-activated protein kinase pathway, transforming growth factor (TGF)-β-like signaling pathway, and Toll pathway (Franzenburg et al. 2012; Pujol et al. 2001; Tenor and Aballay 2008). The Toll signaling pathway, for example, is crucial for the identification of pathogens in flies and mammals (Alegado et al. 2003; Kawai and Akira 2009).

Flatworms (Platyhelminthes) represent one of the most intriguing examples of effective innate immune response in invertebrates, specifically freshwater planarians, which are members of the Lophotrocozoans and have the capacity to rapidly eliminate microbes that infect humans, such as *Mycobacterium tuberculosis* and *Staphylococcus aureus*, among others (Abnave et al. 2014; Tsoumtsa et al. 2017). These findings are particularly exciting because planarians rely on an innate immune response that actively recognizes self versus foreigner microbes and it only requires a few days to eliminate massive amounts of human pathogenic bacteria (Abnave et al. 2014; Peiris et al. 2014). This is hugely significant in the sense that

tuberculosis alone caused approximately 10.4 million infections and 1.7 million deaths worldwide in 2016 (https://www.cdc.gov/tb/statistics/default.htm). In this chapter, we discuss some of the most recent findings about planarian immune response with the aim of understanding mechanisms of innate immunity that effectively fight pathogenic microorganisms of human relevance.

The Planarian Model System

Planarians are widely used to understand fundamental biological questions associated with stem cell-mediated tissue regeneration and maintenance of adult tissues. These flatworms possess a large pool of adult stem cells called neoblasts, which are instrumental for their regenerative capabilities, developmental plasticity, and cell turnover (Gentile et al. 2011; Newmark and Alvarado 2002; Pellettieri and Sánchez Alvarado 2007; Reddien and Sánchez Alvarado 2004). In recent years, the immune system of planarians has received additional attention based on its potential to regenerate tissues and the ability to clear a wide spectrum of pathogenic bacteria within a short period of time (Abnave et al. 2014; Peiris et al. 2014). Modern studies in freshwater planaria are mostly conducted in two species (*Schmidtea mediterranea* and *Dugesia japonica*), which in their natural habitat (i.e., ponds, rivers, etc.) are expected to deal with a wide variety of microbial pathogens. Planarians have evolved a remarkably regenerative response that is pertinent to asexual reproduction in different strains. This reproductive process involves naturally splitting the planarian body in two or more fragments that regrow multiple animals resembling the original worm after a few days. Remarkably, during asexual reproduction or accidental injury, there is little to no traces of infection in open wounds (Abnave et al. 2014; Pang et al. 2016; Petersen 2014). These observations provide an opportunity to study roles of innate immunity in clearing and preventing growth of microbes during tissue repair. There are important resources for data mining and genomic analysis of possible homologs and predicted function of innate immunity in planaria (Abnave et al. 2014; Arnold et al. 2016; Pang et al. 2016; Peiris et al. 2014). This information can be used to understand basic molecular mechanisms and signaling cascades that can be further exploited to fight pathogens during injury or massive infestations in the adult body.

Planarians are protostomes, members of the phylum Platyhelminthes and are free-living—non-parasitic—organisms closely related to other acoelomates (Gehrke and Srivastava 2016). Planarians are commonly placed between the ecdysozoa clade, which includes *Drosophila melanogaster* and *C. elegans* and non-bilaterian phyla such as the Cnidarians (Sánchez Alvarado 2003; Egger et al. 2009; Hejnol et al. 2009) (Fig. 1). As one of the oldest bilaterians, the planaria, like other acoelomates and the Cnidarians, lack any respiratory and circulatory systems (Gehrke and Srivastava 2016; Newmark and Alvarado 2002; Sánchez Alvarado 2003). This is in striking contrast to other lophotrochozoans, such as the annelids, which include species with a circulatory system and a variety of immune competent, and even connection with various nutritional and medicinal, benefits (Bailey et al. 1971; Bilej et al.

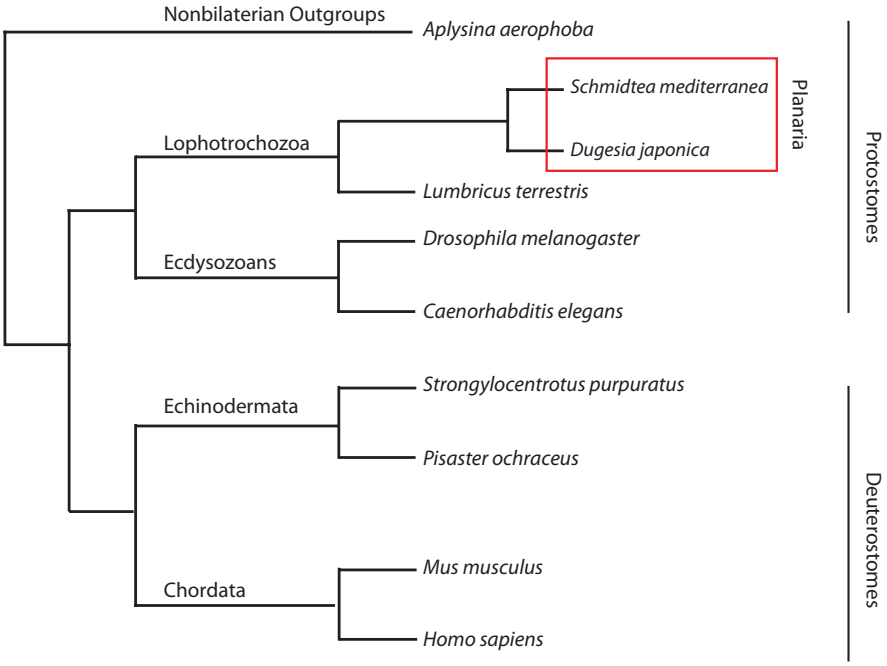

Fig. 1 Phylogenetic distribution of vertebrates and invertebrates across bilaterians. The tree only represents some members of the Lophotrochozoa, Ecdysozoans, Echinodermata, and Chordata. The red lines highlight two planarian species members of the Platyhelminthes

1990; Cooper et al. 2001, 2004; Engelmann et al. 2002; Nakao 1974; Salzet et al. 2006). Intriguingly, the planarians rely on diffusion to obtain oxygen, and this provides evidence of an absence of any blood- or lymph-related immunity (Peiris et al. 2014; Reddien and Sánchez Alvarado 2004).

Planarians have a digestive system comprised of three main branches: triclads (Fig. 2). We pay particular attention to phagocytic cells in planarians, which engulf food or pathogens and participate in the innate immune response (Forsthoefel et al. 2011, 2012; Garcia-Corrales and Gamo 1988; Roberts-Galbraith and Newmark 2015). Additional features of the planarian anatomy and further characterization of their different cell types can be found elsewhere (Gentile et al. 2011; Reddien and Sánchez Alvarado 2004; Roberts-Galbraith and Newmark 2015; Zhu and Pearson 2016). The innate immune system in planarians is starting to receive attention among investigators of planaria, with about a dozen manuscripts being published in the last few years (Abnave et al. 2014; Arnold et al. 2016; Gao et al. 2017; Han et al. 2017; Lu et al. 2017; Morita 1991; Pang et al. 2010, 2012, 2016, 2017; Peiris et al. 2014; Torre et al. 2017). Here, we attempt to provide a succinct summary of the most recent literature, while presenting the most prominent molecular structures that potentially play a role in the planaria immune response. Some of the most marked features include the mucous membrane, phagocytic cells, pattern recognition receptors (PRRs), complement, and recent research on the planarian microbiome.

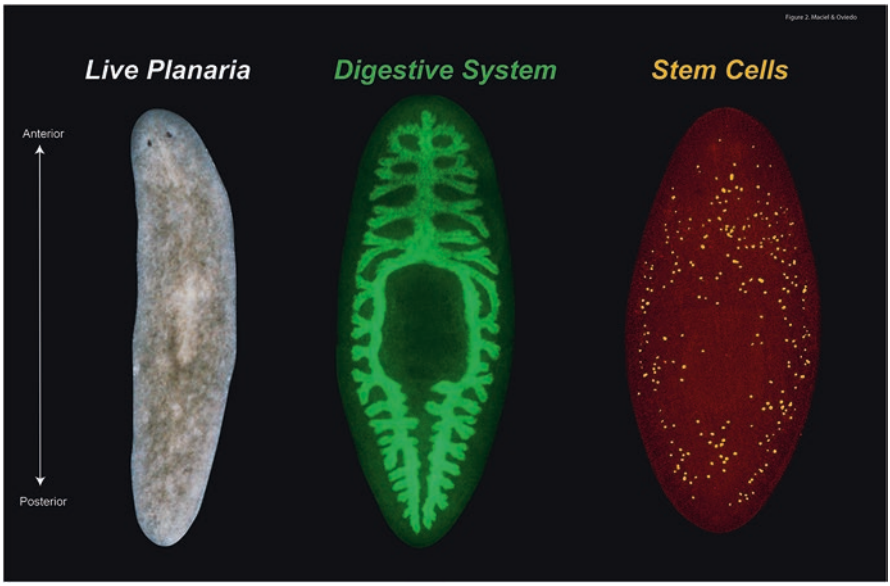

Fig. 2 The planarian model system. Representative images of the planarian species *Schmidtea mediterranea*. Left image is a dorsal view of a live intact planaria with the pharynx located in the middle of the animal. The middle image highlights the digestive tract of the planaria evidenced by whole-mount fluorescence in situ hybridization using antisense probe for *Smed-innexin-9*.The right image demonstrates the spatial distribution of a group of dividing stem cell (neoblasts) labeled with immunostaining using anti-phospho-histone3 (H3P)

The Mucous Barrier

The mucus generally provides an extra barrier against pathogens and contains digestive and immune properties. In mammals, the mucus coats microorganisms, making it more difficult to adhere and easier to expel (Cone 2009; Fahy and Dickey 2010; Zanin et al. 2016), while in invertebrates, the mucus contains antimicrobial properties that are mainly used as a microbicide or to inhibit microbial growth (Pitt et al. 2015; Ramírez-Gómez et al. 2008). In the echinoderm sea urchin, the intestinal tract that secretes mucus also expresses immune-related genes to combat pathogenic bacteria, monitor microbiota, and aid in digestion (Bavington et al. 2004; Ramírez-Gómez et al. 2008, 2009; Zasloff 2002). The mucus in most organisms may also trap bacteriophages, which brings a derived immunity outside of the host by using cross-linked glycoproteins, allowing for the interaction of specific bacteria with predators (Barr et al. 2013a, b; Meyer 2013). This mechanical barrier also aids in the ability to regulate and control commensal bacteria in the mucus (Barr et al. 2013a, b; Meyer 2013).

Planarians have a thin mucous layer covering the exterior planarian body and the lining of the digestive system (Fig. 3). This mucous layer represents the first physical barrier to pathogenic microorganisms. The mucous membrane aids in digestion,

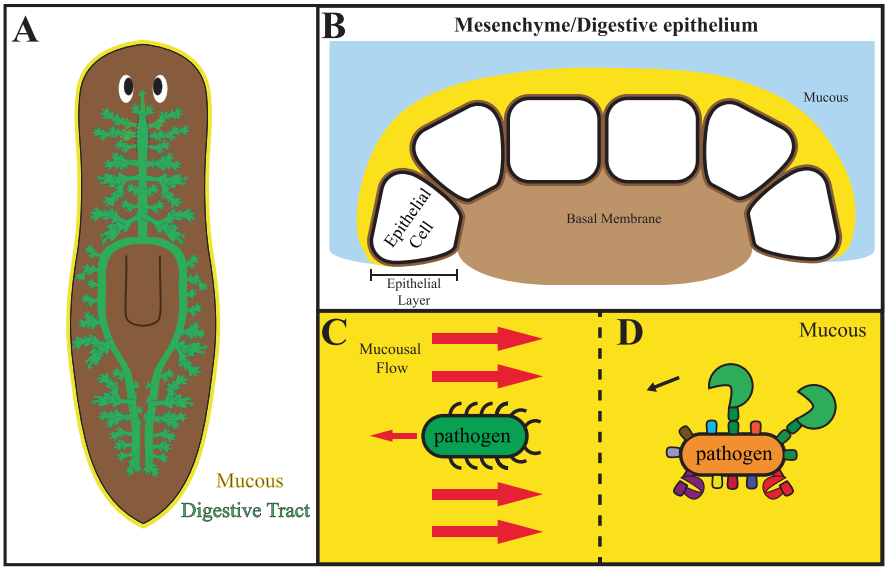

Fig. 3 The mucous barrier in planarians. (**a**) The mucous layer covering the whole animal (see yellow outline of the planaria). (**b**) Magnified illustration of the digestive epithelium and connective tissue underneath (basal membrane), highlighting the mucous layer covering epithelial cells (yellow outline). (**c**) The mucous membrane creates resistance and a countercurrent that prevents microbes to colonize and penetrate the epithelial layer. (**d**) Mucous proteins: zymogens and proteases (purple and red structures, respectively) participate in degradation of microbes and initiate inflammatory responses. Green structures are predicted C-type lectin functions that may participate in the elimination of microbes

locomotion, adhesion, and innate immunity (Bocchinfuso et al. 2012). The mucus is secreted by gland cells, which are found in the pharynx and subepidermal regions as insunk epithelial cells (Bocchinfuso et al. 2012; Pedersen 1963). The mucus is a dynamic semipermeable barrier that allows for movement of vital molecules but obstructs the passage of invading pathogens (Bocchinfuso et al. 2012; Pedersen 1963).

Although the mucus poses an immense challenge for pathogens, some microbes have evolved an ability to penetrate this physical barrier (Ribet and Cossart 2015; Schrank and Verwey 1976). Recent proteomic analysis of the planarian mucus revealed a total of 1604 proteins, some of which are antimicrobial peptides, zymogens, and proteases that possess digestive and immunological roles (Bocchinfuso et al. 2012).

Zymogens are proteases activated by proteolytic cleavage and are commonly found in the gut (Muta and Iwanaga 1996). During an infection these zymogens could be activated locally, triggering a cascade of inflammatory responses. In the invertebrate horseshoe crab, zymogens are used to form a gel to entrap microbes in their hemolymph (Muta and Iwanaga 1996; Salzet 2001). The cascade starts with pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), which activates zymogen factor C, which then activates factor B, leading to

the activation of a clotting enzyme. In parallel to this cascade, zymogens activate other clotting enzymes creating insoluble coagulin (Kawabata and Muta 2010).

An active zymogen in planarians is the phenoloxidase that originates from its precursor prophenoloxidase. Phenoloxidase facilitates the formation of the pigment melanin, leading to the sequestration of foreign material when phagocytosed (Lu et al. 2014) The phenoloxidase cascade is also widely distributed in invertebrates catalyzing monophenols and diphenols to quinones to polymerize them into melanin (Taleh et al. 2014). Melanin is produced around invading pathogens, which promotes phagocytosis and enhances cytotoxic activity (Nappi and Christensen 2005). Though presence of phenoloxidase has been found in planaria, there is no clear understanding about its functional roles in immunity and regeneration. Current work in our laboratory is characterizing the spatial distribution of the prophenoloxidase as well as its functional role with RNA interference (RNAi) to better understand whether it contributes in the fight against microbial pathogens in planaria.

Proteases are involved in a large number of processes involving digestion of proteins, apoptosis, and immune responses. Planaria contain several proteases in their mucus covering the intestinal system (Bocchinfuso et al. 2012). Proteases such as cysteine, aspartyl, and trypsin-like proteases are relevant for digestion, but also contribute to the defense reaction against a bacterial challenge (Goupil et al. 2016; Zhou et al. 2012). For example, serine proteases in *Drosophila* are known to activate the Toll/Spätzle pathway that leads to secretion of a variety of antimicrobial peptides (Imler and Hoffmann 2000). The implications of activation of the prophenoloxidase cascade, which leads to melanization of invaders, have been shown in crustaceans (Liu et al. 2007). In Protochordata, the serine protease shows various roles in immunoreactive cells (Feng and Zhang 2012). In the planaria model, there is an increase in expression of trypsin-like proteases during regeneration (Zhou et al. 2012). Cysteine and aspartyl proteases also found in planaria contribute to digestion of protein in the digestive tract, and in regeneration (Goupil et al. 2016). However, functional analyses are needed to rule out the potential role of these proteases during bacterial infection and to confirm whether they indirectly activate zymogens.

Pattern Recognition Receptors

Once invading microbes gain access to a cellular layer, phagocytic cells are activated to eliminate them (Morita 1991). Phagocytic cells must have effective mechanisms in order to identify self- versus non-self, and one potential candidate may involve PRRs (Abnave et al. 2014; Peiris et al. 2014). The innate immune system contains several PRRs that can be found on the surfaces of cells, intracellular compartments, or free floating in tissue fluids (Wang and Wang 2013). The functions of PRRs among all organisms fall under a combination of opsonization, activation of proinflammatory or immune response, and induction of apoptosis (Mogensen 2009; Yang et al. 2011). There is evidence of multiple types of PRRs in invertebrates, including a long range of Mannan-binding lectin, C-reactive protein, serum amyloid

protein, C-type lectin, peptidoglycan recognition proteins (PGRPs), thioester-containing proteins, Gram-negative binding proteins, scavenger receptors, galectins, and leucine rich repeats (LRRs) (Christophides et al. 2002; Hultmark 2003; Pang et al. 2016). This list can be even longer, but shows the variety of different strategies to detect pathogens in invertebrates. The diversity of PRRs in planarians is unclear, but it has been found that the planarian species *D. japonica* contains C-type-like lectin.

The C-type-like lectin in *D. japonica* was found in the mucus covering external and internal epithelia (Gao et al. 2017; Pang et al. 2012). These types of lectins recognize several bacterial species and have an agglutinating activity (Gao et al. 2017; Pang et al. 2012; Shagin et al. 2002) which requires Ca^{2+}, as shown in crude homogenates of injury and bacteria-induced planarians (Gao et al. 2017; Pang et al. 2012). In addition, Ca^{2+}-independent C-type lectin has also been observed in *D. japonica* and is known as *DjCTL* (Pang et al. 2012). The spatial distribution of *DjCTL* expression is found in parenchymal cells surrounding the pharynx (Fig. 3), which is similar to the C-type lectin reported in *Girardia tigrina* (Shagin et al. 2002). Interestingly, *DjCTL* displays important molecular conservation with Reglllγ, a C-type lectin found in mice and amphioxi CTLs, which have both demonstrated antimicrobial properties (Gao et al. 2017). This is consistent with recent results implicating Ca^{2+}-dependent upregulation of *DjCTL*, which has agglutination activity against Gram-negative bacterium (Gao et al. 2017). Collectively, these findings strongly support the idea that C-type lectins play an important immunological role in planarians. These C-type lectins show PRR properties towards bacteria as they can bind bacterial sugars, but further experiments are needed to determine if C-type lectin-induced agglutination activity aids in opsonizing the bacteria. It would also be interesting to find out what induces *DjCTL* over-expression when challenged with bacteria. Lastly, what specific cells are secreting the *DjCTL*?

Additional results in planaria have implicated the expression of PGRPs during a *Pseudomonas* and *S. aureus* infection (Arnold et al. 2016; Torre et al. 2017). PGRPs induce prophenoloxidase cascade, activation of Toll and immune deficiency (IMD) signaling pathways, and phagocytosis (Kang et al. 1998). This finding strongly supports the theory that a component of an inflammatory response pathway is activated during bacterial infection in planaria. Furthermore, the planarian homolog of PGRP4 displays a threefold increase after a few days of exposure to *Pseudomonas* (Arnold et al. 2016). The expression of PGRP4 has been shown in the digestive tract, and resembles the expression of phagocytic cells (Fig. 3) (Arnold et al. 2016). It is tempting to speculate about a potential PRR on the surface of planarians' reticular cells that upon activation may lead to a variety of inflammatory pathways in response to an infection, but further experiments are needed to test this possibility. Nonetheless, 32 homologous genes of conserved inflammatory signaling in the mammalian and IMD pathways of *Drosophila* have been found in planaria (Arnold et al. 2016). Arnold et al. (2016) found that conserved inflammatory signaling mediates tissue degeneration after bacterial infection. Specifically, the authors of this study identified the transforming growth factor-β–activated kinase 1 and p38 mitogen-activated protein kinase (TAK1/MMK/p38), which modulates apoptosis

during infection and regeneration (Arnold et al. 2016). Together, these studies demonstrate evolutionarily conserved molecular pathways in planaria that can identify bacteria and trigger the innate immune response.

Other PRRs of interest involve LRRs. There are more than 6000 proteins identified with LRR motifs that can identify various microbial-associated molecular patterns in animals and plants (Jones et al. 2016; Matsushima et al. 2007). In mammals, Toll-like receptors and Nod-like receptors contain the LRRs that aid in triggering immune responses when a pathogen is detected (Mogensen 2009; Ng and Xavier 2011). Planarians possess LRRs without the Toll- or Nod-like proteins and can be considered as LRR-only proteins (Peiris et al. 2014). These LRR-only proteins are found in sea urchins, amphioxus, and many other invertebrates (Buchmann 2014). Even with the high numbers of LRR-only proteins found in invertebrates, the exact role they play in an immune response are still not well-defined. Thus, additional studies in planaria may provide further opportunities to understand the role of LRRs as PRRs.

The PRRs themselves can lead to a variety of inflammatory responses once an infection is detected (Mogensen 2009). Planarians over-express homologs of tumor necrosis factor-associated factors (TRAFs) and Toll/interleukin-1 receptor with connection to the myeloid differentiation primary response gene 88 (MyD88) and NF-κB pathway (Arnold et al. 2016; Peiris et al. 2014). This interesting finding suggests that PRRs induce a specific inflammatory response in respect to the location and pathogen/damage-associated molecular patterns. The PRRs within the digestive tract are expected to be different to those found within the epithelial layers in the parenchyma. These predicted differences might help to discover novel strategies planaria use to survey the type and location of microbes present at a given time. This can also lead to the discovery of different effector cells that ignite a response dependent on the activated PRR and its location. Future studies will need to answer whether the same cell type contains different PRRs, in particular the phagocytic cells. This may involve analysis of the mechanistic responses of the different PRRs in planaria.

Phagocytic Cells

Phagocytic cells were one of the earliest observed host defenses found in the animal kingdom (Abnave et al. 2017). However, the overall process by which phagocytic cells in invertebrates discriminate, reject, and eliminate microbes or react against exogenous cells is not well-understood. Classic work in various worms including annelids and nemerteans addressed mechanisms of graft rejection by the innate immune system (Cooper 1969; Cooper and Roch 1992; Dales 1978; Langlet and Bierne 1984; Salzet et al. 2006). In these worms a type of phagocytic cell (amoebocytes or coelomocytes, respectively) mediates graft rejection (Cooper 1969; Cooper and Roch 1984, 1986, 1992; Dales 1978; Langlet and Bierne 1984; Salzet et al. 2006). However, most of these initial studies about mechanisms of innate immune cellular rejection have not been favored by modern molecular biology and genomic approaches. Although planarians possess phagocytic cells that associate with the

intestine, as in nemerteans (Ishii and Sakurai 1991; Langlet and Bierne 1984; Morita 1991), there is no experimental evidence regarding an elaborate graft rejection response noted during tissue transplant experiments in planaria (Guedelhoefer and Alvarado 2012; Santos 1929, 1931). It is possible that the planarian innate immune system is more amenable to the engraftment of tissues due to its developmental plasticity and regenerative properties but further experiments are required to define the mechanisms of cellular rejection among Lophotrocozoa. A response mediated by phagocytic cells that is used to recognize non-self tissues in worms has been elaborated; however, this has not been characterized in planarians.

Phagocytic cells of invertebrate animals also represent the main effectors in the defense against microbes (Abnave et al. 2017; Loker et al. 2004). This process is based on recognition of self versus non-self to allow phagocytic cells to effectively bind, engulf, and kill pathogens (Bayne 1990). Among the evolutionarily conserved functions in phagocytic cells are digestion and elimination of pathogenic microbes (Bayne 1990; Hildemann et al. 1979). In order to respond to non-self entities and initiate a response, the phagocytic cells likely contain receptors with an affinity to conserved structures in pathogens (Gordon 2016a). Examples of conserved structures allowing phagocytic cells to detect pathogen may include LPS or chitin for Gram-negative bacteria and fungi, respectively (Bayne 1990; Gordon 2016b). During an engagement with a foreign microorganism, the phagocyte will respond by engulfing with pseudopods (Aderem and Underhill 1999; Gordon 2016a). This resembles the first descriptions of the planarians' phagocytic cells that roam the digestive tract, which are involved in intracellular digestion (Forsthoefel et al. 2011; Ishii and Sakurai 1991; Morita 1995), where they were described as labyrinthine and pseudopodia during the ingestion of food (Bowen 1980). These pseudopods encapsulate objects and internalize them in a phagosome, which will mature and fuse with a lysosome-containing reactive chemical species and proteolytic enzymes (Bowen 1980). The planaria phagocytic cells (Fig. 4), called reticular cells, can migrate towards heat-shocked bacteria and phagocytose them (Bayne 1990; Ishii and Sakurai 1991; Morita 1995). The mechanisms of cellular-mediated elimination of pathogens are still unclear, but Abnave et al. (2014) suggested that autophagy, a selective engulfment of cytoplasm, also contributes to the antibacterial immunity of the planaria phagocytes. This work also provides a strong demonstration of the use of the planarian system to provide information about unknown evolutionarily conserved molecular targets associated with the immune response.

Autophagy is the leading mechanism of interaction with immune signals in some invertebrates (Kuo et al. 2017). For example, tectonin proteins (TECPR1 and TECPR2) with β-propeller folds recognize pathogens and may interact with autophagy proteins that contribute to form pathogen-induced autophagy of selectively targets (Levine et al. 2011). The evolutionary conservation of autophagy as an effective strategy for microbial clearance is shown through most of the animal taxa (Lionaki et al. 2013; Ren et al. 2009). Functional disruption of the murine autophagy protein atg5 leads to colonization of pathogenic group A streptococcus in embryonic fibroblasts. However, in wild-type cells the streptococci are eliminated by autophagosomes, suggesting autophagic proteins are mediators of the immune response

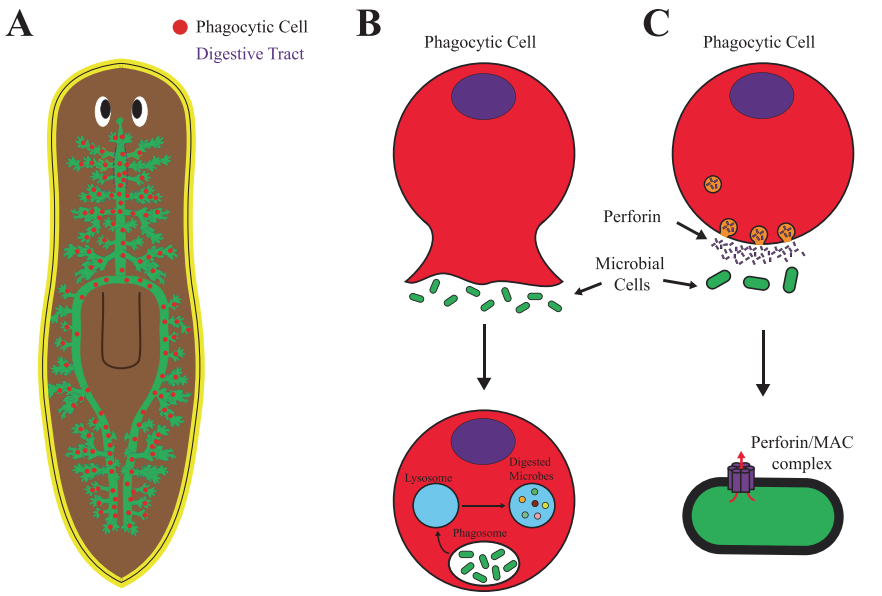

Fig. 4 Phagocytic cells along the digestive tract and their potential function as effectors of microbial clearance in planarians. (**a**) The digestive tract contains phagocytic cells (red dots). (**b**) Representation of the potential mechanisms used by phagocytic cells to eliminate microbes. The process may involve fusion of the phagosome with a lysosome-containing degradative enzymes. (**c**) Hypothetical scenario whereby phagocytic cells secrete perforin, which binds to microbes and creates a pore structure, diffusing all of its cytoplasmic contents

leading to bacterial clearance (Nakagawa 2004). In *C. elegans,* the induction of autophagy, through a mammalian ortholog TFEB, known as a helix–loop–helix transcription factor EB, HLH-30, is necessary for epithelium intrinsic defense and protection from bacteria (Chen et al. 2017; Tiller and Garsin 2014). The work on autophagy in the planaria model has shown its importance for supplying energy for proliferation and differentiation during starvation periods (González-Estévez et al. 2007). Autophagy is also required to recapture escaped bacteria from phagosomal membranes with double-membrane compartments that fuse with lysosomes (Casanova 2017). Consistently, autophagy-related genes in planaria are overly expressed during a bacterial infection, such as *S. aureus* and *Legionella pneumophila*, suggesting that autophagy is used to encapsulate and inhibit proliferation of bacteria. Abnave et al. (2014) found an increased number of genes for bacterial clearance as they primary focused on MORN2 (membrane occupational recognition nexus 2). MORN2 has been linked to binding and recruitment of autophagy protein LC3 to phagosomes, aiding in the elimination of internalized bacteria (Abnave et al. 2014; Bah and Vergne 2017). This is an interesting finding from the evolutionary perspective of the immune system because the mechanisms found by Abnave et al. (2014) are conserved between humans and planarians but absent in ecdysozoa (e.g., *D. melanogaster* and *C. elegans*).

The silencing of MORN2 in planaria resulted in the persistence of and a reduction in the ability to eliminate pathogenic bacteria (Abnave et al. 2014). Indeed, over-expression of MORN2 significantly reduced the survivability of bacteria by human macrophages (Abnave et al. 2014). The research performed by Abnave et al. (2014) provided a clear demonstration of evolutionarily conserved mechanisms obtained in planarians and their potential translational impact in humans. Thus, it is likely that, in addition to MORN2, other signaling pathways may act together to effectively activate phagocytic cells in planaria and remove bacteria over a short period of time. The elegant work performed by Abnave et al. (2014) at the Ghigo Laboratory paves the way to a greater understanding of phagocytic cells; nonetheless, additional experiments involving alternative methods of bacterial infection (i.e., injection, soaking, feeding) may be required to better assess the role of autophagy and the activation of alternative innate immune responses in planarians. Among the most intriguing questions are the mechanisms by which the reticular cells recognize non-self in order to phagocytose bacteria.

In addition to phagocytosis, phagocytic cells may also deliver humoral responses to destroy the membrane integrity of target cells during bacterial infection (Abnave et al. 2017). A conserved effector molecule that is expressed and secreted by phagocytic cells is perforin and the membrane attack complex: the MAC/perforin module (McCormack and Podack 2015). Perforin is highly conserved in invertebrates, as seen in sponges, and has a major role in clearing bacterial infections. The majority of invertebrates express a basal level of perforin throughout the body and its expression increases in response to inflammation (McCormack and Podack 2015). If this is so in planarians, we hypothesize that perforin expression (a) is located in reticular cells (digestive system); and (b) increases upon exposure to pathogenic bacteria. Testing these hypotheses may provide further insights regarding the mechanisms used by the innate immune system that lead to effective and accelerated clearance of pathogens.

Complement

Complement proteins are instrumental in identifying and eliminating foreign microbes; these free-floating proteins can be found in the first metazoan ancestors and are expected to be present in planaria (McCormack and Podack 2015). Nearly all multicellular organisms have the ability to deploy pore-forming proteins (McCormack and Podack 2015) which disrupt the membrane of intruding microbes and allow access to cytotoxic molecules to eliminate the target (Lukoyanova et al. 2015). In organisms with an adaptive immunity, the complement system functions to identify pathogens and proceeds to clear and induce an immune response by triggering an inflammatory cascade (Strbo et al. 2003). Homologies of the complement system have been found in the planaria that are a part of the perforin pore-forming proteins (Peiris et al. 2014). The planaria, as do many other invertebrate models, contain the perforin homolog known as perforin-2/macrophage expressed 1 (MPEG1), which contains a membrane attack complex/perforin (MACPF) protein

domain (McCormack and Podack 2015). Invertebrates such as the sponge upregulate the induction of gene and protein expression of perforin when exposed to LPS (Wiens et al. 2005). Gastropods produce perforin proteins through phagocytic cells to recognize pathogens (Mah et al. 2004). The phagocytes can use perforin as a cellular and humoral response, which can opsonize the pathogen (Humphries and Yoshino 2003; McCormack and Podack 2015). Since phagocytic cells are largely associated with the digestive tract, we speculate that perforin expression may be present along the planaria intestine and the surrounding mesenchyme.

The Planarian Microbiome Contributes to Innate Immunity

All animals harbor a variety of bacterial populations that contain many beneficial properties for the host organism (McFall-Ngai et al. 2013). Some of these benefits include digestive aid and nutrient gain such as cellulose digestion and nitrogen acquisition (Harris 1993). Interactions between the host and its flora are important to set up and control the microbial communities in order to keep a positive symbiosis relationship (Silver et al. 2007). The host PRR, phagocytic cells, humoral, specific compartments/organs, and the microbial community itself all contribute to maintaining a commensal microbial community (Chu and Mazmanian 2013; Nyholm and Graf 2012). Some studies suggest that even though invertebrates can shelter many bacterial species, only a relatively small number of resident species are maintained (McFall-Ngai 2007). The role of the microbiome is particularly relevant during adult tissue homeostasis and in situations where tissue injury and wounding are present because they may play a critical role in the healing process. However, the general role of the microbiome in the establishment of disease and tissue repair is a subject of intense research (Bosch et al. 2015; Grasis 2017; Hibbing et al. 2010). This is significant because disrupting the bacterial community or shifting in the bacterial populations can lead to tissue degradation and colonization of pathogenic microbes (Berezow and Darveau 2011; Hibbing et al. 2010).

Recent work by the Sánchez Alvarado group demonstrated that the planaria *S. mediterranea* is particularly well-suited to address the role of the innate immune system and the microbiome during tissue regeneration (Arnold et al. 2016). The authors of this study identified the composition of the planarian microbiome and revealed that many of the bacteria present in planarians are commonly found in humans. Planarians have a high ratio of the population of bacteriodetes to protobacteria when healthy, and when that ratio is disturbed the animals are more susceptible to tissue degradation and impediment of regeneration (Arnold et al. 2016). This finding links a particular group of microorganisms in planaria with tissue homeostasis and regeneration. Thus, the planarian model could provide a simple paradigm for identifying how resident bacteria are sensed and regulated by the innate immune system and what the contribution of particular microbial communities is to maintaining homeostasis at the organismal level. Answering these questions can also spark interest in the potential translational application in humans, specifically whether manipulation of particular bacteria species within the microbiome may lead to tissue deterioration in mammals.

Arnold et al. (2016) also showed through a candidate gene approach that a TAK1 signaling mechanism underlies the interplay between changes in the microbiome and the possibility to repair tissues. Specifically, the authors implicated TAK1/MKK/p38 signaling as mediator of programmed cell death during bacterial infection and regeneration (Arnold et al. 2016). This is significant because it connects evolutionary conserved inflammatory signaling and the regeneration of complex tissues. This supports other studies showing that changes in the microbiome can lead to abnormal inflammatory responses and tissue damage in the host (Belkaid and Hand 2014; Round and Mazmanian 2009). Consistently, experiments in *S. mediterranea* have demonstrated that increased activation of inflammatory genes coincides with expansion of protobacteria, which together impedes regeneration (Arnold et al. 2016). Thus, additional research in planaria may lead to better understanding of the organismal effects that changes in the microbiome through usage of antibiotics, diets, or change in environments may have.

Similar to mammals, the microbiome of invertebrate organisms is sensitive to changes in diet (Bahrndorff et al. 2016; Egert et al. 2004). This is an important consideration for the experimental design of studies in planaria, which in some cases use feeding of genetically modified bacteria to induce RNAi. Further experiments are needed to determine whether bacteria-fed RNAi affects the ability of planaria to eliminate pathogenic bacteria, repair tissue or introduce changes in host inflammatory responses.

In addition, the complexity of the microbiome regulation is compounded by the fact that physical forces can also alter the proportion of bacteria within the body (Morokuma et al. 2017). The Levin Group at Tufts University found that microgravity and hypomagnetic environments have important effects on behavior and the microbiome after deploying planarians (*D. japonica*) to the International Space Station (Morokuma et al. 2017). Interestingly, the absence of normal gravitational and geomagnetic fields for 5 weeks had multiple effects on the establishment of regenerative polarity, the proportions of Proteobacteria and Bacteroidetes, and behavioral changes associated with planarian-negative phototaxis that lasted for many months after worms returned to Earth. This pioneering work exposing planarians to extra-terrestrial conditions opens up new possibilities for determining the specific mechanisms by which physical forces affect the microbiome and innate immunity, and whether these changes result in alterations of body polarity. The findings underscore how sensitive the microbiome is to gravitational changes during space travel.

Final Remarks

The study of the immune system in invertebrates provides not only unique possibilities to understand their biology but also to learn about diverse strategies aimed at enhancing the first line of the defense against pathogens. Among invertebrates, the planarian model system presents fascinating opportunities to evaluate the innate immune system in the context of the whole organism undergoing renewal and repair of adult tissues. Planarians are traditionally known for their astonishing regenerative capacities, but there is an emerging interest in their immune system. From an

evolutionary standpoint, studies in planarians could inform about the immune system of the understudied group of Lophotrocozoans. Indeed, there is evolutionary conservation between planarians and mammals that involves both signaling pathways of the innate immunity and shared components of the microbiome. Uniquely, studies in planarians can shed some light on the remarkable conservation of the innate immune system between Platyhelminthes and humans that are absent in Ecdysozoans.

The physical barriers and mechanisms of defense against pathogens also show noteworthy conservation between planarians and mammals. The mucus covering planarian epithelia immobilizes and prevents colonization of pathogens and plays a similar role to the mucus layer covering the epithelial surface of many mammalian organs. In addition, the mucus contains a variety of protein-like proteases and zymogens with immune functions. This is an interesting aspect of planarians that could be further explored in the near future with the goal of identifying mechanisms promoting the regeneration of tissues. Once pathogens overcome the mucous barrier and reach epithelial layers there is activation of mechanisms aimed at agglutinating bacteria likely to induce an inflammatory response and opsonizing bacterial targets. Obtaining mechanistic understanding of this process could lead to innovative strategies to prevent bacterial infection in humans. Moreover, the effective and rapid strategy used by planarians to recognize and remove pathogens is particularly significant to millions of patients affected with tuberculosis and in an era of increased antibiotic resistance that also kills millions of patients around the globe.

Finally, we believe that the study of the planarian immune system will bring important basic understanding of innate immunity and has the potential for future clinical applications to fight human pathogens. Thus, planarians represent an important model for comparative immunology of the innate immune system.

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Nematoda: The *Caenorhabditis elegans* Model for Innate Immunity – Interactions Between Worms and Pathogens, and Their Responses to Immunogenic Damage

Ashley B. Williams and Björn Schumacher

Introduction

Caenorhabditis elegans is a small, generally free-living, non-parasitic nematode whose natural habitats include soil, compost, rotting fruit, and snails. The worm has been a powerful model for the study of many important biological processes, many of which can be very effectively transferred into studies of higher organisms. For example, the core apoptotic signaling pathway was first dissected in the worm (Horvitz and Lecture 2002), and a mutant screen revealed the first longevity-control pathway (Kenyon et al. 1993). Much of the power of *C. elegans* in basic research comes from its small size and easy handling in the laboratory, as well as a vast array of resources and infrastructure, including mutant and RNA interference libraries, a very active and open community of researchers, facilities for strain collection and distribution, and data curation. Another remarkable feature of the worm that increases its power as a model system is that the adult worms (which develop via the transition between four main larval stages), with the exception of the germline, are entirely post-mitotic. Following the fourth (and final) larval stage, further development consists only of growth, with no additional change in the number of somatic cells. Furthermore, the number and identity of the cells are invariable from worm to worm, and the full lineage for each cell, from fertilized oocyte to terminal differentiation, has been completely dissected. For this reason, some of the complexities of working with a multicellular organism in which cells turn over quickly are eliminated. The worm has been found to be susceptible to several human

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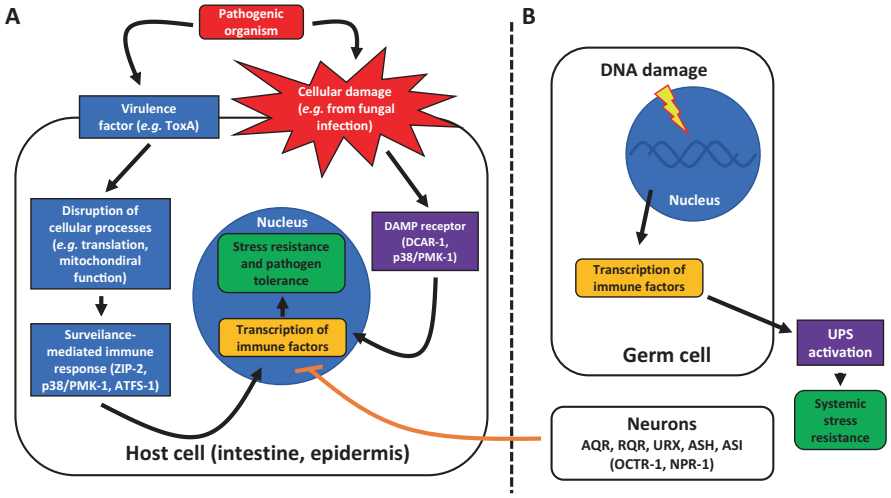

Fig. 1 Overview of the *Caenorhabditis elegans* innate immune response. The worm innate immune response consists of both cell-autonomous processes (**a**) and systemically disseminated responses (**b**). The transcription-based response can be stimulated via disruption of cellular processes (surveillance mediated immunity) or by direct cellular damage (damage-associated molecular pattern [DAMP]-mediated immunity). Signals from neurons appear to limit the immune response and DNA damage in the germ cells stimulates a robust immune response; however, most of the molecular mechanisms for these pathways remain to be clarified

pathogens, including *Pseudomonas aeruginosa*, *Serratia marcescens*, *Salmonella enterica*, and *Enterococcus faecalis*, as well as fungi, including *Cryptococcus neoformans* (Marsh and May 2012). This feature of the worms' response to pathogens spawned the productive and active field of nematode innate immunity. Furthermore, several nematode-specific pathogens have been identified, which allow the analysis of aspects of the immune response that may be part of the natural existence of the animals. These organisms include the bacteria *Microbacterium nematophilum* (Hodgkin et al. 2000), the fungus *Drechmeria coniospora* (Jansson 1994), and a positive-strand RNA virus (Orsay virus) (Félix et al. 2011).

The *C. elegans* immune system (see the overview in Fig. 1) evolutionarily predates those of higher organisms and seems to be relatively simple, particularly in that it lacks an adaptive immune system. Furthermore, the worms have no specialized or mobile immune cells. While they possess three pairs of cells involved in detoxification (the coelomocytes), these cells have not been assigned any immune functions. Because the worm relies only on its innate immune response to resist and tolerate pathogens, the complex interactions that exist between innate and adaptive immune responses in higher organisms do not cloud the study of specific features of innate immunity.

Signaling Pathways in the *Caenorhabditis elegans* Innate Immune Response

The worm innate immune response generally occurs at the level of transcriptional regulation (Shivers et al. 2008) and is controlled by several signaling cascades, depending on the type and location of the pathogen challenge. To date, at least four pathways have been identified:

1. p38/PMK-1 signaling
2. ERK/MPK-1 signaling
3. Insulin-like signaling (DAF-16 and DAF-2)
4. DBL-1 pathway

The p38/PMK-1 Signaling Pathway

Genetic screens to identify mutants with increased sensitivity to *P. aeruginosa* uncovered the p38 mitogen-activated protein kinase (MAPK)–related pathway as an important regulator of immunity in the worm (Bolz et al. 2010; Kim et al. 2004; Troemel et al. 2006). The signaling cascade underlying this pathway consists of several players: the neuronal symmetry family member 1 (NSY-1), stress-activated protein kinase (SAPK)/extracellular signal–regulated kinase (ERK) kinase 1 (SEK-1), and PMK-1, the worm p38 homolog. Signal propagation occurs via sequential phosphorylation of SEK-1 by NSY-1 and PMK-1 by SEK-1 in a strictly linear fashion (Fig. 2). The primary downstream effector of the pathway is the transcription factor ATF-7, which, when activated, induces a large repertoire of putative immune factors (Pujol et al. 2001; Shivers et al. 2010). As discussed in more detail in section "Missing Links", the nematode's genome encodes only one Toll-like receptor (TLR) protein (TOL-1), which has been only loosely associated with the immune response (Tenor and Aballay 2007). In mammals, all characterized TLRs seem to rely on adaptor proteins that contain Toll/interleukin-1 receptor (TIR) domains for the signal transduction that ultimately leads to the nuclear factor (NF)-κB-mediated proinflammatory response (i.e., TRAM [Trif-related adaptor molecule], TICAM [TIR domain-containing adapter molecule]/Trif [TIR-domain-containing adapter-inducing interferon-β], TIRAP [TIR domain-containing adapter protein]/Mal, and myeloid differentiation primary response gene 88 [MyD88]). A fifth TIR-containing protein, SARM (sterile alpha and TIR motif-containing protein), also exists and, while it is the least understood in mammals, it is also the only one that has a direct ortholog in *C. elegans* (*tir-1*) (Liberati et al. 2004). The *tir-1* gene does, in fact, have important roles in worm immunity: in particular, it acts together with NSY-1 and SEK-1 in the p38 pathway. TIR-1, NSY-1, and SEK-1 can be co-immunoprecipitated and probably form a protein complex (Chuang and Bargmann 2004), and phosphorylation of p38/PMK-1 depends on *tir-1* (Liberati et al. 2004); thus, it seems to be firmly situated in the p38 signaling pathway. Data suggest that TIR-1 is likely upstream in the pathway (Liberati et al. 2004), placing it in closer proximity

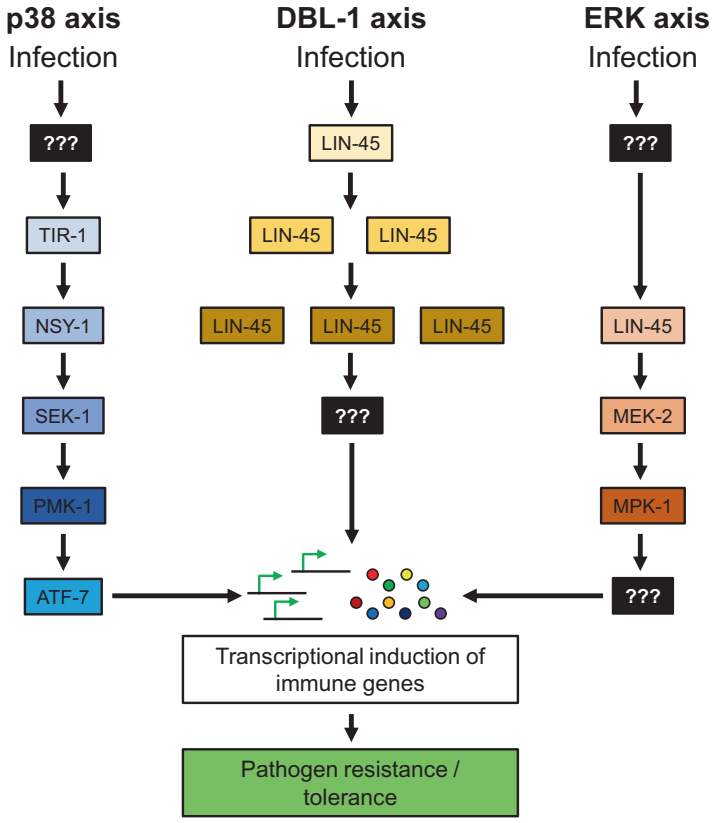

Fig. 2 Signaling in the *Caenorhabditis elegans* innate immune response. The worm immune response consists of three currently known signaling pathways, the p38 axis (*C. elegans* PMK-1), the DBL-1 axis, and the extracellular signal-regulated kinase (ERK) axis (*C. elegans* MPK-1). The outcomes of these pathways following pathogen exposure are the transcriptional induction of many genes thought to be involved in pathogen tolerance and resistance, although the functions of these genes remain mostly unknown (see text)

to the initiating events of the signaling cascade; however, what signals lead to TIR-1 activation remain entirely unknown.

The ERK/MPK-1 Signaling Pathway

The p38 MPK signaling cascade seems to be the most important in terms of innate immune function in *C. elegans*; however, the ERK1/ERK2 MAPK homolog MPK-1 can also be activated by some pathogens, in particular infection by *M. nematophilum* leads to an MPK-1-dependent immune response (Gravato-Nobre et al. 2005; Hodgkin et al. 2000; Nicholas and Hodgkin 2004). The upstream factors in this signaling cascade are LIN-45 and MEK-2, which have also been assigned various

functions in development and fertility (Fig. 2). Thus, it is clear that this signaling cascade is not exclusively dedicated to the immune response pathway, but instead regulates a plethora of processes ranging from stress responses to multiple aspects of the animal's development. The most upstream element of the pathway and the terminal effector protein remain unknown.

Insulin-Like Signaling (DAF-16 and DAF-2)

The insulin-like signaling (IIS) pathway involving the insulin-like growth factor receptor DAF-2 and the forkhead box O (FOXO) transcription factor DAF-16 were first identified as regulators of the extraordinarily long-lived dauer larval stage (Kenyon et al. 1993), an alternative developmental fate of worms under starvation stress, as well as determinants of adult longevity in *C. elegans*. Subsequently, they have been shown to be part of a larger network of pathways that confer stress resistance, which is intimately intertwined with the worm's immune response (Cezairliyan et al. 2013; Mahajan-Miklos et al. 1999). In the presence of its ligand DAF-28, DAF-2 is activated, which goes on to activate the phosphatidylinositol-3 OH kinase AGE-1 (Li et al. 2003; Malone et al. 1996). AGE-1 then catalyzes the conversion of phosphatidylinositol bisphosphate (PIP2) into phosphatidylinositol triphosphate (PIP3) (Tazearslan et al. 2009). PIP3 then binds to the AKT-1/AKT-2 complex to reveal two phosphorylation sites that are phosphorylated by the PDK-1 kinase (which also depends on PIP3 binding for its function). The AKT complex then phosphorylates the transcription factor DAF-16, which is blocked from entering the nucleus (Paradis and Ruvkun 1998). In contrast, in the presence of an antagonistic ligand (e.g., INS-1 [Insulin-like peptide]), the pathway is inactive and DAF-16 is not phosphorylated, leading to its translocation into the nucleus, where it activates stress response and putative antimicrobial genes. Genetic inactivation of *daf-2* leads to the same outcome as DAF-16 remains constitutively hypophosphorylated. Not entirely unexpectedly, loss of *daf-2* leads to pathogen resistance and this effect seems to be primarily rooted in the intestinal cells (Garsin et al. 2003; Hsin and Kenyon 1999; Libina et al. 2003; Lin et al. 2001). As is the case for ERK signaling, the outcomes of DAF-16 activation extend far beyond immune function, indicating that the pathway is not a dedicated immune pathway.

The DBL-1 Signaling Pathway

The gene *dbl-1* encodes one of four transforming growth factor (TGF)-β-like ligands in *C. elegans* and is (in part) required for resistance to both *P. aeruginosa* and *S. marcescens* (Kurz and Tan 2004; Mallo et al. 2002). The DBL-1 protein binds to the DAF-4/SMA-6 heterodimeric receptor and, via the SMA-2/SMA-3/SMA-4 complex, controls gene expression levels (Fig. 2), while it also has diverse functions independent of immunity (e.g., body size regulation and structural patterning). In fact, loss of the *sma* genes leads to increased sensitivity to *P. aeruginosa* (Kurz and

Tan 2004; Mallo et al. 2002; Roberts et al. 2010). Interestingly, TGF-β signaling in mammals leads to immunosuppression, demonstrating a remarkable divergence of function during evolution.

The *C. elegans* Viral Defense Strategy

To date, only a single virus that can infect and replicate in *C. elegans* has been identified (Félix et al. 2011). This virus, called Orsay after the site of its discovery in France, is a member of the *Nodaviridae* family and is a positive-strand RNA virus. Infection leads to easily observable morphological defects in the worm's intestine. The first indication of an antiviral response came from the observation that viral load was increased when factors of the RNAi pathway were deactivated, such as RDE-1, RDE-4, MUT-7 (RNaseD), and DRH-1 (Dicer) (Ashe et al. 2013; Félix et al. 2011; Guo et al. 2013). The implication of the RNAi pathway in viral defense (also supported by viral infection experiment using isolated worm cells) provided a sturdy scaffold for considering the evolutionary origins and conservation of the pathway, which is consistent with its function in plants. The current model for antiviral immunity in *C. elegans* (Fig. 3) proposes that the double-stranded RNA (dsRNA) intermediates produced during viral replication are bound by the dsRNA-binding complex RDE-1/RDE-4 responsible for initial detection and sequestration of exogenous dsRNAs. The canonical RNAi pathway is subsequently recruited: the dsRNA is passed to the DExD box RNA helicase DRH-1, which when interacting with RDE-1/RDE-4 unwinds the molecule to provide accessibility by the dicing complex. The Dicer homolog DCR-1 then produces small RNAs that go on to serve as templates for the RNA-directed RNA polymerase to produce a pool of secondary antisense small RNAs, which mediate the degradation of the full length viral RNA genome.

Some mammalian viruses have mechanisms to avoid detection by the host immune system; for example, by blocking the major histocompatibility complex (MHC) class I antigen processing and presentation pathway to escape T-killer cells (Horst et al. 2011). In *C. elegans*, the Flock house virus protein B2 can robustly downregulate the RNAi machinery to increase the sensitivity of worms to Orsay virus (Guo and Lu 2013). Another shared element of mammalian and nematode antiviral pathways is the similarity between DRH-1 and the RIG-I (retinoic acid inducible gene I) RNA helicase, an important sensor of dsRNA in mammals (Ashe et al. 2013; Coffman et al. 2017; Guo et al. 2013). While the RNA-binding domains are highly similar, the proteins do have different functions: DRH-1 presents RNA to Dicer for processing, while RIG-I activates an inflammatory antiviral immune response. Nevertheless, it is plausible that the two antiviral responses are connected over the span of evolutionary time.

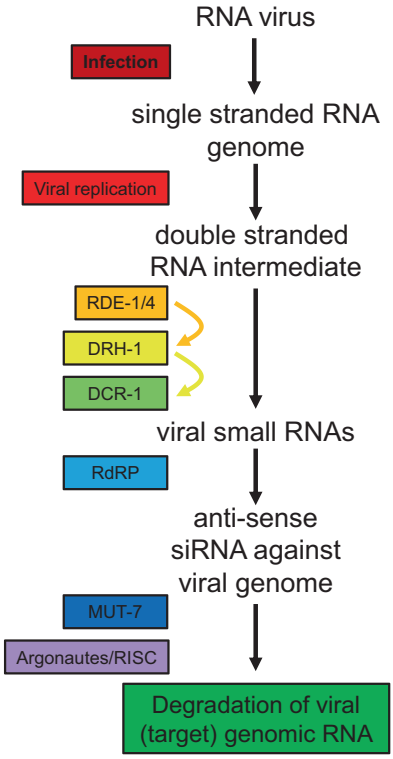

Fig. 3 The *Caenorhabditis elegans* antiviral response. Protection against viral invasion is mediated by components of the RNA interference (RNAi)-mediated gene-silencing pathway. Following infection by an RNA-based virus, the RNA is bound and processed to yield antisense small interfering RNAs (siRNAs) against the viral genome, ultimately leading to degradation of the viral genetic material to limit the infection

Neuronal Regulation of Immunity

The simple body plan and limited number and type of cells has led to functional multitasking in various cell types. As already mentioned, the intestinal cells are a key site of immune function; it turns out that neurons are also key players in worm immunity. *C. elegans* is an extremely powerful model for studying the nervous system, as the morphology, identity, and synaptic connectivity of all its 302 neurons is entirely understood; furthermore, a detailed catalog of the relevant neurotransmitters for most of the neurons has also been compiled. In addition to the production of antimicrobial factors, worms also seem to respond to pathogen exposure via neuron-driven behavioral programs, most notably pathogen avoidance. A polymorphism in the gene encoding the G-protein-coupled receptor (GPCR) protein NPR-1 caused decreased survival during *P. aeruginosa* infection by limiting the ability of the worms to avoid the pathogen (Reddy et al. 2009); however, it turned out that this is not the sole function of *npr-1* in worm immunity. Worms lacking NPR-1 exhibited altered expression of intestinally expressed, PMK-1-regulated genes during infection (Styer et al. 2008). Remarkably, elimination of the sensory neurons AQR, RQR, and URX rescued this phenotype, suggesting that in the absence of NPR-1 these neurons become hyperactive and disturb immune

pathways. Worms lacking these neurons are more pathogen resistant, suggesting that they have a negative regulatory function on the immune response.

The neuron-expressed GPCR OCTR-1 also plays a role in worm innate immunity (Sun et al. 2011). Through action in the ASH and ASI neurons, OCTR-1 suppresses the pathogen-dependent activation of PMK-1 and blocks the induction of a non-canonical UPR in distal tissues, indicating a non-cell-autonomous function. While the increased resistance of *npr-1* mutant worms to *P. aeruginosa* involved alterations in pathogen avoidance behavior, *octr-1* mutant worms exhibit increased pathogen resistance without such a behavioral change. How the neurons are stimulated by pathogens and the mechanisms underlying these phenotypes remain open questions in the field. Interestingly, the OCTR-1 protein is related to vertebrate adrenergic receptors that bind to their ligand noradrenalin. The outcome of this binding is a response to acute stress that can be accompanied by immune suppression (Aballay 2013).

The Interface Between Innate Immunity and DNA Damage

Study of the innate immune response of *C. elegans* has primarily focused on host–pathogen interactions; however, it is also now clear that the system can also respond to damaged self-DNA through a process called germline DNA damage-induced systemic stress resistance, or GDISR (Fig. 4) (Ermolaeva et al. 2013). *C. elegans* has been an especially valuable tool for dissecting the distinctive features and roles of DNA damage responses (DDRs) in the germline versus somatic tissues. In this discussion, we focus on germline processes, as they have so far been shown to be intricately intertwined with the innate immunity of *C. elegans*.

The majority of tissues in the adult worm are post-mitotic, as the cellular lineages are invariable and somatic development is generally completed by the last larval stage. The exception is the germline, which contains mitotic cells in a stem cell niche. Once cells leave the stem cell niche, they proceed through meiosis to generate mature germ cells—in hermaphrodites, the most common sex in worms—the production of sperm and oocytes are temporally separated during growth. In hermaphrodites, diakinesis-arrested oocytes are fertilized by sperm produced earlier during development to generate clonal offspring (Kimble and Crittenden 2005). Following DNA damage in germ cells, conserved cell cycle checkpoints are robustly activated to arrest mitotic proliferation, allowing time for DNA repair pathways to remove destabilizing lesions (Ahmed and Hodgkin 2000; Gartner et al. 2000). In a case where checkpoint activation cannot be resolved in a timely manner, apoptosis mediated by the CEP-1, the *C. elegans* homolog of the highly conserved p53 tumor suppressor, occurs through CEP-1-dependent transcriptional induction of the BH3-only-domain genes *egl-1* and *ced-13*, the protein products of which then trigger the apoptosome (Derry et al. 2001; Hofmann et al. 2002; Schumacher et al. 2001, 2005).

These processes are themselves cell autonomous; however, it is now clear that the GDISR pathway leads to non-cell-autonomous effects via factors associated with the innate immune response (Ermolaeva et al. 2013). The systemic aspects of

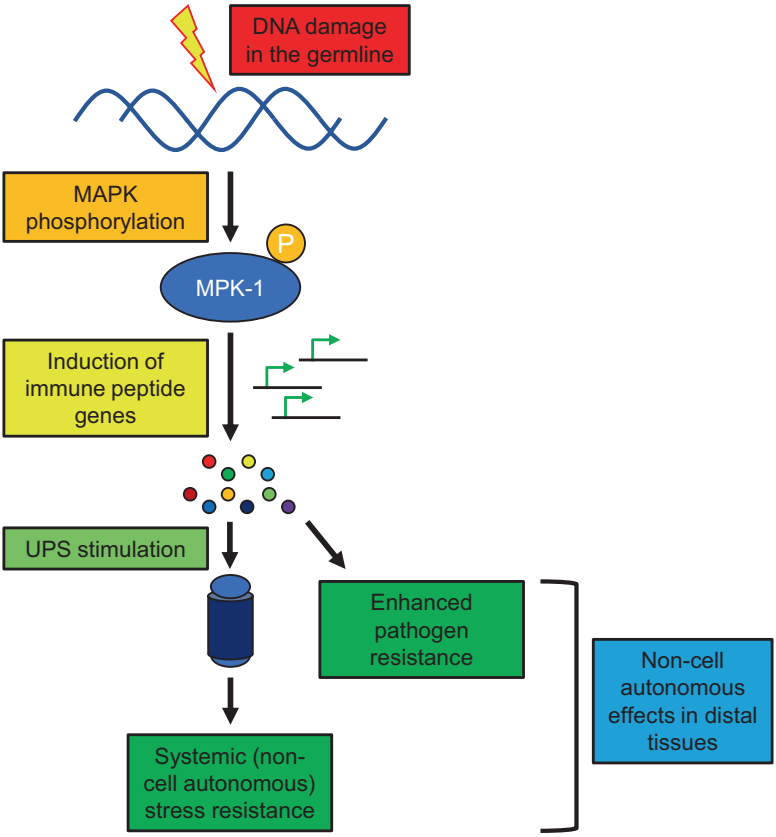

Fig. 4 Non-cell-autonomous stress resistance following immune induction. DNA damage in germline cells leads to the activation of immune genes, stimulating the ubiquitin proteosome system (UPS). This enhanced activity confers systemic stress and pathogen resistance. The mechanism for the detection of the DNA damage and the intermediate signaling pathway leading to gene induction remains unknown

the DDR were initially observed in animals that were defective for global-genome nucleotide excision repair, which fail to remove UV light-induced lesions in germ cells, ultimately leading to accumulation of DNA damage. Quite remarkably, the somatic tissues of UV-exposed animals developed profound resistance to both heat and oxidative stress. Importantly, this effect was not specific to UV-induced damage, as DNA damage induced by ionizing radiation or hydroxyurea, as well as endogenously generated DNA double-strand breaks in pachytene germ cells, was sufficient to elicit the stress resistance phenotypes. This induction of stress resistance through damage of endogenous DNA suggests that damaged DNA can be recognized as a damage-associated molecular pattern (DAMP) by *C. elegans*. The molecular basis of GDISR was shown to depend on the activation of the ERK homolog MPK-1, which subsequently induced expression of a repertoire of immune

genes. This added burden of such a broad induction of gene expression subsequently stimulated the ubiquitin proteasome system (UPS), which conferred systemic stress resistance. Importantly, this pathway is distinct from the p38/PMK-1 pathway discussed earlier and the first tendency may be to attribute the effect to CEP-1 activity; however, this was not the case and, in fact, no components of the canonical DNA damage checkpoint signaling were necessary for GDISR. Therefore, GDISR is a distinct response to DNA damage, independent of checkpoint signaling. A connection between UV irradiation and immunity has been demonstrated in human skin cells where UV irradiation leads to a complex and highly coordinated range of immune-associated processes, ranging from localized inflammation to systemic outcomes mediated by cytokines and other growth factors—highly reminiscent of GDISR. It is likely that further study of GDISR in *C. elegans* may lead to the discovery of fundamental features of such systemic responses and how damaged DNA is recognized as a DAMP. Immune reactions to DNA damage have also been reported following infection with bacteria such as *Escherichia coli* and *Helicobacter pylori*, which can both cause DNA damage in eukaryotic cells (Nougayrede et al. 2006; Toller et al. 2011), suggesting that GDISR—at least conceptually—may be an ancestral version of extant mammalian pathways.

Missing Links

A conspicuously missing component of innate immunity in *C. elegans* is a repertoire of mechanisms for the detection of pathogen-associated molecular patterns (PAMPs) and DAMPs. In higher organisms, a number of pathways have been characterized for the specific detection of a broad list of foreign elements (e.g., Toll-like signaling, cGAS-STING [cyclic GMP–AMP synthase–stimulator of interferon genes], among others). Despite efforts by a number of groups over many years, similar pathways have not been identified in the nematode.

The sole putative DAMP detection mechanism identified in the worm to date is DCAR-1, a GPCR protein that was previously assigned a function in chemosensory neurons (Zugasti et al. 2014). It was subsequently shown to be expressed in the epidermis, a major site of innate immune responses in the worm. In this tissue, DCAR-1 can detect the tyrosine derivative hydroxyphenyllactic acid (HPLA), which accumulates when worms experience wounding or fungal infection. In these situations, HPLA accumulates triggering a signaling cascade that culminates in the canonical p38 (PMK-1)-mediated innate immune response.

While the *C. elegans* gene encodes one TLR, TOL-1, it has not been assigned a role in detection; rather, it seems to be involved primarily in developmental processes and tissue maintenance (Pujol et al. 2001; Tenor and Aballay 2007). Furthermore, the genome also encodes a large collection of leucine rich repeat (LRR)-containing proteins. It is reasonable to conjecture that such genes may function in detection based on the well-characterized functions of LRRs in ligand binding in the Toll-like and nucleotide-binding oligomerization domain (NOD)-like receptors; however, while one LRR protein has been assigned a function in pathogen resistance (FSHR

[follicle-stimulating hormone receptor]-1) (Miller et al. 2015; Powell et al. 2009), it has not been shown to function as a sensor.

As discussed in the section "The Interface Between Innate Immunity and DNA Damage", worms induce a potent innate immune response to DNA damage in the germline that then confers systemic somatic stress resistance (GDISR). Pathways for the detection of damaged DNA, including double-strand breaks, single-strand breaks, and stalled replication forks at sites of damage have been identified and well-characterized in *C. elegans.* Quite unexpectedly, such pathways were clearly shown not to be involved in the induction of this immune response (Ermolaeva et al. 2013).

Another significant gap in our understanding of worm innate immunity is the function of the specific proteins induced as part of the innate immune response. As discussed earlier, the *C. elegans* innate immune response is characterized in general by the transcriptional upregulation of a large regulon of genes that encode putative secreted factors, many of which have structures indicating that they may function as antimicrobial agents; however, to date we know very little about how they function to combat pathogenic challenges. The one example in which a specific function is coming into focus is a C-type lectin gene (of which the genome encodes hundreds), which is induced following exposure to the Gram-positive bacterial pathogen *Bacillus thuringiensis* (Pees et al. 2017). Mutants for several C-lectin genes were shown to have either decreased or, surprisingly, increased resistance to the pathogen. Specifically, loss of one gene in particular resulted in enhanced avoidance behavior, which prompts the worms to leave the lawns of the pathogen. Furthermore, the same mutant animals also had increased periods of feeding cessation; thus, in this case, an immune-regulated gene was shown to actually be a negative regulator of pathogen resistance via behavioral modulation. Even with this bit of insight, the roles not only of the C-lectin proteins but essentially of the other immune-regulated genes remain entirely unknown. The elucidation of their functions is particularly hampered by the dazzling similarities between many of the genes, likely resulting in robust redundancy. Overcoming this experimental challenge remains a stubborn block in furthering research in this area.

Surveillance-Mediated Immunity

As mentioned in the section "Missing Links", our understanding of the sensors and effectors of the *C. elegans* innate immune response remains rudimentary, as several fundamental components have yet to be identified, most notably pathways for sensing PAMPs and DAMPs. One theory proposed to resolve this issue is that the nematode relies on an alternative approach for immune activation, independent of direct sensing, called "surveillance immunity" (Pukkila-Worley 2016)—similar in principle to the long-studied effect-triggered immunity in plants. The basis for surveillance immunity is that instead of monitoring for pathogens directly, the animals monitor for disruptions in endogenous processes that could be caused by the

presence of a pathogen, for example, translation, cellular homeostasis, or structural integrity.

The first identified and best understood surveillance pathway is involved in monitoring host translation. Many bacteria can produce protein toxins that interfere with efficient translation of mRNAs, including ToxA, produced by *P. aeruginosa*. This protein blocks polymerization of nascent peptides by blocking host elongation factor 2 function via ribosylation in intestinal cells following *P. aeruginosa* infection (Dunbar et al. 2012). Following this disruption, the transcription factor ZIP-2 (bZip transcription factor) accumulates and, in concert with the conserved protein CEBP-2, regulates an innate immune response (Estes et al. 2010; McEwan et al. 2012; Reddy et al. 2016). What is clear is that both the pathogen and toxin are functionally invisible to the animals and instead disruption of translational function stimulates the response. Quite remarkably, genetic ablation of host-encoded functions can induce a similar response, even in the absence of a pathogen, reinforcing this validity of this concept.

A conceptually similar pathway has also been reported in the mitochondria. Siderophores, toxins produced by pathogens (including *P. aeruginosa*), can interfere with mitochondrial homeostasis (Kirienko et al. 2015). The unfolded protein response in the mitochondria (UPR^{mt}) helps to ensure mitochondrial function by inducing the expression of nuclear-encoded, mitochondrially targeted chaperone molecules. A central player in this pathway is the transcription factor ATFS-1. ATFS-1 is normally taken up by functionally intact mitochondria, thus limiting cytosolic levels; however, upon disruption of the mitochondria, this uptake is reduced, leading to cytosolic accumulation. Subsequently, the protein can enter the nucleus, where it induces a repertoire of genes encoding putative antimicrobial factors. ATFS-1 also enters the nucleus during *P. aeruginosa* infection, leading to the expression of genes that confer resistance to the infection (Nargund et al. 2012), while loss of ATFS-1 leads to reduced resistance (Pellegrino et al. 2014). Further work remains to fully understand the interplay between the UPR^{mt}, ATFS-1, and bacterial infection, but as in the case of translation, this pathway provides a satisfying mechanism by which the animals can indirectly sense the presence of a pathogen.

In a large-scale study of the microbiome of the worm's natural habitat, nearly 20% of isolates examined (a total of 560) induced mitochondrial stress (Liu et al. 2014). This observation strongly supports the broad usefulness of such a surveillance pathway in responding to bacterial challenges. Much work remains to be done to understand the implications and complexity of surveillance immunity, but the concept is already becoming a useful framework in which to consider the innate immune response of worms, given the gaps in the more conventional mechanistic pathways.

To What End: Immunity or General Stress Resistance?

Expression of genes associated with the *C. elegans* innate immune response can be controlled by pathways that are generally discussed in the context of distinct biological processes (i.e., MAPK signaling in response to pathogen infection and DNA damage and DAF-16 as part of the IIS pathway). Furthermore, activation of overlapping innate immune genes by DNA damage confers resistance not only to pathogens but also to heat and oxidative stresses (Ermolaeva et al. 2013). While the latter two cases seem to be secondary effects due to activation of the UPS driven by the enhanced expression of putative immune factors, rather than effected directly by the immune peptides, the net outcome of the activation of the innate immune response is enhanced stress resistance.

An important conceptual consideration is whether what is studied in *C. elegans* and labeled as "innate immunity" is rather a complex set of interconnected stress responses, which happen to confer pathogen resistance. The label applied to these responses certainly does not negate the value and usefulness of this field of research in *C. elegans* as broadly applicable biological processes have been clarified; however, oversimplification of the conceptualization of these responses could lead to missed opportunities for study and interpretation of results. Importantly, however, stress responses appear to comprise an essential component of not only ancestral but also of mammalian immune responses, such as when natural killer cells need to survive their own rampage against infections, during which they produce reactive oxygen species. The nematode might therefore turn out to be particularly instructive for the understanding of how the stress responses could balance the consequences of immune defenses. Given their intimate involvement in the regulation of longevity, stress response pathways could play a central role in alleviating the consequences of innate immunity driving the chronic inflammation during human aging. Deeper insight into the regulation of stress responses during the activation of innate immunity in *C. elegans* might therefore yield new conceptual avenues for counteracting the pathological consequences of chronic inflammation.

Future work on the responses of *C. elegans* to environmental challenges, from pathogens to chemicals, and even to radiation, will surely shed light on these questions and provide new and exciting avenues for further research.

Glossary

AGE-1 Ortholog of phosphoinositide 3-kinase (PI3K) p110 catalytic subunit.

AKT-1/-2 Homologs of serine/threonine kinase Akt/PKB ortholog of the serine/threonine kinase Akt/PKB.

ATF-7 Leucine zipper transcription factor; ortholog of CREB/activating transcription factors.

ATFS-1 bZip transcription factor involved in UPR^{mt}.

CEBP-2 Ortholog of human CCAAT7 enhancer binding protein gamma (CEBPG).

CED-13 BH3 domain-containing protein involved in apoptosis.
CEP-1 Ortholog of human tumor suppressor p53.
DAF-2 Receptor tyrosine kinase; insulin/insulin growth factor receptor ortholog.
DAF-4 Serine/threonine kinase; ortholog of type II transforming growth factor (TGF)-β receptor.
DAF-16 Forkhead box O (FOXO) transcription factor in insulin-mediated signaling.
DAF-28 Beta-type insulin; homologous to human insulin.
DBL-1 Member of transforming growth factor (TGF)-β super family.
DCAR-1 Ortholog of human neuropeptide FF receptors (1 and 2) and pyroglutamylated RF amide peptide receptor.
DCR-1 Ribonuclease involved in RNA interference.
DRH-1 Dicer-related helicase involved in RNA interference.
EGL-1 BH3 domain-containing protein involved in apoptosis.
LIN-45 Ortholog of vertebrate RAF protein.
MEK-2 Mitogen-activated protein kinase (MAPK) kinase involved in Ras-mediated signaling.
MPK-1 Mitogen-activated protein kinase (MAPK); ortholog of human extracellular signal-regulated kinase (ERK).
MUT-7 RNaseD homolog involved in RNA interference.
NPR-1 G-protein-coupled neuropeptide receptor; homolog of mammalian neuropeptide Y receptor.
NSY-1 Neuronal symmetry family member 1. Mitogen-activated protein kinase (MAPK) kinase; ortholog of mammalian ASK family of proteins.
OCTR-1 G-protein-coupled receptor involved in neuronal signaling.
PMK-1 Mitogen-activated protein kinase (MAPK); ortholog of human p38 MAPK, orthologous to human mi MAPK (OMIM:600289); MAPK, orthologous to human MAPK (OMIM:600289).
RDE-1 Argonaute and PIWI family protein.
RDE-4 Double-stranded RNA (dsRNA)-binding protein involved in RNA interference.
SEK-1 Ortholog of human mitogen-activated protein kinase kinases (MAPKK) 3 and 6.
SMA-2/-3/-4 Orthologs of SMAD proteins.
SMA-6 Serine/threonine protein kinase; orthologous to type I transforming growth factor (TGF)-β receptors.
TIR-1 Toll/interleukin-1 receptor domain adapter protein; ortholog of human SARM.
TOL-1 Toll-like receptor protein.
UPR^{mt} Mitochondrial unfolded protein response.

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Annelida: Oligochaetes (Segmented Worms): Earthworm Immunity, Quo Vadis? Advances and New Paradigms in the Omics Era

Péter Engelmann, Kornélia Bodó, József Najbauer, and Péter Németh

Immune Surveillance of Earthworms: A Brief Introduction

Morphological and Physiological Background

More than 8000 earthworm species have been described; their sizes vary greatly and they dwell in soil and freshwater habitats (Pirooznia et al. 2007). They possess an elongated, segmented body shape that bears numerous setae to help their locomotion. Most of these segments contain the same set of organs, except the shared digestive, circulatory, and nervous system along their body. Several important anatomical and physiological structures are specific to these animals, such as a secondary body cavity (e.g., coelomic cavity) and the closed, complex circulatory system with blood vessels that contain hemoglobin in suspension (Mill 1978). A specialized tissue compartment surrounds the gut (e.g., chloragogenous tissue) and has multiple important functions (e.g., carbohydrate metabolism, storage, detoxification and immune properties) resembling vertebrate hepatocytes (Jamieson 1981). The coelomic cavity is filled with a protein-rich fluid that contains various free-floating cellular components, termed coelomocytes (Fig. 1). Coelomocytes are considered to be the mesodermal effector cells of an earthworm's immune system. The body cavity is connected with the outer environment through nephridiopores and dorsal pores that ensure continuous microbial exposure and promote effective immune mechanisms in earthworms.

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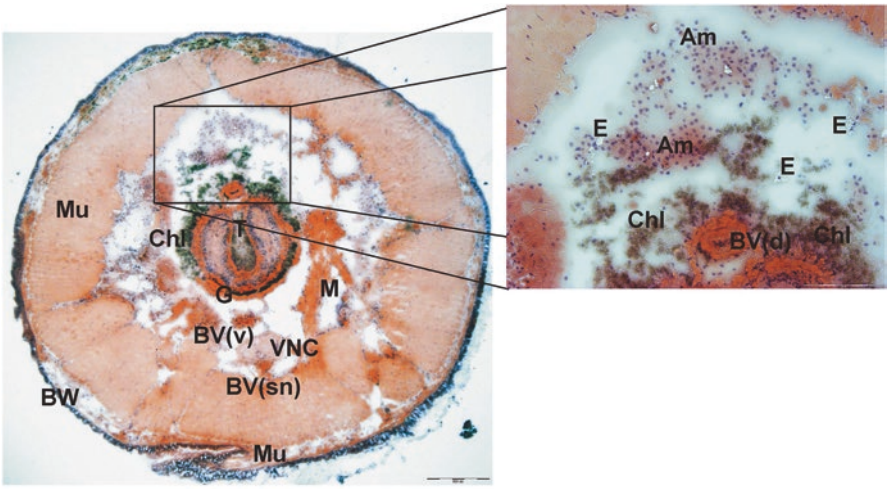

Fig. 1 Cross-section of *Eisenia andrei* earthworm. Main organs and tissue compositions are marked on the photomicrographs. Inset shows the distinct coelomocyte subpopulations (amoebocytes and eleocytes) of earthworm located in the coelomic cavity. Hematoxylin-eosin staining. Scale bars: 500 μm (main image) and 200 μm (inset). Am amoebocytes, BW body wall, BV (d) dorsal blood vessel, BV (sn) subneural blood vessel, BV (v) ventral blood vessel, Chl chloragocytes, E eleocytes, G gut, M metanephridium, Mu muscle layers, VNC ventral nerve cord

Back to the Roots

In the past century the field of immunology, including comparative immunology, gained enormous amounts of novel and important knowledge relevant to basic and applied biological sciences. Comparative immunological studies date back to Metchnikoff, who established the field of cellular immunology (Cooper et al. 2002; Silverstein 2001). In the late 1800s several pioneering morphological observations in earthworms identified the cellular constituents (e.g., coelomocytes) of the coelomic cavity (Liebmann 1942; Rosa 1896). Indeed, the nomenclature of these cell types is based on the categorization of vertebrate leukocytes, which is sometimes misleading because no direct homology exists among the immune cells of invertebrate and vertebrate organisms. Following early ground-breaking observations, several research groups studying earthworm immunity were initiated in the USA (established by Edwin L. Cooper), Germany (begun by Werner Mohrig), France (launched by Pierre Valembois), and Czech Republic (led by Jaroslav Rejnek). These research groups have carried out in-depth studies of earthworm immune mechanisms, ranging from transplantation experiments to the analysis of humoral and cellular immune components. Now earthworms are even used in biomedical applications and as an alternative food source (Cooper and Balamuragan 2010; Cooper and Hirabayashi 2013). With regard to the University of Pécs, Hungary, in the 1970s Ernő Fischer initiated several experiments investigating chloragogenous tissues and their physiological role in earthworms after exposure to different toxicants (Fischer 1977; Fischer and Molnár 1992).

Many excellent reviews and book chapters (including those present in this volume) summarize the recent advances of earthworm immunity (Bilej et al. 2010; Cooper et al. 2002, 2006; Engelmann et al. 2016a). In the following section we provide a brief survey of our contribution in this research area. Furthermore, we discuss some novel observations that might open new research directions in the field of earthworm immunity.

Application of Monoclonal Antibodies in Earthworm Immunity

Monoclonal antibodies (mAbs) are major scientific tools used to study vertebrate (mainly mouse and human) immune functions. Interestingly, very few studies are available about the application of mAbs in the field of earthworm immunology.

According to Cossarizza et al. (1996), mouse mAbs raised against mammalian antigens unequivocally identify one subgroup of coelomocytes denoted as small, electron-dense cells. This cell group showed cross-reactivity with a-CD90 (Thy-1), a-CD54, a-CD11a, a-CD45RA/CD45RO, a-CDw49b, and a-β_2-microglobulin, whereas the large, electron lucent cells were negative for these reactions. Furthermore, these phenotypic differences were mirrored in their functionality because small coelomocytes were cytotoxic against certain tumor cell lines, whereas the large cells were phagocytic. As a follow-up study, first we applied several mouse mAbs developed against conserved mammalian cell surface molecules (Thy-1, CD24), cytokines (tumor necrosis factor [TNF]-α, transforming growth factor-α), oxidative stress enzymes (Cu/Zn superoxide dismutase), and hormones (thyroid-stimulating hormone) (Engelmann et al. 2002; Wilhelm et al. 2006). Among the obtained results, we believe that the cross-reactivity of a-TNF-α mAb with earthworm cellular structures is a unique observation (Engelmann et al. 2002). Our immunoserological, immunohistochemical approach proved that a conserved epitope of a TNF-like molecule might exist in earthworms. Since our study, several TNF-family homologs have been cloned and described in various invertebrates (Parinello et al. 2008; Sun et al. 2014). Considering these supportive data, it seems that cytokines with an essential role in inflammation might have emerged early during evolution (Beschin et al. 1999; Engelmann et al. 2005a; Wiens and Glenney 2011). Interestingly, leech immune cells also show cross-reactivity with a panel of mAbs specific against mammalian myeloid antigens (de Eguileor et al. 2000). The current data should be supported by further experiments, including isolation and identification of cell surface antigens and adhesion molecules in annelids.

The aforementioned studies used antibodies generated against mammalian antigens because of the lack of coelomocyte-specific reagents, which potentially raises criticism about the specificity of these antibodies to antigens in annelids.

To fill this gap we developed a panel of specific mAbs (EFCC [*Eisenia fetida* coelomocyte cluster] clones) against earthworm (*Eisenia* spp.) coelomocytes to further analyze the distinct features of coelomocyte subsets (hyaline amoebocytes, granular amoebocytes, and eleocytes) (Engelmann et al. 2005b). With the aid of these molecular tools we were able to phenotypically and functionally characterize

different subsets of coelomocytes combining cell sorting with imaging and functional assays (Engelmann et al. 2016b). We confirmed that hyaline and granular amoebocytes are capable of phagocytosis and encapsulation, although granular amoebocytes engulf fewer foreign particles than do hyaline cells (Engelmann et al. 2005b; Fuller-Espie 2010). Eleocytes show no phagocytic properties (Stein et al. 1977), but they possess several metabolic functions including the production of bioactive molecules such as lysenin (Kobayashi et al. 2004; Lassegues et al. 1997; Opper et al. 2013; Valembois et al. 1982). Using a novel mAb (a-EFCC5) reacting with lysenin, we obtained data in support of the view that eleocytes and large amoebocytes, but not sessile chloragocytes, are the major source of lysenin. We observed that lysenin production in earthworm coelomocytes is modulated in vitro by exposure to Gram-positive bacteria (Opper et al. 2013). Along with other studies we demonstrated that coelomocytes exert a very strong cytotoxicity in addition to their phagocytic activity (Engelmann et al. 2004). As a follow-up to our initial cytotoxicity study, we showed in extensive downstream experiments that, indeed, lysenin is one essential, but not exclusive, member of the earthworm cytotoxic molecular palette (Mácsik et al. 2015).

Recently, we obtained insightful data about the in vitro interactions of coelomocytes and silver nanoparticles (Hayashi et al. 2012). With the aid of recombinant lysenin and our lysenin-specific mAb (a-EFCC5), we observed that lysenin is an essential molecular component of the protein corona formed around silver nanoparticles. Furthermore, lysenin-rich protein corona directly facilitates the cellular uptake of silver nanoparticles (Hayashi et al. 2013).

Indeed, we gathered a handful of phenotypic and functional information about the cellular immune components of earthworms; however, there are several unexplored areas that remain to be investigated further. For instance, some publications discuss the proliferation capacity and recovery of coelomocytes in the coelomic cavity (Dvořák et al. 2016; Engelmann et al. 2011; Homa et al. 2013; Roch 1979). There is still no clear consensus whether all coelomocyte subtypes are able to proliferate or only certain subtypes. Is there lineage commitment in these cell types, and which coelomocyte types can be considered as progenitor cells?

Transcriptomics in Earthworm Immunity

Digging for Information: Earthworm Nucleotide Databases

This chapter focuses on earthworm immunity, but we think it is essential to have a short overview of the molecular databases dealing with earthworms. Currently, earthworms are frequently used in studies testing specific target genes, isolating Expressed Sequence Tags (ESTs), or performing whole transcriptome analysis to uncover the effect of certain toxicants (Gong and Perkins 2016).

These “omics” studies cover multi-platform genomics, transcriptomics, proteomics, and metabolomics (Brulle et al. 2010). Simultaneous informatics and statistical approaches allow the extraction of information from the multivariate datasets

to illustrate how interdependent pathways of genes respond to different stimuli. For this research purpose, publicly available nucleotide databases (in addition to the NCBI [National Center for Biotechnology Information]) are extremely relevant.

In the case of earthworms, *Lumbricus rubellus* was initially used to build an EST library (Stürzenbaum et al. 2009). More than 17,000 tags that represent approximately 8000 genes are incorporated in this database. This information was the basis to construct a publicly available genome library, the LumbriBASE (www.earthworms.org). Certain genes show significant homologies with their correspondent genes isolated from *C. elegans*, *Drosophila melanogaster*, and *Homo sapiens*. Interestingly, from the different gene cohorts of earthworms, the highest numbers of significance were evidenced with the human counterparts. Later, the abovementioned *L. rubellus* database was further developed into another database incorporating the EST sequences of *Helobdella robusta* leech and *Capitella teleta* polychaete species (http://badger.bio.ed.ac.uk/earthworm/) (Elsworth et al. 2013); however, this database is currently not accessible. Recently, another available earthworm nucleotide database has been released based on the draft genome of *Eisenia fetida* (http://ryanlab.whitney.ufl.edu/genomes/Efet/) (Zwarycz et al. 2015). These databases are invaluable bioinformatics tools to those researchers who plan to target certain genes in their studies.

Transcriptomics and (Eco)Toxicology of Earthworms

Due to human industrial and economic activities, environmental contamination is rapidly increasing. Earthworms are eco-engineers that are responsible for the quality control of our terrestrial environment (Darwin 1881). These soil sentinels are more frequently applied in various (eco)toxicological studies than in other scientific fields. Indeed, *Eisenia* spp. is recommended by the Organisation for Economic Co-operation and Development (OECD) as a well-established organism for ecotoxicological testing of certain toxic chemicals (OECD 1984, 2004). Using the standard in vivo readouts (e.g., survival, reproduction, growth, soil avoidance test) these animals are the practical bioindicators for different environmental toxicants (Velki and Ečimović 2017). During the last two decades the aforementioned classical ecotoxicological toolbox has been complemented with novel molecular biological approaches aimed at untangling the questions concerning ecological and environmental stressors (Brulle et al. 2010; Thunders et al. 2017). Ecotoxicological experiments now frequently apply transcriptomic, proteomic, metabolomic, and bioinformatic approaches to monitor the ecological effects of polluted soils on earthworms (Stürzenbaum et al. 2009). Transcriptomic analysis has certain benefits over the classical ecotoxicological tools. First, sensitivity: gene expression will be more sensitive; fluctuation of expression patterns can be detected at lower exposure concentrations. Second, specificity: gene expression will be specific to the triggering stress mechanisms, unlike reproduction or soil avoidance. Third, rapidity: the gene expression pattern changes quickly, on the order of hours to days, while classical tests could last weeks (Gong et al. 2010). On the other hand, transcriptomic

information may not always be reliable at the protein level, so the experiments are more complete if the transcriptomic results are complemented with proteomic and metabolomic studies. To this end, earthworms can provide sensible and reliable biomarkers (e.g., metallothionein, phytochelatin synthase, riboflavin) to monitor the health status of the soil ecosystems (Brulle et al. 2006; Stürzenbaum et al. 2004; Plytycz et al. 2010). Recently, several regulatory agencies have discussed how genomic tools could fit into the risk assessment process.

In the last 20 years approximately 60 studies have dealt with earthworms in the field of toxicology and genomics. Within these studies a broad array of toxicants have been tested, ranging from metals, insecticides, pharmaceutical drugs, and organics to explosives. Indeed, these studies highlight the recently emerged interest in these areas of research. It is quite striking that certain toxicological studies applying parallel metabolomics and transcriptomics resulted in a significant overlap between the effects elicited by different compounds (Van Straalen and Roelofs 2008).

Immunotranscriptomics of Earthworms

Why is transcriptomic information important in studies of earthworm immunity? Largely because stimuli that reach coelomocytes can be measured at the gene expression level and this is among the first biological parameters to change upon exposure to stressful conditions. Some specific studies have directly analyzed the transcriptome of coelomocytes; however, this was mostly in the ecotoxicological and not immunological context (Brulle et al. 2006).

One initial study analyzed 1151 ESTs directly from a complementary DNA (cDNA) library of coelomocytes and found 659 unique genes, among which 24 were immune- and defense-related genes (Tak et al. 2015). Overall, the most abundant genes were related to energy metabolism; lysenin-related protein has evidenced the highest frequency among the immune-related genes. Furthermore, they proved that mRNA expression of lysenin-related protein was modulated in vivo only by Gram-positive bacteria (*Bacillus subtilis*) challenge, which is consistent with our findings on the protein level of lysenin expression following in vitro exposure to bacteria (Opper et al. 2013).

Another transcriptomic study applied next-generation sequencing (NGS) on an Illumina platform to identify characteristic genes for the earthworm immune system (Mikami et al. 2015). For this purpose, coelomocytes were isolated from *E. fetida* reared in a sterile environment. Isolated coelomocyte mRNAs were subject to NGS and resulted in 151,929 contigs. From these contigs 41,423 unigenes were identified, and among those 12,285 unigenes had annotations in NCBI. Based on gene ontology assignments several unigenes were assigned to immune system processes, notably to immune receptor signaling pathways. Indeed, the Toll-like receptor (TLR) adaptor protein myeloid differentiation primary response gene 88 (MyD88) was found to be a unigene, confirming other previous datasets (Gong et al. 2008; Hayashi et al. 2016), while a homolog for Janus kinase (JAK) 2, a member of JAK-STAT

(Signal Transducer and Activator of Transcription) signaling, was also identified (Mikami et al. 2015). Several lines of evidence suggest that ancient natural killer (NK)-like activity exists in earthworms (Cossarizza et al. 1996; Engelmann et al. 2004; Kauschke et al. 2001); however, so far the NK-specific molecular fingerprints (e.g., homolog for perforin, granzymes, etc.) have not been available. Mikami et al. (2015) found an *Eisenia* homolog of the β-1,3-glucuronyltransferase 1 (*GlcAT-P*) gene that participates in the synthesis of the CD57 NK cell marker of vertebrates. In fact, this gene shows high similarity to human *GlcAT-P*; however, the molecule has other functions (e.g., neurobiological processes).

There have been only a few transcriptomic studies dealing with earthworm immunity; however, this field is rapidly developing. The high number of genes that remain to be identified could shed more light on the physiology of coelomocytes. On the other hand, earthworm coelomocytes represent a mixed population of eleocytes and amoebocytes. This dichotomy could manifest at several biological levels (Hayashi and Engelmann 2013) that should be taken into account during the (gen)omic approaches. Eleocytes and amoebocytes can be separated by cell sorting (Engelmann et al. 2016b) to resolve this issue and apply the well-characterized single populations in various downstream 'omic' applications.

Epigenetics: From Basic Concepts to Worms as Pivotal Model Organisms

Basic Concepts in Epigenetics: DNA Methylation, Histone Modifications, and RNA Interference

The eukaryotic cell nucleus contains the genomic DNA, associated nuclear proteins (histones, transcription factors, etc.), and various RNA species that comprise the chromatin. The structure of chromatin determines the expression of genes and the flow of genetic information to a large degree, and many of these processes are regulated by epigenetic mechanisms. Epigenetics means above/upon (epi) genetics that consist of heritable changes in gene expression or phenotype that are stable between cell divisions, and sometimes between generations, but do not involve changes in the underlying DNA sequence of the organism. The combination of histone and DNA post-translational modifications and related interacting proteins helps to define the transcriptional program in a given cell. In the context of epigenetic signaling, post-translational modifications are often called 'marks' or 'tags' (Arrowsmith et al. 2012).

DNA methylation involves covalent modifications of CpG (cytosine–guanine) dinucleotides. The majority of methylated CpG dinucleotides in vertebrates are in heterochromatic regions, often in promoter CpG islands (of note, the worm *C. elegans* lacks detectable levels of 5-methylcytosine, while the fly *D. melanogaster* contains very low levels of 5-methylcytosine) (Bird 2002). The cytosines in CpG dinucleotides become 5-methylcytosines as a result of DNA methylation. Among its functions, DNA methylation in mammals is required for gene silencing. As a result

of DNA methylation, the chromatin becomes more condensed (transcription repressed). DNA demethylation results in chromatin expansion (transcription permitted) (Gaspar-Maia et al. 2011).

Another type of DNA modification, N^6-methyladenine (6 mA), was identified first in prokaryotes and some unicellular eukaryotes but has recently been found in metazoans. The function of this modification in metazoans is not well-defined; however, it is postulated that it may be involved in gene activation and transposon suppression (Luo and He 2017). It is likely that future studies will identify proteins involved in 6 mA writing, reading, and erasing, which should help in determining the functional relevance of the this type of DNA modification.

Covalent modifications of histones are essential to regulation of chromatin dynamics, and thus affect numerous biological processes. One of the most frequent post-translational modifications of histones is methylation of lysines and arginines (Greer and Shi 2012). Lysines in histones can be non-methylated, or mono-, di-, or trimethylated on the ε-amino group of the lysine side chains. Arginines can be non-methylated, N-monomethylated or N,N-dimethylated on the side chain guanidino group. Lysines in histones can also be acetylated and ubiquitinated, whereas arginines in histones can be citrullinated. Other modified amino acids in histones include phosphoserine, phosphotyrosine, and ribosylated glutamic acid (Campos and Reinberg 2009). Propionylated and sumoylated (SUMO [small ubiquitin-like modifier]) amino acids can also be found in histones. Many of these molecular 'tags' that establish the so-called 'histone code' are in a dynamic state, i.e., such tags can also be removed (Arrowsmith et al. 2012). These tags and proteins, together with DNA methylation and demethylation, comprise the dynamic components of the epigenome.

The Nobel Prize in Physiology or Medicine in 2006 was awarded to American scientists Andrew Z. Fire and Craig C. Mello for their discovery of RNA interference and gene silencing by double-stranded RNA. The discovery was made in the nematode worm *C. elegans* by observing the phenotypic effect of injection of single-stranded or double-stranded *unc-22* RNA into the gonad of *C. elegans* (the *unc-22* gene encodes a myofilament protein). Injected double-stranded RNA, but not single-stranded RNA, induced the twitching phenotype in the progeny. RNAi-based regulatory processes are involved in numerous biological processes, including protection against viral infections, securing genome stability by keeping mobile elements silent, epigenetic regulation, chromatin remodeling, genome integrity/stability, developmental gene regulation, and so on. The RNA species that regulate such processes are, among others, long non-coding RNAs (lncRNAs), small non-coding RNAs (sncRNAs), microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), and short interfering RNAs (siRNAs) (Gomes et al. 2013) (Fig. 2). RNAi can be used to modulate gene expression and has provided a powerful tool for studying gene function, and is likely to have great impact on treatment of diseases (Castanotto and Rossi 2009; Pecot et al. 2011).

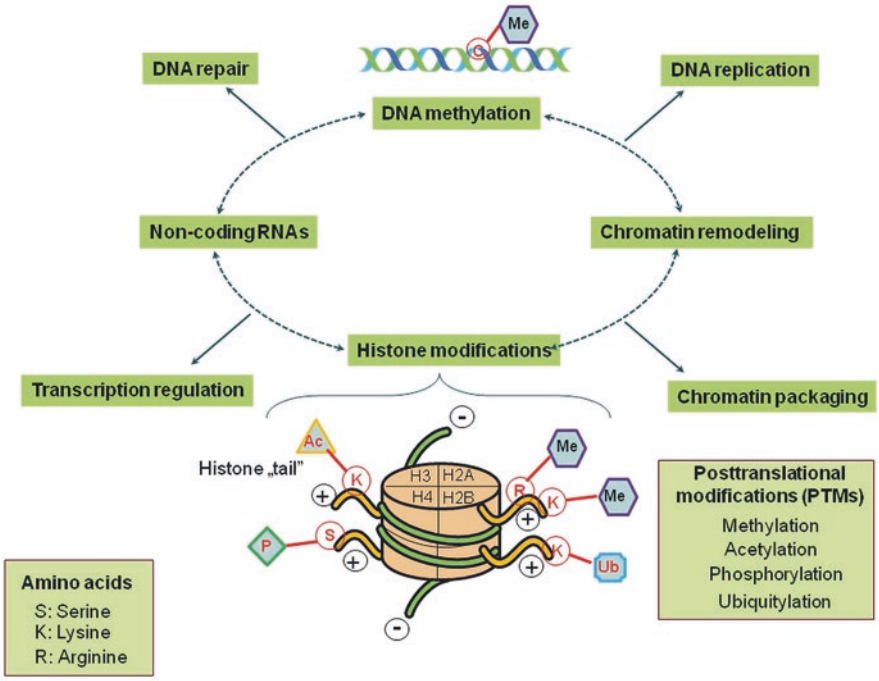

Fig. 2 Schematic summary of epigenetic mechanisms that affect the nuclear structure, function, and gene expression. Epigenetic components include DNA methylation, histone post-translational modifications, chromatin remodeling, and non-coding RNA targeting. These components and their interplays contribute to the regulation of crucial nuclear activities such as DNA replication, repair, chromatin packaging, and transcription. The nucleosome subunit is shown with schematic summary of histone modifications that comprise the histone code

Epigenetic Mechanisms in Earthworms

MicroRNAs in Earthworms

Investigation of epigenetic mechanisms in earthworms has been advancing at a significantly slower pace than in the fruit fly *D. melanogaster* or the nematode worm *C. elegans*. Nevertheless, earthworm epigenetics are starting to emerge, especially in the context of ecotoxicology, but also at the basic science level, such as concerning miRNAs as regulators of the epigenome. miRNAs are an abundant new class of sncRNAs that average 22 nucleotides in length and which play important roles in regulating gene expression in a wide range of processes from cell differentiation and development and immunological defense mechanisms to learning and memory (Follert et al. 2014; Kosik 2009; Mehta and Baltimore 2016; Self-Fordham et al. 2017). Gong et al. identified 32 miRNAs belonging to 22 families using earthworm EST databases and homology-based EST analysis with secondary structure requirements (Gong et al. 2010). They found that these earthworm miRNAs share multiple features with known animal miRNAs, such as the nucleotide U being

dominant in both mature and pre-miRNA sequences. Using in silico analysis they identified 436 unique target ESTs for 30 of the 32 earthworm miRNAs. Interestingly, the potential target genes were predicted to be involved in biological processes and pathways related to development, growth, and reproduction, as well as the response to stress, particularly oxidative stress. The authors suggested that deep-sequencing technologies are likely to help in further characterization of both conserved and species-specific earthworm miRNAs (Gong et al. 2010). In another study, Huang et al. investigated the role of miRNAs in tissue regeneration in the earthworm *E. fetida* (Huang et al. 2012). The authors cloned and characterized 17 non-coding RNAs from this species and found four small RNAs (efe-let-7, PB1–1, PB7, PB10) that showed higher expression in regenerating tissues than in normal control tissues, suggesting their role in the regeneration process (Huang et al. 2012).

Earthworm Epigenetics in an Ecotoxicological Context

Earthworms, especially *Eisenia* spp. and *Lumbricus* spp., are widely used as sentinel species and serve as bioindicators/biomonitors for soil ecosystems due to their close contact with the environment. Earthworms have also been used extensively to assess environmental risk and chemical toxicity in laboratory and field settings (Gong and Perkins 2016). With advances in epigenetics, earthworms are increasingly used to determine the effects of ecotoxicological factors and environmental stressors on the epigenome (Vandegehuchte and Janssen 2014). In an earlier study, Santoyo et al. studied global DNA methylation in earthworms and suggested that it may serve as a candidate biomarker for epigenetic risks related to the presence of metals in soil (Santoyo et al. 2011). The authors have found a direct correlation between soil and tissue content of As, Se, Sb, Zn, Cu, Mn, Ag, Co, Hg, and Pb, and have observed an inverse correlation between the percentage of methylated DNA cytosines and total tissue and some of these metals (individually or in combination). A recent study showed that low levels of Cd (a common soil pollutant) induced persisting epigenetic modifications, which may mediate acclimation of the earthworm *Lumbricus terrestris* (Šrut et al. 2017). As mentioned, these and other studies highlight the use of earthworms as sentinel species in studying the ecotoxicological effects on the epigenome, and aid in formulating important questions and tasks: (1) Are the epigenetic changes causal to phenotypic alterations?; (2) The long-term persistence of transgenerationally transferred epigenetic and phenotypic alterations needs to be assessed in more detail; and (3) What are the consequences of interaction between the environment and the epigenome at the population level? (Vandegehuchte and Janssen 2014).

Innate Immunity, Epigenetics, and Invertebrates

Macrophages are ancient phagocytes that are prevalent in invertebrates as well as vertebrates. Monocytes exposed to different pathogens differentiate into macrophages with various functional properties. There is compelling evidence that this differentiation process is regulated by epigenetic mechanisms (Logie and

Stunnenberg 2016). Macrophages exposed to β-glucan (fungal cell wall component) undergo H3K27 acetylation affecting several thousand gene enhancers. Similarly, lipopolysaccharide (LPS) or bacillus Calmette-Guérin (BCG) stimulation also induces such events in the promoter and enhancer regions of certain genes. Engagement of pattern recognition receptors (TLR, nucleotide oligomerization domain [NOD], scavenger receptors, etc.) is a prerequisite for microbe engulfment. Emerging data suggest that TLR expression and the downstream macrophage activation is dependent on histone deacetylation (Hennessy and McKernan 2016).

A novel concept suggests the existence of innate immune priming or innate immunological memory (also termed trained immunity) that involves epigenetic mechanisms (e.g., trimethylation of H3K4 in monocytes). These epigenetic modifications enhance intracellular activation and cytokine release and modify the cellular metabolism of monocyte/macrophage lineage (van der Meer et al. 2015). In addition, chromatin remodeling is essential to orchestrate the balance between the classically and alternatively activated macrophages (Quintin et al. 2012).

Similarly to the vertebrate counterparts, *Drosophila* macrophages (e.g., plasmatocytes) show extreme diversity in function, ranging from microbe phagocytosis to the clearance of apoptotic bodies during embryonic development. This type of activity is controlled by certain epigenetic mechanisms and is associated with immune priming (Weaver and Wood 2016). Hence, a memory component of innate immunity in insects and other invertebrates has been debated for a long time (Hauton and Smith 2007; Kvell et al. 2007; Milutinovic and Kurtz 2016).

Interestingly, host–parasite co-evolution provides solid experimental evidence for the existence of immune priming or even transgenerational immune priming (Vilcinskas 2016). In an extensive experimental set-up, the wax moth *Galleria mellonella* larvae were fed on different bacteria-containing diets. The microbe-contaminated diet induced a biased expression of immune response-related genes in various tissues (including the eggs). Proteomic analysis of the eggs revealed unique immune-related gene products similar to those in the exposed larvae. These findings were also verified in the beetle *Tribolium castaneum*. To define the mechanism of these transcriptional changes, more attention was focused on epigenetics during host–pathogen interactions. Interestingly, it seems DNA methylation events were not involved in the course of transgenerational priming (the parenteral generation was exposed to different bacteria strains), but appeared during the parasitic fungi infections. In contrast, histone deacetylation was evident in a parasitic fungi infection of *G. mellonella*, resulting in immune suppression of the host organism (and delay of larval development). As for miRNA, several small RNA species were identified and differentially expressed in the parasitic fungus-infected *G. mellonella* (Vilcinskas 2016). It seems histone modifications and miRNA biogenesis are both involved in the transgenerational immune priming.

We currently have little information concerning earthworm immunity and epigenetics; thus, this field is wide open for future studies which may generate valuable data in basic research and biomedical sciences.

Co-evolution of Microbiota and the Immune System in Invertebrates

Animals in a Bacterial World: The Metazoan–Microbial Holobiont

It is increasingly evident that animal biology is affected by the broad range of animal–bacterial interactions that involve shared ecosystems and symbiotic relationships. New thinking starts to prevail about fundamental questions such as: How have bacteria facilitated the origin and evolution of animals? How do animals and bacteria affect each other's genomes and epigenomes? How does animal development depend on bacterial partners? How do animals and their symbionts maintain their homeostasis in a symbiotic relationship? And how can ecological and other approaches deepen our understanding of the different levels of animal–bacterial interaction (McFall-Ngai et al. 2013)? A new scientific field is emerging named "ecological evolutionary developmental biology" (Eco-Evo-Devo), which has the goal of incorporating the rules governing the interactions between an organism's genes, epigenome, development (developmental plasticity), and environment (biotic and abiotic) into evolutionary theory. The metazoan host plus its persistent symbionts that form the holobiont may be considered as an important unit of evolutionary selection (Gilbert et al. 2015).

Microbial symbionts can exert multiple effects on the host, including development (e.g., induction of developmental transitions and affecting developmental plasticity), nutrition (e.g., processing and transformation of nutrients), and host protection by competition against detrimental/pathological microbes and induction of immune system maturation and modulation of immunity (Selosse et al. 2014). The immune system, in return, has evolved largely to maintain the symbiotic relationship of the host with these useful, highly diverse, and evolving microbes while protecting the host from potentially harmful microbial agents (Belkaid and Hand 2014).

Metazoan Innate Immunity and Microbiota: Insight from *Hydra*

Cnidaria represent a sister group to Bilateria, and are divided into five classes, one of which is the Hydrozoa, which include several *Hydra* species. Symbiotic relationships between *Hydra* and photosynthetic algae have been studied for decades. More recent research has dealt with bacteria, which are important symbiotic components of the *Hydra* holobiont (Bosch 2013). These research data show that the innate immune system of the epithelium (mobile phagocytes are absent) in the *Hydra* largely determines the bacterial colonization of the epithelial surfaces. A highly conserved signaling cascade that involves TLR, MyD88, and nuclear factor (NF)-κB is of central importance in sensing microbes. Different species of *Hydra* are associated with species-specific microbiota, which suggests that the host selectively shapes the symbiotic bacterial community and underscores the

importance of host genetic (and likely epigenetic) factors in determining microbial colonization of epithelial surfaces. Antimicrobial peptides are major effector components of the innate immune system of the *Hydra*. These peptides, in addition to protecting the host from microbial pathogens, are key regulators of the composition of microbiota that colonize the *Hydra* host. The forkhead box O (FOXO) transcription factor, which is involved in the maintenance of stem cells, might also be involved in controlling the synthesis of antimicrobial peptides, and thus could serve as a link between tissue homeostasis, the innate immune system, and composition of resident microbiota (Bosch 2013, 2014; Schröder and Bosch 2016).

Host–Microbiota Interactions in Insects

All insects are colonized by microbiota that comprise bacterial, archaeal, and eukaryotic organisms (e.g., fungi and various unicellular eukaryotes), and viruses that impact multiple aspects of insect biology (Douglas 2015). These microorganisms can establish residence in multiple organs of the insect, including the insect exoskeleton, gut lumen, and hemocoel, and within cells of the insect. For example, the body surface of *D. melanogaster* contains ~1000 bacterial cells. The insect gut, especially the hindgut, is favorable for colonization by microorganisms. The midgut is less favorable for microbial colonization because it secretes numerous enzymes and has a heightened immune response. A pH < 3 region is present in the midgut that likely kills many microbial cells. Thus, the factor that influences the composition and abundance of insect microbiota is to a large degree the immune system of the insect, but physico-chemical conditions also play a role, as well as interactions among the colonizing microorganisms. Microbiota can modulate the insect immune system and can protect their hosts against pathogenic microbes, including viruses, bacteria, and parasitoids (Douglas 2015).

Insect viruses, especially the arthropod-borne viruses (arboviruses) such as Dengue, West Nile, Chikungunya, and others can cause disease in humans and pose a major global health burden (Jupatanakul et al. 2014). The resident microbiota of insects can potentially be exploited as modulators of insect vector competence.

Gut microbial communities have been extensively studied in social bees (Kwong and Moran 2016). The gut microbiome of honey bees (*Apis mellifera*) shows similarities to the mammalian, including human, microbiota (both are mostly composed of host-adapted, facultatively anaerobic and microaerophilic bacteria), but the microbiome of the bee gut is much simpler than the mammalian microbiota (only nine bacterial species clusters are specific to bees, and these bacteria are transmitted through social interactions between individual bees) (Kwong and Moran 2016). In addition to serving as model organisms in microbiome research, bees are also of global economic importance because they are (together with bumble bees) the main pollinators of many crops and wild flowering plants. For these reasons, studying the bee gut microbiome is of great importance.

The Earthworm Microbiome

Earthworms are oligochaete annelids used in ecotoxicology, comparative biochemistry, physiology, regeneration, and innate immune system studies, and, more recently, studies related to the earthworm microbiome. In 2013, Dvořák and colleagues reported data on the microbial environment and its effects on innate immunity in two closely related earthworm species *E. andrei* and *E. fetida*, which inhabit different ecological niches (*E. andrei* lives in compost and manure, *E. fetida* can be found in the litter layer of forests) (Dvořák et al. 2013). Coelomic cytolytic factor (CCF; a pattern recognition protein) and lysozyme showed only slight differences in expression level and activity, whereas fetidin/lysenin expression and hemolytic activity was higher in *E. andrei* than in *E. fetida*. After exposure of *E. fetida* to compost microbiota, expression of fetidin/lysenin was not affected (the reasons for which are not well-understood). Importantly, genomic DNA analyses revealed an approximately twice as high number of fetidin/lysenin gene copies in *E. andrei* than in *E. fetida*. The authors hypothesized that *E. andrei*, by colonizing and adapting to compost as a new habitat, may have acquired an evolutionary advantage that is reflected in higher expression of antimicrobial proteins. In another study, the same group reported on the earthworm's immune defenses against infection (Dvořák et al. 2016). This latter work suggested that microorganisms are sensed by pattern recognition receptors in the gut, and this information might be transferred into the adjacent mesenchymal lining that contains precursors to coelomocytes (Dvořák et al. 2016).

Liu et al. carried out a comparative study of gut microbiota of earthworms (*E. fetida*) that were fed in three different substrates: nutrient-poor soil, mineral soil, or organic-rich compost soil (Liu et al. 2017). High-throughput sequencing was used to characterize the earthworm gut microbes. Data showed that the microbial community in the earthworm gut was dominated by microbial taxa Proteobacteria, Firmicutes, Actinobacteria, Chloroflexi, and Bacteroidetes. There was a core microbial community in the earthworms' gut, but the total gut microbial composition differed between substrates. Proteobacteria and Bacteroidetes were more abundant in earthworms kept on organic-poor substrates than in the gut of earthworms kept in organic-rich compost soil. It was postulated that these bacteria may help earthworms survive in a nutrient-poor environment. In an unrelated study using *E. andrei* earthworms, we analyzed the microorganisms on the body surface and in the coelomic cavity and have identified numerous microbes (Table 1). Interestingly, despite the use of different species, different anatomic sites, and different environments, there was a partial overlap between the microbial species identified by Liu et al. (2017) and those identified in our study (Table 1) (overlapping microbes were *Acinetobacter* and *Aeromonas*). Procházková et al. studied the activity of digestive enzymes and non-self recognition in the gut of *E. andrei* earthworms. They showed a correlation between expression of CCF, lysozyme, and other enzyme activities in the gut of earthworms, following a challenge with various microbes. The data suggested that enzymes that play important roles in molecular pattern recognition and effector pathways are modulated during microbial challenge.

Table 1 Isolated microorganisms from *Eisenia andrei* earthworms

Body surface	Coelomic cavity
Aeromonas hydrophila	*Aeromonas hydrophila*
Aeromonas media	*Aeromonas media*
Pseudomonas alcaliphila	*Aeromonas caviae*
Serratia marcescens	*Serratia marcescens*
	Pseudomonas anguilliseptica
	Acinetobacter lwoffii
	Comamonas testosteroni
	Comamonas aquatica

Laminarinase (β-glucanase) and glucosaminidase activities were increased in addition to upregulation of CCF and lysozyme expression (Procházková et al. 2013).

Another study reported that *L. terrestris* earthworms fed a crystalline cellulose diet had a longer lifespan than high energy-fed earthworms (Rudi and Strætkvern 2012). Further, independent of the feeding regimen, earthworms that contained Ferrimonadaceae bacteria showed an increased lifespan.

Finally, it should be noted that in addition to the exciting research summarized herein, network-based analytical approaches are starting to be used to disentangle and better understand the interactions between the microbiome and the host that comprise the holobiont, as well as to reveal the complex interactions within the polymicrobial microbiome (Layeghifard et al. 2017).

Regeneration, Immunity, and Earthworms

About Regeneration: A Concise Introduction

Since the pioneering observations of Abraham Trembley, Charles Bonnet, and Lazarro Spallanzani, studies on animal regeneration have been extensively reported (Dinsmore 2001). Indeed, the mystery of restoration mechanisms was what excited Thomas Hunt Morgan most in his early scientific career (Morgan 1901). He also determined the ways that the injured body parts are regenerated in various organisms (earthworms, fish, and tadpoles). Organs and tissues are restored by a regeneration process called epimorphosis. During this type of regeneration, stem cells/progenitor cells (named neoblasts in some model species) become activated, proliferate, and form a regenerating blastema. Subsequently, this blastema differentiates into an emerging bud to form the restored body parts. Additionally, other regenerating mechanisms such as morphallaxis are also involved in the body restoration. In this case, intact cells reorganize the injured tissue or severed organ/organism without stem cell involvement and proliferation (Mainschein 2011). According to the recent literature, morphallaxis operates mainly in invertebrates, while epimorphosis exists in both invertebrate and vertebrate species. Surprisingly, there is a strong variation in the regeneration capacities even within closely related species. For instance,

annelids, like many other invertebrate animals, are able to replace lost body parts (segments) by regeneration (Dinsmore 2001). However, this ability is prominent in certain annelid species but not in others; for example, leeches do not regenerate their lost segments. Most of the *Oligochaeta* earthworms can regenerate both anterior and posterior body parts (Bely 2006).

On the other hand, regeneration is considered to be an alternative process of development because both regeneration and development are terminated at the same endpoint; the adult body plan will be built or restored. Until recently, only a few studies tried to directly compare regeneration with embryogenesis, but it is unlikely that there are distinct programs between these two biological processes (Myohara 2004; Zattara and Bely 2011). For better understanding of these mechanisms, additional molecular biological approaches need to be carried out.

Indeed, multicellular organisms have variable regenerative capacity. Considering the ancestries of regeneration it is frequently noted that in some invertebrates the capability to reproduce asexually is tightly combined with regeneration. It seems that restorations of certain injured body parts (e.g., limbs and eyes) are restricted in the course of evolution (Fig. 3).

Regenerative Capacity and Immune Response

Interdependency of inflammation and regenerative capacity dates back to some invertebrates appearing early in phylogenesis. Recent data claim that regeneration of injured body parts in various organisms is inversely correlated to the complexity of their immune response (Eming et al. 2007; Godwin et al. 2013). One of the first clues on the crosstalk between regeneration and immunity was derived from the lens restoration process of newts (Godwin and Brockes 2006); in a specialized sequence of events (so-called transdifferentiation), the dorsal iris cells mediate the replacement of the damaged lens. Additionally, the immune response is triggered by a damaged lens structure. On the other hand, fin regeneration in zebrafish is not dependent on the recruitment of inflammatory cells (e.g., neutrophils and macrophages) to the site of injury (Mathew et al. 2007). Upon injury, the host immune response not only defends against infections, but facilitates the removal of cellular debris at the site of the wound. Among tailed Amniotes, only lizards are able to regenerate their tail; in contrast, their removed limbs are passed into a scar or tail-like outgrowths. Transcriptome analyses revealed strong downregulation of immunoglobulin and surface B and T receptor genes in the tail blastema of *Podarcis muralis.* In contrast, upregulation of inflammatory genes was observed in the limb blastema. It is probable that the regenerating tail demands immune suppression. The development of adaptive immunity is hypothesized to induce scarring instead of the regeneration process, which might be a significant obstacle to tissue regeneration in higher vertebrates (Vitulo et al. 2017).

Despite its vital importance in physiology, the positive, negative, or neutral mechanisms that orchestrate the regeneration process and the immune response are poorly understood.

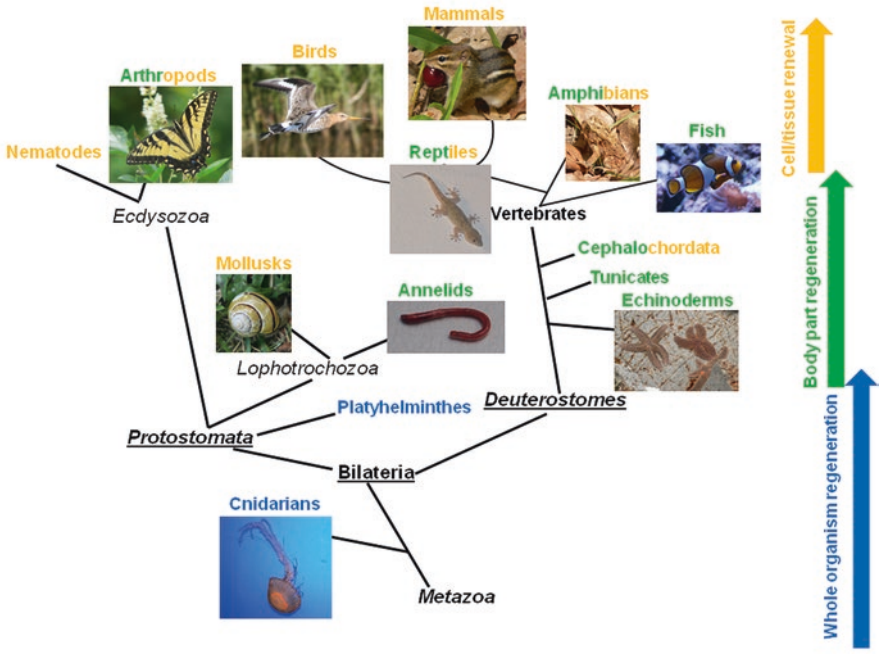

Fig. 3 Schematic illustration of the regenerative capacity in a phylogenetic tree of various invertebrate and vertebrate phyla. Distinct organisms have different regeneration capacities, ranging from whole-body regeneration to cell renewal, which is illustrated by the different colors in the animal groups according to available publications. Photographic images show the representatives of various animal groups: cnidarians (*Chrysaora fuscescens*), mollusks (*Cepea nemoralis*), annelids (*Eisenia andrei*), arthropods (*Papilio glaucus*), echinoderms (*Asterias vulgaris*), fish (*Amphiprion ocellaris*), amphibians (*Bufo americanus*), reptiles (*Tarentola mauritanica*), birds (*Limosa limosa*), and mammals (*Tamias striatus*). (All images were taken by Péter Engelmann except for *Tarentola mauritanica*, which was captured and provided generously by Mariann Szabó)

Earthworm Regeneration

Earthworms have enormous ability to regenerate their lost/injured body segments (Liebmann 1943). Interestingly, the ability to restore body segments is present in certain annelid groups, while it is limited in others such as leeches (Bely 2006). In the course of evolution, earthworms have probably acquired this regeneration capacity because of their high likeliness to be attacked by natural predators resulting in the injury or loss of body segments. The regeneration process depends on numerous factors such as ambient conditions, nourishment, and developmental stages as well as the position and/or numbers of segments that are removed. It seems that regeneration of anterior segments is significantly limited as more segments are abolished. For instance, *E. fetida* is not capable of anterior regeneration if more than 20 segments are amputated (removed) (Berrill 1952). In addition, in contrast to the

anterior body parts, posterior regeneration is a robust and almost universal process in various annelid groups. Moreover, during anterior regeneration, the survival rate of regenerating earthworms is dramatically reduced if the cerebral ganglion is involved in the injured/lost segments (Xiao et al. 2011).

Generally, the newly produced segments are regenerated by epimorphosis. During this process stem cells/progenitor cells (e.g., neoblasts) are activated and form a regenerating blastema. Subsequently, this blastema turns into an emerging bud to form a new segment either at the head or tail part. Interestingly, other regenerating mechanisms such as morphallaxis are also involved in the body restoration. In this case, the intact cells reorganize the body segment without stem cell involvement (Zoran 2010). Molecular aspects of regeneration are largely unexplored in oligochaete annelids. Most of the molecular data (e.g., *hox* and *hedgehog* genes) are derived from regeneration experiments involving *Platynereis* polychaete and *Pristina* naidid annelid species (Nyberg et al. 2012; Tessmar-Raible and Arendt 2003).

Immune Response During the Course of Earthworm Regeneration

It has long been known that coelomocytes participate in the regeneration and wound healing of injured earthworms (Liebmann 1943). Following tissue transplantation, phagocytic coelomocytes migrate into the injured tissue and eliminate damaged epithelial and muscle cells during wound healing (Cooper and Roch 1984). Moreover, it is speculated that migrating coelomocytes have supporting functions providing hormones, nutritive factors, and growth factors for the regenerating segments (Jamieson 1981; Somogyi et al. 2009). However, this hypothesis has been challenged by arguing that coelomocyte deprivation did not affect the kinetics of segmental regeneration (Moment 1974). This discrepancy might be solved if we consider not only the restoration of the segments but also the organ-specific regeneration. Straightforward experiments proved that immune cells actively participate in the regeneration of the crustacean central nervous system (Chaves da Silva et al. 2013). In addition, we now know that production of certain antimicrobial factors (theromacin, neuromacin, lumbricin) can also enhance the restoration of injured axons in the leech *Hirudo medicinalis* (Schikorski et al. 2008). Recent experiments demonstrated that coelomocyte depletion could significantly delay the restoration process of the cerebral ganglion in *Dendrobaena veneta* (Molnar et al. 2015; Okrzesik et al. 2013). These findings underline the possible involvement of coelomocytes in tissue regeneration.

According to our recent preliminary observations, regenerated segments (Fig. 4a) are abundant in certain types of coelomocytes (Fig. 4b). Coelomocytes are frequent in the lacunae of the regenerating blastema and in the neighboring intact segments; however, coelomocytes are less frequent in the appropriate location of intact animals. Immunostaining with the a-EFCC5 mAb (lysenin-specific) has revealed that these coelomocytes are mostly lysenin-producing eleocytes (Fig. 4c, d). Whether the role of lysenin-producing cells is related to the clearance of apoptotic

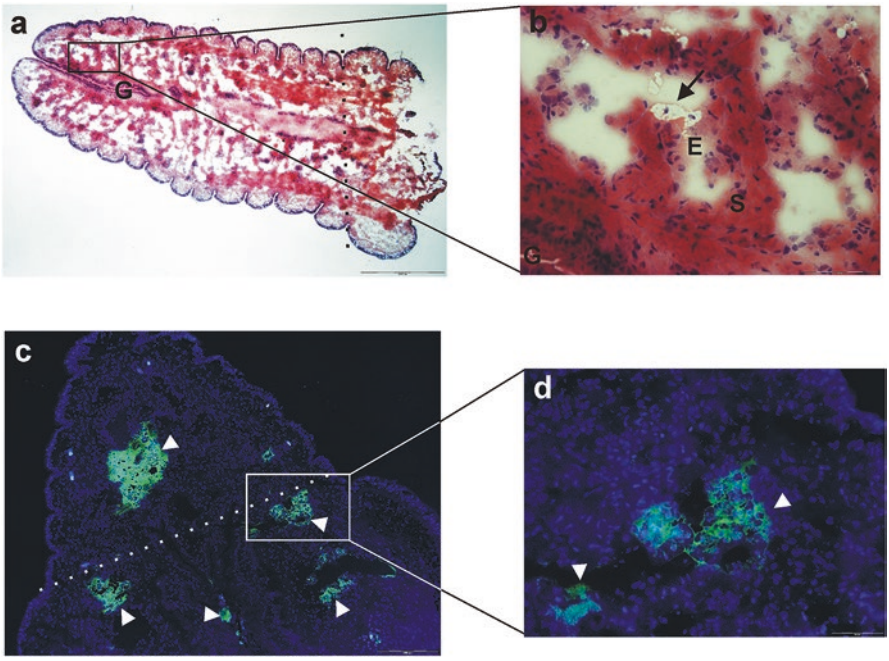

Fig. 4 Parasagittal sections of regenerating blastema in *Eisenia andrei* earthworms. (**a**) Hematoxylin-eosin staining of 2-week-old regenerating blastema. (**b**) Inset representing a larger magnification of the parasagittal section where eleocytes (arrow) are evidently present in the newly developed segments. Immunofluorescence analysis (**c**, **d**) using a lysenin-specific a-EFCC5 monoclonal antibody (fluorescein isothiocyanate [FITC] conjugated) shows that the lysenin-rich eleocytes (arrowheads) are abundant in the regenerating segments. DAPI (4′,6-diamidino-2-phenylindole) counterstain was used. The dotted line marks the beginning of intact segments. Scale bars: 1000 μm (**a**), 100 μm (**b**), 200 μm (**c**), and 50 μm (**d**). E eleocytes, G gut, M metanephridium, S septum

cells and/or the immune response against injury-related infections is a matter of debate and could be addressed with a new set of experiments.

Future research in this field may shed more mechanistic insights into the possible involvement of coelomocyte subpopulations in the earthworm regeneration process.

Conclusions and Perspectives

In this chapter we have given a short overview of the advances and interconnectedness of various fields of research on the innate immune system, transcriptomics, epigenetics, microbiota, and regeneration in invertebrate organisms, focusing on earthworms. It is becoming clear that in the "omics" era new and important questions can be addressed both on the evolutionary scale (e.g., role of microbiota in the emergence of innate immunity) and processes occurring on shorter time scales (e.g., regeneration). Invertebrate model organisms continue to yield valuable data in these

fast-advancing fields of research. Earthworms are also increasingly studied as sentinel organisms in the context of ecotoxicology and effects of soil pollution on the epigenome. A highly complex network of epigenetic modifications plays fundamental roles in the development and immunity in various organisms from worms to humans, which are viewed as holobionts of host organisms and symbiotic microbiota. Finally, this chapter summarizes the current state of research of earthworm (and invertebrate) immunity, but the "Quo vadis?" of this field is only just becoming discernible. We expect that the main directions for future research will likely be determined, in part, by the currently available data and the "omics" methodologies that are becoming more powerful, as well as by the application of "network science", which will help in integrating the current and future research data, thus revealing new horizons in this exciting field.

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Annelida: Recognition of Nonself in Earthworms

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Introduction

The ability to recognize self and nonself exists in all animal species. Unicellular animals, such as protozoans that often engulf living microorganisms, must discriminate between nutrition proteins and their own cell structures. The mechanism of discrimination at this level remains unknown, but one can assume that the specificity is based on the substrate specificity of proteolytic enzymes. Together with the evolution of multicellular organisms, the necessity to recognize self and nonself emerged to prevent the undesirable intrusion of pathogenic microorganisms or cells originating from another multicellular organism that could cause serious damage to the host. Besides a histocompatibility polymorphism enabling the rejection of xenografts by means of cytotoxic reactions evidenced already in the evolution of sea sponges, the innate immune system evolved several strategies of discrimination between self and nonself, leading to an immune response. The response is triggered upon pathogen recognition by a set of pattern recognition receptors (PRRs). These receptors recognize conserved molecular patterns shared by large groups of microorganisms. Recognition of these patterns allows the innate immune system not only to detect the presence of an infectious microbe but also to determine the type of the infecting pathogen. PRRs then activate conserved host defense signaling pathways that control the expression of a variety of immune response genes (Janeway 1989, 1992).

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Earthworms as Experimental Model for Comparative Immunology

Earthworms became a model of comparative immunology in the 1960s when in transplantation experiments (see Cooper and Roch 1994 for a review) cell-mediated short-term memory was observed (Bailey et al. 1971; Valembois 1971). All these experiments proved the existence of self and nonself recognition in earthworms and initiated extensive studies of earthworm immune mechanisms. The ability to recognize and reject xenografts as well as allografts and, on the other hand, the ability to accept or not destroy autografts was observed in model species of earthworms. The graft rejection begins like the reaction to an injury. After wounds are healed, regardless of the graft origin, coelomocytes accumulate near graft sites and infiltrate into the matrix. The response to the xenografts results in a complete encapsulation of the graft and its destruction (Parry 1978). The number of invading coelomocytes during the autograft transplantation is substantially lower than in the case of xenograft transplantation (Cooper 1970; Hostetter and Cooper 1973), but the reaction seems to be more rapid with the maximum number of coelomocytes surrounding the graft in 24 h, returning to the normal level within 3 days. In contrast, the peak response to xenografts occurs on day 3 or 4, and normal levels are not reached before day 7. The destruction of xenografts is completed approximately by day 17 following transplantation. If a second graft is transplanted at this time, an accelerated rejection within 6 and 7 days occurs. Moreover, the number of the invading coelomocytes is 20–30% higher compared to the first set's reaction. The increased number of coelomocytes during the second-set transplantation is most likely due to an increased proliferating activity of mesenchymal lining of the coelomic cavity and the septa. These data might explain the existence of short-term and very limited memory based solely on cells because the transfer of either the coelomic fluid or other soluble substances does not induce any accelerated reaction (Bailey et al. 1971; Hostetter and Cooper 1973).

Pattern Recognition Receptors in Earthworms

CCF

Twenty years ago, the first PRR from earthworms called a coelomic cytolytic factor (CCF) was characterized and cloned (Beschin et al. 1998). CCF was originally described as a 42-kDa cytolytic protein responsible for the proteinase-independent cytolytic activity of the coelomic fluid of *Eisenia andrei* earthworms (formerly *Eisenia fetida andrei*) against the tumor necrosis factor (TNF)-sensitive tumor L929 cell line (Bilej et al. 1995).

CCF recognizes and binds microbial pathogen-associated molecular patterns (PAMPs), namely O-antigen of the lipopolysaccharide (LPS) of Gram-negative bacteria, muramyl dipeptide and muramic acid of peptidoglycan from the cell walls of Gram-positive bacteria, and β-1,3-glucans and *N*, *N*′-diacetylchitobiose of yeast

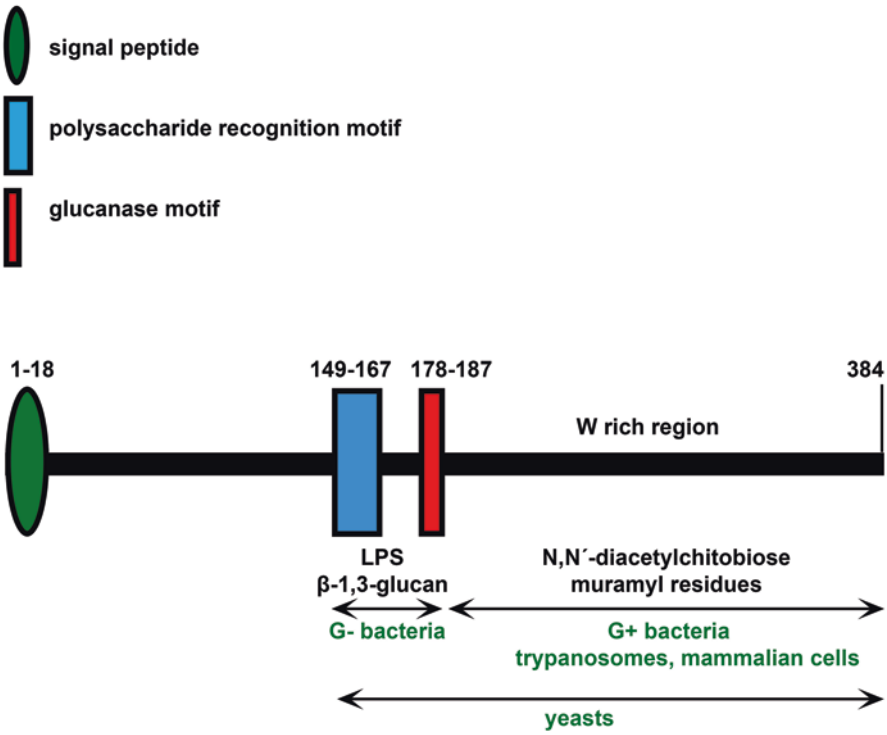

Fig. 1 Structure of *E. andrei* CCF. The broad specificity of CCF for PAMPs results from the presence of two distinct domains. One domain, which shows homology with the polysaccharide-binding motif and glucanase motif of β-1,3-glucanases and invertebrate defense molecules, is located in the central part of the CCF polypeptide chain and interacts with LPS and β-1,3-glucans. The C-terminal domain mediates the interaction of CCF with *N,N′*-diacetylchitobiose and muramyl constituents. (Based on Beschin et al. 1998; Bilej et al. 2001)

(Bilej et al. 2001). The broad specificity of CCF for PAMPs results from the presence of two spatially distinct pattern recognition lectinlike domains. One domain, showing homology with the polysaccharide and glucanase motifs of β-1,3-glucanases and invertebrate defense molecules, is located in the central part of a CCF molecule and interacts with LPS and β-1,3-glucans. The C-terminal tryptophan-rich domain mediates interactions of CCF with *N*, *N′*-diacetylchitobiose, muramyl dipeptide, and muramic acid (Fig. 1) (Beschin et al. 1998; Bilej et al. 2001).

Upon binding such molecular patterns, CCF triggers the activation of the prophenoloxidase (proPO) cascade, resulting in the formation of cytotoxic and antimicrobial compounds (Beschin et al. 1998; Bilej et al. 2001; Prochazkova et al. 2006). The proPO-activating cascade is an important invertebrate defense mechanism triggered by the presence of minute amounts of PAMPs (see Söderhäll and Cerenius (1998) and Cerenius and Söderhäll (2004) for a review). Their recognition leads to the activation of serine proteases converting the inactive proenzyme called proPO to its active state – phenoloxidase (PO) (monophenol monooxygenase). Subsequently,

the active enzyme catalyzes the oxygenation of monophenols to *o*-diphenols and further oxidation of *o*-diphenols to *o*-quinones. Quinones are nonenzymatically polymerized to melanin, which exhibits antibacterial and antifungal properties (Ashida and Yamazaki 1990; Söderhäll et al. 1994). ProPO and PO have been isolated and characterized in many invertebrate species. Their molecular weight varies between 70 and 90 kDa, and all of them contain two functional copper-binding sites.

The finding of the PO activity in the coelomic fluid of *E. fetida* proved the presence of proPO-activating cascade in annelids (Seymour et al. 1992; Beschin et al. 1998). A proPO cascade of *E. fetida* is directly activated by Gram-negative bacteria and yeasts, while Gram-positive bacteria must be treated with lysozyme to activate the cascade (Bilej et al. 2001). Later on, PO activity was detected in a 90-kDa fraction of the coelomic fluid of the earthworm *E. fetida*. Amino acid sequencing of peptides from the active fraction revealed a partial homology with invertebrate POs and hemocyanins. However, the level of PO activity is lower and the activation slower compared to other invertebrates (Procházková et al. 2006).

Large particles, for example, agglutinated bacteria or parasites, are eliminated by encapsulation (Ratcliffe et al. 1985), leading to the formation of the fibrous capsule called brown body (Valembois et al. 1992). Brown bodies contain two important pigments, lipofuscin and melanin, final products of the proPO cascade. The presence of pigment might inactivate the effect of free radicals released to segregate unwanted particles (Valembois et al. 1994). When the brown bodies reach about 1–2 mm in diameter, they slide toward the posterior segments of the coelomic cavity, often being eliminated by the autotomy of caudal segments followed by wound healing (Keilin 1925; Alonso-Bedate and Sequeros 1985). The process of autotomy enables the elimination of highly toxic organic (Paris-Palacios et al. 2010) and inorganic (Mendez-Fernandez et al. 2013) residues. The autotomy process seems to be under neurohormonal control (Alonso-Bedate and Sequeros 1985).

CCF displays amino acid sequence homology with bacterial and animal β-1,3-glucanases (Yamamoto et al. 1993; Bachman and McClay 1996; Kozhemyako et al. 2004; Pauchet et al. 2009), but it does not exhibit enzyme activity. More importantly, CCF shows homology with the α subunit of the β-1,3-glucan-sensitive factor G from the horseshoe crab *Tachyplesus tridentatus* (Seki et al. 1994), with the Gram-negative bacteria-binding proteins of various animals (Lee et al. 1996; Dimopoulos et al. 1997; Shin et al. 1998; Kim et al. 2000; Zheng et al. 2011) and β-1,3-glucan recognition protein of arthropods (Ma and Kanost 2000; Ochiai and Ashida 2000; Cheng et al. 2005; Glazer et al. 2015; Amparyup et al. 2016). Accordingly, it has been suggested that all these invertebrate homologs play a role in invertebrate innate immunity by acting as pattern recognition molecules.

Comparative analysis of CCF molecules from six different Lumbricidae species (*Aporrectodea caliginosa*, *Aporrectodea icterica*, *Aporrectodea longa*, *Aporrectodea rosea*, *Dendrobaena veneta*, *Lumbricus rubellus*, and *Lumbricus terrestis*) revealed the unique ability of CCF molecules from *E. fetida* to activate a proPO cascade in

the presence of *N,N′*-diacetylchitobiose and β-1,3-glucan laminarin, in contrast to CCF molecules from other earthworm species, which activate this pathway only in the presence of laminarin (Silerova et al. 2006). Cytolytic or trypanolytic activity of the coelomic fluid of these other species, except that of *E. fetida,* was not detected. This broad recognition repertoire of *E. fetida* CCF probably reflects the particular microbial environment in which this species lives.

Indeed, CCF shares functional analogies with mammalian TNF. Previously, it was shown that some activities of TNF, namely the trypanolytic and ion-gating activities, are mediated by its lectin-like domain with *N*, *N′*-diacetylchitobiose specificity (Lucas et al. 1994; Magez et al. 1997; Fontt et al. 1998). Similarly, CCF efficiently lyses African and American trypanosomes (Beschin et al. 1999; Fontt et al. 2002) and affects membrane conductance (Bloc et al. 2002; Bilej et al. 2006) due to the lectin-like interactions. Despite the functional analogies of CCF and TNF, these molecules do not show any gene or amino acid sequence homology, indicating a lack of common evolutionary origin (Beschin et al. 1999) and suggesting the convergence of function.

LPS-Binding Proteins/Bacterial Permeability-Increasing Proteins

LPS-binding proteins (LBPs) and bacterial permeability increasing proteins (BPIs) are closely related proteins involved in innate immunity. When LPS occurs in the blood system of mammals, its lipid A part is recognized by both LBP and bactericidal permeability-increasing protein (BPI). Both LBP and BPI, members of the LBP/BPI family, are pattern recognition molecules that play an important role in organism protection against Gram-negative bacteria. Although these proteins have a similar structure, they have antagonistic biological functions. Whereas LBP mediates the inflammatory response (Fenton and Golenbock 1998), BPI has anti-inflammatory and antimicrobial effects (Elsbach and Weiss 1998). LBP/BPI-related genes have also been identified in non-mammalian vertebrates (see Imler and Hoffmann 2002 for a review) and in invertebrates (Gonzalez et al. 2007; Baron et al. 2013).

LBP/BPI proteins are predicted to have the basic “boomerang” two-domain fold, similar to human BPI and LBP molecules. It is believed that the ancestor of LBP and BPI was a single-domain protein, which was cis-duplicated over the course of evolution (Beamer et al. 1998). Some invertebrates, for example arthropods, completely omitted these molecules. For instance, *D. melanogaster* uses for the detection of Gram-negative bacteria peptidoglycan recognition proteins and Gram-negative bacteria-binding protein (Gottar et al. 2002; Choe et al. 2002).

A molecule of the LBP/BPI family was identified in *E. andrei* earthworms (Skanta et al. 2016). This molecule was found to be expressed in all tissues, with the highest level in coelomocytes and seminal vesicles. It consists of two conserved domains with the potential ability to bind LPS, which is supported by the observation that bacterial stimulation of earthworms leads to its upregulation.

Toll-Like Receptors

Originally, Toll was identified as a molecule playing a role in embryonal development in fruit fly *Drosophila melanogaster* (Nusslein-Volhard and Wieschaus 1980). Later on, Toll was found by Jules A. Hoffman and his colleagues to have an essential role in the fly's immunity to fungal infection (Lemaitre et al. 1996) that is achieved by activating the synthesis of antimicrobial peptides. Soon after the characterization of the immune function of Toll and its ligand S*pätzle*, a family of Toll-like receptors was described. The Toll-like receptors (TLRs) are membrane PRRs recognizing specific conservative molecules expressed by bacteria, fungi, or viruses (PAMPs).

Generally, molecules of TLRs are membrane glycoproteins consisting of three domains: the extracellular N-terminal domains with leucine-rich repeats (LRRs) for the binding of antigens, the transmembrane domain, and the intracellular domain known as the Toll/Il-1 receptor (TIR) domain required for the interaction and recruitment of various adaptor molecules to activate the downstream signaling pathway. Based on the arrangement of the extracellular domain with LRRs, TLRs can be classified as vertebrate-like type (V-type), including all described deuterostome and some insect TLRs, or as protostome-like type (P-type), comprising nearly all TLRs found in insects and in other protostomes (Hibino et al. 2006).

The first earthworm TLR was isolated from an oligochaete annelid *E. andrei* (*Ea*TLR) (Skanta et al. 2013), which belongs to the V-TLR type. This receptor has very high intraspecies variability, suggesting the presence of a high number of TLR genes in the genome of *E. andrei*. Phylogenetic analysis revealed the highest similarity of *Ea*TLR with TLR from polychaete annelid *Capitella teleta* and with TLRs of mollusks and echinoderms. *Ea*TLR is expressed in all tissues of the earthworm body, with the highest constitutive expression in the digestive tract. Further, its expression in coelomocytes can be upregulated by a bacterial challenge (Skanta et al. 2013).

Recently, we found a new P-TLR in oligochaete *E. andrei*. This molecule is expressed primarily in earthworm seminal vesicles and seminal receptacles, suggesting their connection with sperm cells. Stimulation experiments with particular antigens indicated indirect recognition of antigens via some mediator, as was described for the fruit fly (Leulier et al. 2003).

The presence of different PRRs in *E. andrei* earthworms recognizing/binding the same or similar target molecular patterns suggests a division of the function and different tissue distribution. While CCF is expressed mainly in the intestine and in free coelomocytes, mRNA coding for another LPS-binding protein, LBP/BPI, can be detected mainly in the proximal part of the digestive tract and in the coelomocytes and, to lesser extent, in crop, gizzard, and intestine (Fig. 2). The microbial pattern for TLR from earthworms remains unknown. Interestingly, mRNA encoding V-TLR was detected mainly in the digestive tract, while P-TLR transcripts were evidenced mostly in seminal receptacles and seminal vesicles. Taken together, a model of the earthworm defense against infection can be proposed. The increased number of microorganisms is sensed by PRRs in the digestive tract, and this danger

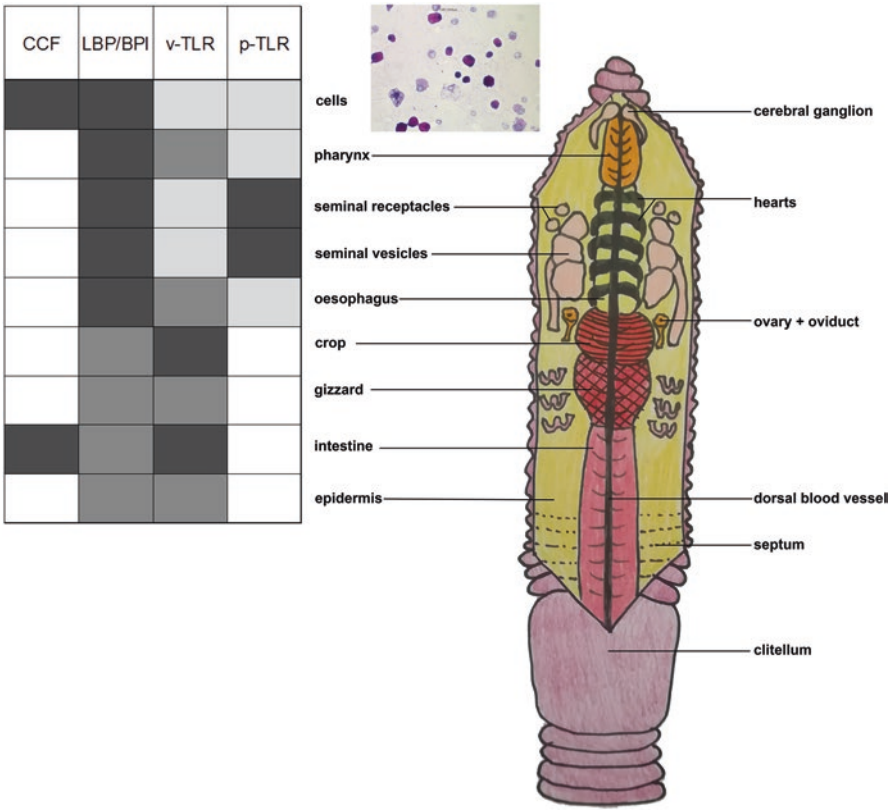

Fig. 2 Distribution of PRRs in earthworm tissues and organs. The level of mRNA transcripts of genes coding for PRRs was assessed by quantitative polymerase chain reaction in various tissues, organs, and free coelomocytes. The darkness of the box corresponds to the level of mRNA (black: relative maximum, white: not detected). (Based on Bilej et al. 1998; Skanta et al. 2013; Dvorak et al. 2016; and Skanta et al. 2016)

signal is transferred to the adjacent mesenchymal lining representing the precursor tissue of free coelomocytes and the site of their release. The released coelomocytes with immune function act as phagocytes or produce antimicrobial proteins and opsonins that reduce the number of microorganisms in the coelomic cavity, thereby keeping the infection under control (Dvorak et al. 2016).

Pattern Recognition Receptors in Polychaeta and Hirudinea

The first TLRs from Annelida were retrieved from in silico analyses of whole genomes of polychaete *Capitella capitata* and leech *Helobdella robusta* (Davidson et al. 2008). The researchers found 105 TLR homologs in *Capitella* and 16 TLR homologs in *Helobdella* genomes. TLR molecules of *Capitella* have vertebrate-like

domain organization, in contrast to TLRs of *Helobdella*, which display the protostome-like architecture. A large number of TLRs in *Capitella* resemble those in sea urchins, where 222 TLR homologs were described (Rast et al. 2006). All *Capitella* sequences are very similar, indicating the likely occurrence of gene duplication, in contrast to *Helobdella* TLRs, which are very different from each other.

PRRs and their downstream signaling were well described in the medicinal leech *Hirudo medicinalis*. In this leech, a number of these molecules are expressed in their central nervous system (CNS). *Hm*-TLR1 was found to be expressed by both microglia and neurons, and it evinced a functional homology with the mammalian TRL3 in brain (Cuvillier-Hot et al. 2011). Besides this molecule, an additional three *Hm*-TLRs were found by the screening of the *Hirudo* expressed sequence tag library (Tasiemski et al. 2007).

Except for TLR, another PRR belonging to the family of Nod-like receptors (NLRs) was found in the CNS of *H. medicinalis* (Cuvillier-Hot et al. 2011). The NLRs are cytosolic receptors recognizing intracellular pathogens. Interestingly, no NLR homologs were found in the genomes of *Drosophila* and *Caenorhabditis elegans*. *Hm*-NLR is localized in the submembranous space of neurons, and it was shown to share a homology with the vertebrate NLRC3 receptor (Cuvillier-Hot et al. 2011). Both *Hm*-TLR1 and *Hm*-NLR were shown to be induced by Gram-positive bacteria and the bacterial cell wall component muramyl dipeptide. Further, *Hm*-TLR1 controls the gene expression of chemokine *Hm*-p43/EMAPII, which is able to attract phagocytic cells to sites of injury (Schikorski et al. 2009).

In addition to these two main pattern recognition molecules, a number of signaling molecules involved in TLR/NLR pathways, including two members of the MyD88 family, was revealed by the screening of *Hirudo* transcriptome and genome databases, suggesting a certain level of conservation (Rodet et al. 2015).

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Annelida: Hirudinea (Leeches): Heterogeneity in Leech Immune Responses

Annalisa Grimaldi, Gianluca Tettamanti, and Magda de Eguileor

Introduction

Examination of fundamental biological principles has revealed a well-preserved regulation of the processes involved in innate immune response and wound healing, which are complex and dynamic processes of restoring cellular structures and tissue layers characterized by the same specific stages in both vertebrates and invertebrates. It is well known that animal models are crucial in immune response, graft, and wound healing research, as they provide a means for studying the complex interactions that occur in living tissues. Currently, rodent or porcine animals are widely used in these studies because they are very well-characterized models at the molecular and cellular levels. However, several factors have led to restrictions in the number of animal species available for experimentation, as well as rapidly rising costs. These factors include ethical considerations, stricter laws and guidelines related to the use of animals for scientific experimentation, and improvements in animal welfare and housing.

The medicinal leech represents an emerging experimental model for investigating the immune system: it is cost effective, easily manipulable, and without significant ethical considerations and regulatory restrictions in relation to its use. In addition, more than in most commonly used invertebrates, such as *Drosophila melanogaster* or *Caenorhabditis elegans*, the immune response processes in leeches have proven to be surprisingly similar to those reported in vertebrates. In fact, these processes involve cellular mechanisms and common equipment of the key effector molecules playing focal roles for regulating hematopoietic cell

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activation and differentiation that are similar to those of vertebrates (Grimaldi et al. 2004, 2006). Another benefit of the medicinal leech—as a powerful model system for studying the basic steps of hematopoiesis, vasculogenesis, angiogenesis, immune response, and wound repair—is linked to its anatomical and physiological characteristics and to a less varied repertoire of cell types involved in immune response. These features allow for easy interpretation of events linked to inflammation and tissue repair (de Eguileor et al. 2003, 2004; Tettamanti et al. 2004). Indeed, the cellular immune response evoked by different stimuli can be easily and unambiguously evaluated in the leech body wall, which has a predominantly avascular muscular region where a few resident immunocompetent cells of myeloid origin (de Eguileor et al. 1999) are detectable (Grimaldi et al. 2006; Schikorski et al. 2008). The effects of lesions or bacterial challenge in this area are rapidly induced and can be studied by morphological and immunohistochemical analyses (de Eguileor et al. 1999). Several publications have indicated the existence of a variety of cytokines in leeches, growth factors, and cluster of differentiation (CD) proteins (commonly used as immune cell markers) acting as modulators of these processes (de Eguileor et al. 2000a, b; Grimaldi et al. 2004, 2006; Tettamanti et al. 2006; Macagno et al. 2010). As a result, for all these reasons the basic steps of the immune responses and wound repair can be easily analyzed in leeches that lack the complex feedback control systems typical of vertebrates.

We have focused our study on the cells recruited at the stimulated sites and on the chemoattractants involved in their recruitment. In order to better understand the processes involved in leech immune response events, we first provide a short description of the animal model anatomy.

Leech Body Organization

The medicinal leech has a parenchymatous body characterized by simple anatomy. Underneath the cuticle and the epithelium, tightly packed bundles of muscle fibers are embedded in a connective tissue and organized in three layers of fibers that are circularly, obliquely, and longitudinally disposed. The body wall in healthy animals is prevalently avascular (de Eguileor et al. 2003, 2004; Tettamanti et al. 2004) and the transport of gas, nutrients, and excretion products occurs via circulation of body fluid in contractile vessels that are longitudinally and transversely disposed (Fig. 1a, b). Close to the musculocutaneous sac and the digestive tube in Hirudinea is the botryoidal tissue, a peculiar tissue involved in vasculogenesis, hematopoiesis, and angiogenesis. It is of mesodermal origin and composed of clusters of two cell types: botryoidal and endothelial cells, which are linked by gap and desmosome-like junctions and well-separated from the connective tissue by a basal lamina. The botryoidal cells are large and roundish, with a cytoplasm rich in granules of different sizes and staining properties (de Eguileor et al. 2001a). In contrast, endothelial-like cells are thin and display a cytoplasm poor in granules (Fig. 1c).

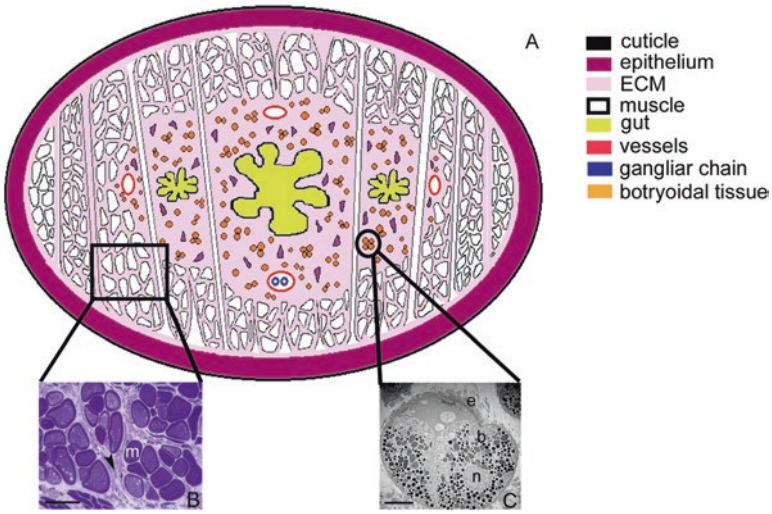

Fig. 1 (**a**–**c**) Representation of a general view of the body of *Hirudo* in cross-section (**a**). Under the cuticle and epithelia, large layers of muscle tissue are visible. The botryoidal tissue (orange) is localized between muscles and gut (yellow) and is immersed in a loose connective tissue (extracellular matrix [ECM], light pink). (**b**) Optical microscope detail of longitudinal muscle fibers surrounded by ECM (arrowhead) in which no vessels are visible. (**c**) Transmission electron microscope (TEM) detail of botryoidal cells showing a granule-filled cytoplasm and endothelial cells characterized by smaller size, flattened shape, and an agranular, electron-dense cytoplasm. Scale bars: 10 μm (**b**) and 5 μm (**c**). b botryoidal cells, e endothelial cells

Vasculogenesis, Hematopoiesis, and Angiogenesis

Immune competent cells in leeches, like those in vertebrates, are of mesodermal origin and the botryoidal tissue has recently been defined as the hematopoietic organ from which myeloid lineage-derived leukocytes arise (Grimaldi et al. 2006). This tissue is surprisingly versatile, since it not only displays myeloid/erythroid and storage functions (Fischer et al. 1976; Sawyer 1986; de Eguileor et al. 2001a), but is also involved in production of hematopoietic cells and angiogenesis (de Eguileor et al. 2001a, b; Tettamanti et al. 2003b; Grimaldi et al. 2006).

Immediately after the induction of stimuli, such as surgical lesions, bacterial infection, or injection of specific growth factors, the botryoidal tissue undergoes a transition from cluster/cord-like structures to a hollow, tubular architecture typical of pre-vascular structures that will give rise to new capillary vessels. In detail, remodeling of botryoidal tissue is characterized by marked flattening, lengthening, and stretching of both botryoidal and endothelial-like cells which define a neolumen. Concurrently with the neo-vessel formation, groups of small, closely associated cells become evident in the center of the immature vessel lumen (Fig. 2a–c). The relevant modification of the botryoidal tissue phenotype during the vasculogenic step is due to the reorganization of actin filaments in both endothelial and botryoidal cells. Strikingly, like vertebrates (Carpenter 2000; Kranenburg and

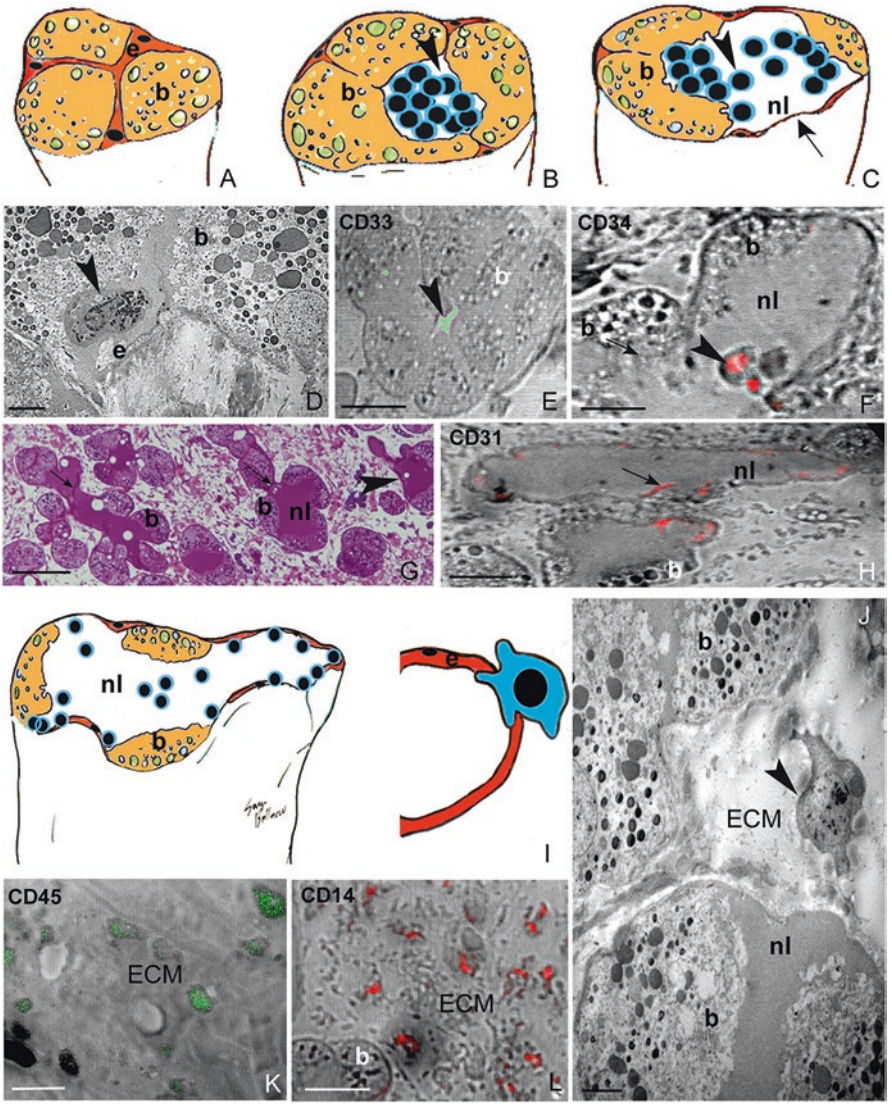

Fig. 2 (**a**–**e**) Representation of the proposed model of the hematopoietic and vasculogenesis process in *Hirudo* (**a**–**c**) (Modified by Grimaldi 2016). Left to right: from solid cords of botryoidal tissue cells (**a**) a neovascular lumen is shaped through a dehiscence process (**b**–**d**). During this process, in the new vessel cavity lined by endothelial cells (arrow), cluster of hematopoietic stem and progenitor cells (HSPCs) (arrowhead) become evident (**b**, **c**). (**d**) TEM detail of an HSPC (arrowhead) in the neolumen lined by botryoidal and endothelial cells. (**e**, **f**) Combined fluorescence/transmission images showing clusters of $CD33^+$ and $CD34^+$ HSPCs inside the botryoidal tissue. (**g**) Optical microscope detail of the newly formed vessel. The neolumen is surrounded by botryoidal and endothelial cells (arrowhead). The latter form transluminal pillars and walls (arrows), leading to neovessel splitting and branching. The immunofluorescent staining (in red) shows that the endothelial cells are $CD31^+$ (**h**). After crawling between endothelial adjacent cells (**i**), the HSPCs leave the bloodstream and migrate into the connective tissue. TEM detail (**j**) and immunohistochemical analysis (**k**, **l**) of HSPCs (arrowhead in **j**) dispersed in the connective tissue adjacent to the botryoidal tissue and expressing the CD14 (in red) and CD45 (in green) markers. Scale bars: 2 μm (**d** and **j**), 10 μm (**e**, **f**, **h**, **k**, and **l**), and 25 μm (**g**). b botryoidal cells, e endothelial cells, ECM connective tissue, nl neovessel lumen

Moolenaar 2001; Sawada et al. 2015), in leeches the Ras GTPase protein is capable of driving an angiogenic switch in the botryoidal tissue by acting on polymerization and spatial arrangements of actin filaments in both endothelial and botryoidal cells (Grimaldi et al. 2013; Grimaldi 2016).

Both botryoidal and endothelial cells contribute to the creation of tissue niches promoting hematopoietic cell maintenance (survival and self-renewal) and regulating precursor cell migration, quiescence, and differentiation. These precursor stem cells (hematopoietic stem and progenitor cells [HSPCs]) are initially adherent to the vessel wall but, as the vessel grows, they lose cell–cell contacts and move freely within the lumen. Leech HSPCs show the typical morphology of blast-like cells (Fig. 2d) and multipotent progenitors, as in vertebrates. As such, they express several surface markers commonly used to identify the endothelium and myeloid hematopoietic progenitors of vertebrates: the CD34, CD117, CD33, and CD45 markers (Wells et al. 1996; Crocker and Varki 2001; Guo et al. 2003; Hildbrand et al. 2004; Raaijmakers and Scadden 2008). These markers have been related to the functional need of maturing cells, and can be up- or downregulated by different cytokines and growth factors. In particular, CD117, CD33, CD34, and CD45 antigens are typically localized on clustered or isolated precursors within the neo-lumen and in cells adhering to the vessel lumen (Fig. 2e, f). The fast recruitment of a large number of HSPCs in the stimulated/lesioned/grafted area is achieved by the formation of new vessels, easily detectable in the stimulated *Hirudo* body wall which is avascular in normal conditions. The newly formed vessel, starting from the gut area and moving between muscle fields towards the lesioned area, consists of a lumen surrounded by botryoidal and endothelial-like cells expressing the typical endothelial marker CD31 and VE-cadherin (vascular endothelial cadherin). Subsequently, endothelial-like cells form transluminal pillars and walls, leading to neo-vessel splitting and branching (Fig. 2g, h). The interposition of newly synthesized extracellular matrix (ECM) cuts off and separates several small portions of the botryoidal tissue from the area of the very same tissue involved in the angiogenic process. The numerous circulating HSPCs, conveyed into the lesioned/stimulated area by vessels, adhere to the luminal wall and, after crawling between adjacent endothelial cells, leave the bloodstream and disperse in the surrounding connective tissue (Fig. 2i, j). As previously reported for vertebrates, several factors play a key role in this interaction, such as L-selectin, talin, and vinculin. In particular, extravasating leech HSPCs express L-selectin, which is a typical marker for hematopoietic cells adhering to the inner vessel wall and destined to leave the bloodstream. Subsequently, HSPCs express talin and vinculin, the role of which is to link collagen fibers during migration across the ECM (de Eguileor et al. 2001a, b; Grimaldi et al. 2006; Grimaldi 2016). These precursor cells in the leech not only express many of the same markers as those commonly used to identify vertebrates' HSPCs and endothelial cells, but also express pattern recognition receptors (PRRs), such as the lipopolysaccharide (LPS) recognition molecule CD14 (Fig. 2k, l) (Kielian and Blecha 1995; Grimaldi et al. 2006), which recognizes conserved and endogenous damage-associated molecular patterns (DAMPs). As in vertebrates, the principal functions of this PRR include opsonization, phagocytosis, and activation of proinflammatory

signaling pathways and respond to conserved microbial molecular patterns. Therefore, as in vertebrates (Nagai et al. 2006; De Luca et al. 2009; Granick et al. 2012), we speculate that leech HSPCs represent cells actively involved in innate immune and inflammatory responses, playing a crucial role in peripheral tissues, where they act as a source of both mature effectors for immediate local leukocyte needs and innate immune cells. Indeed, upon detection of pathogen-associated molecular patterns (PAMPs), HSPCs differentiate into specific lines of mature myeloid immune cells to fight infection.

Innate Immunity in the Leech: Morphological and Functional Characterization of Mature Leukocyte Populations

In the immune response of leeches, which relies exclusively on innate immunity, HPSCs recruited in large numbers from the bloodstream travel towards sites of inflammation and differentiate into mature leukocyte cells (de Eguileor et al. 1999, 2000a, b; Tettamanti et al. 2003a, b, 2006; Grimaldi et al. 2006, 2010; Schorn et al. 2015a, b). These myeloid lineage-derived cells, such as natural killer (NK) cells, macrophages, and granulocytes, which are widely characterized by ultrastructural analyses (de Eguileor et al. 1999), have diverse types of responses in relation to the antigen size (Fig. 3a–c) (Grimaldi 2015, 2016). These data strongly support the hypothesis that a primitive innate immune system appeared early in phylogenies and the same function has been preserved throughout phylogenetic development.

Leech NK-like cells are characterized by a cytoplasm displaying a small nucleus, numerous mitochondria, a well-developed endoplasmic reticulum, and dense granules, resembling those described in vertebrates (Vivier et al. 2008; Mandal and Viswanathan 2015) and in invertebrates such as worms (Porchet-Henneré et al. 1992; Cossarizza et al. 1996; Quaglino et al. 1996) and molluscs (Franceschi et al. 1991). These cells have the ability to respond to a variety of stimuli and to participate in immune responses under different pathological conditions such as bacterial and viral infections, resistance to parasites and regulation of hematopoietic cell growth and differentiation. Indeed, when leech NK cells come in close contact with the membrane of target cells, they exocytate the granules present in their cytoplasm, carrying out their cytolytic role (de Eguileor et al. 1999, 2000a, b).

Leech macrophage-like cells, which are morphologically similar to the mammalian monocyte/macrophage lineage cells, represent the cell population primarily involved in non-self recognition. Bacteria challenge, grafts, or injuries trigger the migration and accumulation of macrophage-like cells in the leech body wall, characterized by pseudopodia and numerous phagolysosomes in their cytoplasm and displaying a strong phagocytic activity (de Eguileor et al. 2000a, b; Schorn et al. 2015a, b). Macrophages simply phagocytize antigens of small dimension, while cumbersome foreign bodies (i.e., groups of spheres, aggregates of nanomaterial such as multiwall carbon nanotubes [MWCNTs], and/or parasites) are encapsulated and then melanized (de Eguileor et al. 2000b; Girardello et al. 2015b). Recent reports have stressed the relationship between melanin synthesis and the production of amyloid fibrils in both vertebrates and invertebrates (Fowler et al. 2006; Falabella

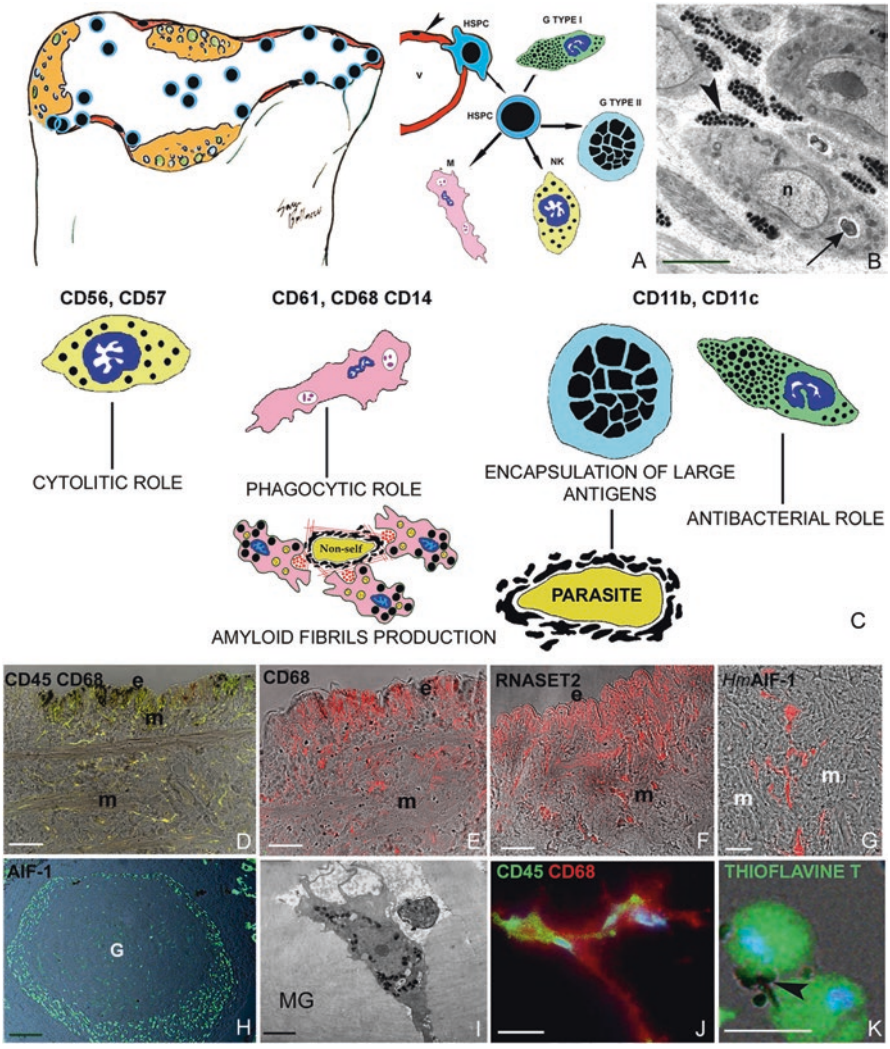

Fig. 3 (**a**–**k**) After leaving the bloodstream, the hematopoietic stem and progenitor cells (HSPCs) differentiate in mature leukocytes expressing specific cluster of differentiation (CD) markers and involved in different types of responses in relation to the antigen size (**a**, **c**: drawing modified by Grimaldi 2015, 2016). (**b**) TEM detail of granulocyte type I (arrowhead) and macrophages (arrow). At 24 h after r*Hm*AIF-1 (**d**) and recombinant ribonuclease T2 (rRNASET2) (**e**) injection, numerous migrating macrophages are stained by the anti-CD45 (in green) and anti-CD68 (in red) antibodies. At 24 h following bacterial injection, numerous ribonuclease T2-positive (RNASET2^{+}) (**f**) and *Hm*AIF-1^{+} (**g**) macrophages migrating towards the injected area were detectable under the epithelium and among the muscle fibers. 24 h after allograft (**h**) numerous macrophages surround the graft and are *Hm*AIF-1^{+}. Ultrastructural analysis at TEM (**i**) and immunohistochemical staining (**j**) of macrophage cells migrated in the matrigel (MG) supplemented with *Hm*AIF-1 and characterized by ruffled surfaces and by the expression of CD45 (in green) and CD68 (in red) markers are shown. Thioflavin T staining (green in **k**) clearly shows the amyloid fibril deposition (yellow) by macrophages strictly associated with multiwall carbon nanotube (MWCNT) aggregates (arrowhead). Nuclei in blue are stained with DAPI (4′,6-diamidino-2-phenylindole). Scale bars: 3 μm (**b**, **i**), 50 μm (**d**–**f**), 20 μm (**g**), 100 μm (**h**), and 10 μm (**j**, **k**). e epithelium, g graft, m muscle

et al. 2012; Grimaldi et al. 2012a, b). In particular, during the encapsulation process leech macrophages produce a large amount of amyloid fibrils which are used as a barrier to restrain non-self material and for packaging and driving melanin towards a non-self invader (Girardello et al. 2015b).

Two different types of granulocytes (type I and type II) have been also identified based on the size and shape of granules present in their cytoplasm and on their different behavior towards the non-self invader. Granulocytes of type I are characterized by small, round granules, which are extruded from the cells in response to a large dose of bacteria (*Escherichia coli*) injected in the leech body wall. Granulocytes of type II are mainly involved in defense against large antigens crossing the epidermis (de Eguileor et al. 1999, 2000a) and are characterized by the presence in their cytoplasm of large, irregularly shaped granules showing complementary profiles.

Strikingly, cells involved in the leech immune system not only display the same features and behaviors of those found in vertebrates but also express the same molecules used as immune cell markers for vertebrates (Fig. 3c). Indeed, several reports (de Eguileor et al. 2000a, b; Grimaldi et al. 2004, 2006; Macagno et al. 2010; Schorn et al. 2015a, b) have provided extensive evidence for the presence in leeches of a wide range of CD proteins that are commonly used as immune cell markers in vertebrates. In both leeches and vertebrates, expression of the surface marker CDs is strictly linked to a specific cell type or a specific function. For instance, the cell surface glycoprotein CD45 is involved in integrin-mediated adhesion of macrophages both in vertebrates (Roach et al. 1997; Zhu et al. 2011; St-Pierre et al. 2013) and in leeches (Schorn et al. 2015a, b) and it regulates the functional responsiveness of cells to chemoattractants (Roach et al. 1997; Mitchell et al. 1999). CD14 and CD68 are specific markers of monocyte/macrophage lineage. CD14, an LPS-recognition molecule, is mainly expressed on the cell surface, promoting phagocytic uptake, whereas CD68 is a protein primarily localized in lysosomes and endosomes and is highly expressed as an intracytoplasmic molecule in human (Holness et al. 1993) and leech macrophages (de Eguileor et al. 1999; Schorn et al. 2015a, b). CD11b (Springer 1990) and CD11c (Cabañas and Sánchez-Madrid 1999) are macrophage and granulocyte markers and mediate phagocytosis adherence to activated endothelium and chemotaxis (Smith et al. 1989; Arnaout 1990; de Eguileor et al. 2000b; Podolnikova et al. 2015; Sándor et al. 2016). CD56 and CD57 are expressed by NK cells (Robertson et al. 1996; de Eguileor et al. 2000b; Gondois-Rey et al. 2017) that, as in vertebrates, have both innate and adaptive immune features and are crucial for second graft rejection (Mandal and Viswanathan 2015; Tang et al. 2017).

Indeed, our previous studies, which were performed on first- and second-set rejection of allografts and xenografts in leeches, revealed the existence of an immune defense system with a suggested memory component. The existence of a sort of positive immune memory is supported by the presence in leeches of two types of cells: a first population that includes $CD56^+/CD8^+$ cells and a second population that includes $CD8^+$/tumor necrosis factor (TNF)-α^+/TNF-β^+. The presence of CD8-, TNF-α-, and TNF-β-positive cells in the grafted area led us to theorize about the existence of a subpopulation of NK cells that respond to antigenic stimulation

and become able to respond rapidly to subsequent antigenic challenges. In vertebrates these antigens are mainly expressed by activated cytotoxic T lymphocytes (CTLs) and NKT cells, which show the same morphology and use similar molecular mechanisms to express their cytotoxicity (Franceschi et al. 1991; Shresta et al. 1998; Seaman 2000). The hypothesis that leeches might possess an effective recognition system has been confirmed by the results obtained from second-set graft rejection experiments in which the same leech received both first and second transplants from the same donor. The responses to the second transplant were highly accelerated: second allograft and xenograft transplants were always faster to respond (usually within 3–4 days) and stronger than those occurring in first-set grafting experiments (Tettamanti et al. 2003a).

Further confirmation that cell recognition and the immuno-defense system share similar characteristics and effector molecules to those of vertebrates is also supported by data from the literature highlighting the presence of CD antigens in other invertebrates also, such as earthworms (Cossarizza et al. 1996), sipunculids, a phylum closely related to annelids (Blanco et al. 1997), and nematodes (Kostich et al. 2000). The highly conserved morphological characteristics and functions of immune cells and the expression of the same CD proteins in both vertebrates and invertebrates is a further confirmation that cell recognition and immune defense systems have been highly conserved during evolution.

Factors Involved in Hematopoiesis, Vasculogenesis, Angiogenesis, and Leukocyte Recruitment

In leeches, as in vertebrates, the various processes that underlie hematopoiesis, neovascularization, inflammation, and immune response are regulated by the same key molecules, such as cytokines and growth factors (Tettamanti et al. 2006; Schikorski et al. 2008; Schorn et al. 2015a, b), and by the same signaling pathways (Grimaldi et al. 2013; Grimaldi 2016).

Interesting outcomes of previous investigations have shown that injection of human cytokines and growth factors in leeches promotes hematopoiesis, vascular growth, immune cell migration, and differentiation (Tettamanti et al. 2003b; Grimaldi et al. 2006). Indeed, administration of angiogenic inducers of some vertebrates such as granulocyte-macrophage colony-stimulating factor (GM-CSF), endothelial growth factor (EGF), and vascular endothelial growth factor (VEGF) stimulate, in unlesioned medicinal leeches, an increase in both vessels and of hematopoietic cells. In particular, injection of the most abundant and biologically active isoform, VEGF165, in the leech body wall promotes the transformation of the botryoidal tissue into new immature vessels from which new vessels can further grow (de Eguileor et al. 2001b) and form an extensive vessel network through the entire avascular body wall. In addition, VEGF165 also induces massive production of circulating cells in the cavities of neo-vessel lumen and their recruitment in the stimulated area. Surprisingly, as in vertebrates, VEGF appears to control survival and repopulation of the hematopoietic stem cells and the angiogenic

process, albeit in a different manner (Damert et al. 2002; Gerber and Ferrara 2003; Wang et al. 2008). The fact that both endothelial cells and HPSCs express the VEGF receptors (VEGFRs) Flt1/VEGFR1 and Flk-1/VEGFR-2 (Tettamanti et al. 2003b; Grimaldi et al. 2006) not only confirms that these cells are responsive to this cytokine, but also that they can activate different genetic programs in response to environmental signals. When $CD34^+$ hematopoietic cells are exposed to a sustained and continuous source of VEGF, mainly produced by muscular cells and slowly diffused from physiological ECM "sinks", they proliferate and maintain an undifferentiated phenotype as precursor cells. In contrast, when the VEGF concentration decreases, these HPSCs enter into a differentiating program and can produce muscular cells (Grimaldi et al. 2009, 2010; Grimaldi 2016). Therefore, as in vertebrates (Chargé and Rudnicki 2004; Cossu and Biressi 2005; Zheng et al. 2007), a direct correlation between hematopoietic/endothelial precursor cells and muscle regeneration processes exists.

Several recent studies from our research group have also demonstrated the importance of macrophages in synthesizing different molecules such as growth factors and cytokines and in inducing vessels and mesenchymal cell recruitment in the injured/grafted or infected area. It has recently been demonstrated that allograft inflammatory factor-1 (AIF-1) and ribonuclease T2 (RNASET2) are molecules produced by macrophages and are highly involved in inflammatory responses in the medicinal leech (Schorn et al. 2015a, b; Baranzini et al. 2017).

AIF-1 was cloned for the first time in rat cardiac transplants subject to chronic rejection and was selectively expressed in macrophages and neutrophils (Utans et al. 1995). Subsequently AIF-1-like factors showing a well-preserved amino acid sequence have been identified in other metazoans, both in vertebrate (Autieri et al. 2000; Deininger et al. 2000, 2002; Watano et al. 2001) and invertebrate species (Kruse et al. 1999; De Zoysa et al. 2010; Zhang et al. 2011; Ovando et al. 2012; Li et al. 2013). Its expression increases significantly after transplantation, wound healing, or bacterial infections, suggesting that it is involved in the inflammatory response and in immune system regulation. A gene showing high similarity with AIF-1 in vertebrates, named *Hm*iba1/alias *Hm*AIF-1, has also been characterized in the central nervous system of the medicinal leech (Drago et al. 2014).

The transferase-type RNASET2 RNases represent the most widespread class among the ribonuclease families (i.e., RNase A and T1), being found in viruses, bacteria, protozoans, plants, and metazoans (Luhtala and Parker 2010). Recently, a large amount of experimental evidence has highlighted a role of T2 ribonucleases in immune responses. In particular, human RNASET2 plays a crucial role as a tumor suppressor, acting as an inducer of the innate immune response. It recruits host macrophages endowed with onco-suppressive properties toward the tumor mass in vivo (Acquati et al. 2011, 2013).

The direct relationship between the expression of *Hm*AIF-1 and RNASET2 and their ability to activate and/or recruit innate immune cells after injury or bacterial infection has not yet been thoroughly investigated in vertebrates and remains unclear, probably because study of the immune response in vertebrates appears to be a difficult challenge, which is primarily due to the complexity of these

organisms. However, recent data obtained in leeches clearly suggest their role in the activation of the innate immune response. Indeed, both *Hm*AIF-1 and RNASET2 have been mainly localized in leech macrophages and are constitutively expressed in untreated animals. Injection of the recombinant proteins rRNASET2 and r*Hm*AIF-1 induced a massive migration of $CD45^+/CD68^+$ macrophages towards the stimulated area (Fig. 3d, e), and the expression of these two factors increases dramatically after microbial infection, inducing macrophage and vessel migration towards the stimulated area. It has been theorized that the recruited macrophages promote the proliferation of other macrophages, suggesting a pivotal role in the initial inflammatory response. Indeed, a steady increase in *Hm*AIF-1 and RNASET2 (Fig. 3f, g) was mainly detected in the initial stages of inflammation, 24–48 h after bacterial infection, and declined by 7–10 days after stimulation (Schorn et al. 2015b; Baranzini et al. 2017). Moreover, in leeches, *Hm*AIF-1 is also elicited during wound healing and allograft tissue recognition and rejection (Fig. 3h). Starting from 24 h after allograft, macrophages, co-expressing *Hm*AIF-1/CD68 and CD45, isolate and infiltrate the graft and produce a large amount of cytokines responsible for mitogenic and chemotactic events (Schorn et al. 2015a). It is interesting to note the co-localization of RNASET2 and *Hm*AIF-1 with the cell surface glycoprotein CD45, a leukocyte-specific member of the transmembrane PTPase family ubiquitously expressed on the surface of all cells of hematopoietic origin (Alkassab et al. 2007; Sommerville et al. 2012; Li et al. 2013; Jeong et al. 2013). In vertebrates, CD45 is implicated in integrin-mediated adhesion of macrophages (Roach et al. 1997; Zhu et al. 2011; St-Pierre et al. 2013) and plays a role in regulating the functional responsiveness of cells to chemoattractants (Roach et al. 1997; Mitchell et al. 1999), affecting the normal feedback mechanisms that are required to maintain adhesion and phagocytic activity.

Collectively, these data clearly demonstrate that in leeches both *Hm*AIF-1 and RNASET2 are involved in early events that trigger inflammation more than they are in the late ones (Autieri et al. 2000).

Use of Medicinal Leeches for Innate Immunity Studies Presents Interesting Potential for Practical Applications

We developed the most original and innovative assay based on the use of the biomatrix matrigel (MG) to achieve in vitro expansion of primary leech cells implicated in immune response and tissue repair (Grimaldi et al. 2008, 2009, 2011; Girardello et al. 2015a). This experimental approach, a kind of in vivo cell sorting, is an important tool used to understand which cells move (and how) and interact during immune response and wound repair and to characterize the genes engaged in these events. Moreover, this method also allows us to achieve in vitro expansion of macrophages in primary leech cells, which is also implicated in the response against abiotic particles such as nanomaterials, and can be used as a quick, sensitive tool for aquatic pollution bio-monitoring.

Selection of Specific Cell Populations for In Vitro Culture, Expansion, and Differentiation Analyses

The MG in vivo cell sorting method is an invaluable tool for studying the features of HSPCs and leukocytes. Starting from the injection of an appropriate combination of the MG supplemented with a selected cytokine/growth factor in the leech body wall, it is possible to isolate in vivo a specific cell population migrating from the tissues in which they reside into the injected biopolymer. Afterwards, the MG containing the migrated cells is removed from the body of animals and can be used as a vector to prepare cell cultures. Notably, unlike standard methods that aim to localize HSPCs in adult tissues to extract and culture them, the use of MG allows specific selection of cell populations for in vitro culture. A comparative analysis of biopolymer in vivo sorted cells indicates that VEGF recruits cells of a hematopoietic/endothelial phenotype expressing CD34, CD117, and the VEGF receptors Flt1/VEGFR1 and Flk-1/VEGFR-2 (Grimaldi et al. 2008, 2009). On the other hand, the cytokines monocyte chemoattractant protein-1 (MCP-1/CC chemokine ligand 2 [CCL2]) (Grimaldi et al. 2008), *Hm*AIF-1, and RNASET2 (Girardello et al. 2015a; Baranzini et al. 2017) recruit monocytes and macrophages expressing CD11c, CD14, CD45, and CD68 (Fig. 3i, j).

Assessment of the Potential Risks to Public Health: Link to Nanomaterial Aquatic Environmental Diffusion

Given that the immune system is the most important natural defense of the organism against external invasions from the environment, the value of the medicinal leech as a reliable, reproducible model for studying the immune response process can also be utilized to assess toxicity of pollutants, such as nanomaterials dispersed in discharged water. The last two decades have seen a steady increase in the production and use of engineered nanoparticles for commercial, industrial, and clinical applications. Unfortunately, intentional or unintentional discharges of these nanomaterials into the environment can occur during their production, transport, use, and disposal. Nanoparticles disperse in water and accumulate in soils through the application of sewage sludge, accidental spills, and deposition from the air, agrochemicals, or soil remediation. Several studies have highlighted the potential impacts of nanoparticles on both aquatic and soil organisms, which can absorb nanomaterials via skin contact or oral uptake through the gastrointestinal tract (Baun et al. 2008; Ali et al. 2014). As the research community is being urged to better clarify the health impact and safety of NMs, risk assessment and ecotoxicology of nanomaterials in the aquatic environment are becoming essential. Indeed, using simple experimental approaches and an anatomically simple model such as leeches, in which the effects of stressful events are unequivocally interpretable, we have been able to monitor the diffusion of MWCNTs in the water environment and the effects of this nanomaterial on the immune system (Girardello et al. 2015a, b). Our results have shown that uptake of MWCNTs by leeches induces toxic effects

even at low concentrations and after a short exposure time, and that it is associated with the induction of an inflammatory response (i.e., macrophage recruitment and amyloid deposition) (Fig. 3k), suggesting novel entry mechanisms and toxicity profiles of NMs. In fact, immunity is an essential function to retain the organism's well-being and represents a sensitive physiological indicator that may be affected even with exposure to low concentrations of nanomaterials (Hayashi and Engelmann 2013).

Closing Remarks

Our data as a whole indicate the following:

1. Leeches share several morpho-functional and cell molecular mechanisms with vertebrates and are a reliable, reproducible tool for screening molecules and studying biological responses, which are surprisingly similar to those occurring in vertebrates, suggesting a remarkable evolutive conservation.
2. Leech hematopoiesis and angiogenesis studies can provide an invaluable tool for functional studies of immune response modulators and may greatly help to better understand tissue stem cells and their role in angiogenesis and tissue regeneration.
3. Comparing the inflammation processes in leech model organisms with mammals provides valuable information about the molecular mechanisms underlying this process faster than if studied in mammal systems.
4. The use of leeches as an experimental model effectively avoids many inconveniences linked to a growing number of restrictions (i.e., ethical considerations, stricter controls for experimental animal engagement, improvements in animal welfare and housing, and the number of animal species available for experimentation).
5. The use of a MG biopolymer is a powerful translation approach aimed at filling the deep gap that exists between the data obtained in vitro and the results observed in vivo.

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Insect Innate Immune Memory

Humberto Lanz-Mendoza and Jorge Contreras Garduño

Innate Immune Memory: History, Controversy, and Attributes

The immune system is a complex network of molecules, cells, and tissues that interact with each other to maintain the genetic and physiological integrity of individuals against invaders (Cadavid 2009). For more than 500 million years of animal evolution, invertebrates and vertebrates have evolved diverse strategies within the immune response to cope with parasites and pathogens (Schmid-Hempel 2011). Some of these strategies, for example, phagocytes evolved in invertebrates and have been conserved in most animal groups, including humans. Other strategies are innovations specific to particular animal groups (e.g., the melanin defense system in invertebrates but not in vertebrates) whose consolidation has responded to environmental conditions or to the presence of parasites (here we use the term parasite as pathogens, viruses, parasites and parasitoids). For example, classical textbooks on immunology propose that differences between the immune response of vertebrates and invertebrates is that immune responses in invertebrates are innate (nonspecific) and confer short-lived protection, and in vertebrates it is innate and adaptive (immune responses that use B and T lymphocytes that develop antigen-specific immune responses and that protect the host for its full life). However, this paradigm is changing because growing evidence proposes that invertebrates and not only vertebrates possess immune memory.

In this paradigm shift, insects have been a cornerstone. In the history of immunology, scientists have found evidence of immune memory in this group, but time

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after time, the evidence has been debated because studies have also supported a nonspecific immune response. For example, in the 1920s, studies on *Galleria mellonella* showed that the injection of attenuated bacteria protect them from a lethal bacteria in a second encounter (Metalnikow 1920) and that aquired resistance in larvae, may protect the pupae and adult moth stages (Chigasaki 1925). These proposals remained dormant for 40 years until studies in *Drosophila* against the bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, and *Aerobacter cloacae* showed that the injection of sublethal doses of bacteria protect them from lethal doses but that such protection was nonspecific (Boman et al. 1972). This evidence convinced immunologists that insects may have long-lasting nonspecific protection rather than immune memory. The possibility that invertebrates may have immune memory was abandoned until new testing was conducted between 1977 and the early 1990s.

In 1977, specificity and memory were demonstrated in coral reefs (Hildemann et al. 1977), and between 1980 and 1992 the group of Richard Karp suggested that the cockroach *Periplaneta americana* may have an immune-memory-like response against bacteria (Karp 1990; Faulhaber and Karp 1992). In 1983, Ann Lackie showed that cuticle transplants in insects were rejected, but she found no evidence of specificity (Lackie 1983). Here it is important to note that studies examined specificity of an innate immune response (see glossary), and the question of whether results supported either long-lasting nonspecific protection or an immune memory (specific) response remained a topic of debate. At the same time, Edwin Cooper established the fundamentals of invertebrate immune memory in annelids. Cooper and his colleagues reported that annelids (earthworms and leeches) showed accelerated rejection, weak specificity, and short-term "memory" mediated by the cellular immune system (Cooper and Roch 1986). The quantitative response of coelomic cells associated with first- and second-set *Eisenia* xenografts transplanted to *Lumbricus* hosts at 20 °C was compared with autografts and nonspecific wounds. Coelomocyte numbers were significantly lower in response to first- versus second-set xenografts. Coelomocytes also increased in association with autografts and nonspecific wounds, but the reaction was short-lived and essential for early wound healing and repair. Such nonspecific increases are different from subsequent specific, immunologic, longer-lasting coelomocyte responses. First-set xenografts induced a relatively slow increase in coelomocytes, which declined after 3–4 days post grafting. By contrast, second-set xenografts caused an accelerated rise in coelomocytes, usually 20–30% greater than the maximum coelomocyte response induced by first-set xenografts. These experiments showed that annelids were able to remember a previous contact with tissues from genetically different organisms, and this phenomenon is analogous to mammalian memory (Cooper 1992). However, the lack of a functional equivalent of B and T cells in vertebrates convinced scientists to accept that the innate immune response of invertebrates was nonspecific and, hence, that invertebrates lacked an immune memory response (Theopold et al. 1996).

It was not until the first decade of the twenty-first century that two seminal studies clearly showed evidence for an innate immune memory at the phenomenological level (without mechanisms). The first study was carried out by the group of Joachim

Kurtz using the crustacean Maxillopoda *Macrocyclop salbidus* against the Cestoda *Schistocephalus solidus* (Kurtz and Franz 2003). In this study, two groups were first challenged with *S. solidus*, and 2 days later one group was challenged with a genetically similar parasite (full sibs) or nongenetically similar parasite (nonfull sibs). The results were very interesting because they revealed a reduced reinfection success of *S. solidus* and less host intensity of reinfection in the former group than the latter, so the priming was specific (Kurtz and Franz 2003). This phenomenon was termed *immune priming* (see glossary) to refer to immune mechanisms of immune memory in invertebrates is different from that of vertebrates (Little and Kraaijeveld 2004). The second study by the group of Tom Little revealed that such protection was also specific, but in this case, the offspring of *Daphnia magna* were protected at strain against strain of *Pasteuria ramosa* that its mothers confronted (Little et al. 2003). This phenomenon is now called *immune priming across generations* or *transgenerational immune priming* (Little and Kraaijeveld 2004). Following the publication of these two studies, the lack of physiological and molecular evidence to support the immune priming concept generated skepticism (Hauton and Smith 2007). However, it was suggested that innate memory might not be mediated through the same mechanisms in different taxa but should be similar at phenomenological and organism levels, since in both invertebrates and vertebrates, self and nonself discrimination may have a strong impact on fitness (Little and Kraaijeveld 2004; Little et al. 2008). This rationale was similar to that in the original proposal of Cooper in 1992 (Cooper 1992). Now immune priming (immune memory) is studied not only in vertebrates (Sun et al. 2009; Paust and Andrian 2011), but also in invertebrates (Kurtz 2005; Schmid-Hempel 2011; Masri and Cremer 2014), plants (Spoel and Dong 2012), and jawless vertebrates (Herrin and Cooper 2010; Buonocore and Gerdol 2016).

Actually, various definitions have been proposed for innate immune memory. Milutinovic and Kurtz (2016) defined immunological memory as *the ability of an immune system to store or simply use the information on a previously encountered antigen or parasite upon secondary exposure*. This may apply to both invertebrates and vertebrates and may be considered as immune memory taking into account that the response *should be specific and long lasting*. In vertebrates, immune priming (immune memory) and immune enhancement (or enhanced protection upon a second nonspecific challenge) has been used sometimes as synonym and defined as immune training (Netea et al. 2011; Netea and van der Meer 2017). We propose that, although immune enhancement, enhanced protection, or immune training could be synonyms of a nonspecific response, immune priming is not part of this definition if it is functionally analogous to vertebrates' adaptive immune response. In this sense, immune training is a broad term that includes immune memory and immune enhancement (Netea et al. 2011; Netea and van der Meer 2017), but immune priming or innate immune memory should be evoked in invertebrates and vertebrates if three attributes are present: (1) specific immune resistance (for review see Milutinovic and Kurtz 2016; Contreras-Garduño et al. 2016) as, for example, in *Tribolium castaneun*, in which it is strain specific (Roth et al. 2009); (2) long-lasting protection (for a review see Milutinovic and Kurtz 2016; Contreras-Garduño et al. 2016), as in the oyster *Crassostrea gigas*, in which

it lasts for at least 5 months (Lafont et al. 2017) or, as in *T. castaneun*, persists from one developmental stage to another (Thomas and Rudolf 2010); and, finally, (3) a biphasic immune response suggestive of an increasing immune response following the first challenge and returning to basal levels but increasing again to a greater degree following the second challenge (Kurtz 2005; Brehélin and Roch 2008; Schmid-Hempel 2011).

Evidence of this phenomenon has only been reported in dipteran insects, particularly mosquitoes (Contreras-Garduño et al. 2015; Vargas et al. 2016) and is the less studied attribute of the three cited here (Contreras-Garduño et al. 2016). We propose that to avoid confusion about the existence of immune memory within an innate immune response, we should be careful in the use of terminology and we should use the term *memory* only if the immune response is specific and long lasting; if not, it should be considered immune enhancement, enhanced protection, or immune training without memory (see a similar rationale in Boraschi and Italiani 2018). In invertebrates, immune priming is a synonym of immune memory (Milutinovic and Kurtz 2016; Contreras-Garduño et al. 2016) and if the term immune training will be used in vertebrates as synonym of immune memory, then the nonspecific response should be not used as evidence for immune training. We should exert care and not include mechanisms in the concept of immune memory or innate memory because some mistakes could occur as occurred with the classical term of adaptive immunity (it included B and T cells). This is because some mechanisms may not exist in a particular taxonomic group. We agree with others (Little and Kraaijeveld 2004; Little et al. 2008; Milutinovic and Kurtz 2016) to define innate memory in a phenomenological sense without invoking mechanisms. For example: *Host-improved protection in terms of immune response, parasite elimination, and survival after being able to respond maximally to a parasite, pathogen, or immune challenge following a first specific exposure; recognized within and across generations* (Milutinovic and Kurtz 2016). Finally, this definition is part of a dichotomy (immune memory vs non-specific immune response upon a second challenge), but could be a gradual immune response (Cooper 2016; Pradeu and Du Pasquier 2018)

Recent reviews (Milutinovic and Kurtz 2016; Contreras-Garduño et al. 2016) have shown that immune priming or innate memory as the adaptive immune memory in vertebrates occurs within or across generations, and since 2003 there has been increasing interest in both topics in invertebrates, with studies in insects dominating the field (Tables 1 and 2). As Tables 1 and 2 show, most experiments support the immune priming phenomenon, but negative results are also encouraged in order to clarify how effective is the immune priming against parasites (Contreras-Garduño et al. 2016). Recently, Milutinovic and Kurtz (2016) published an interesting review of invertebrate immune memory indicating that immune memory has been observed in various invertebrates, including, for example, sponges, mollusks, annelids, crustaceans, insects, echinoderms, and tunicates. However, the mechanism of innate immune memory remains unknown. Pham and Schneider (2008) proposed three features. First, the molecules involved in recognition and protection that confer memory should be induced by an immune challenge. Induction may occur at

Table 1 Studies showing immune priming within generations

Study subject	Pathogen	Measured response	Evidence of priming	References
Macrocyclops albidus (Crustacea, Cyclopoida)	*Schistocephalus solidus* (Cestoda)	Prevalence	Yes	Kurtz and Franz (2003)
Penaeus monodon (*Crustacea*)	Viral proteins	Viral clearance Survival	Yes	Witteveldt et al. (2004)
Bombus terrestris (Insecta, Hymenoptera)	*Pseudomonas fluorescens* (Gram−)	Survival Bacterial clearance	Yes	Sadd and Schmid-Hempel (2006)
	Paenibacillus alvei (Gram+)		Yes	
	Paenibacillus larvae (Gram+)		Yes	
Drosophila melanogaster (*Diptera*)	*Streptococcus pneumoniae* (*Gram+*)	Survival	Yes	Pham et al. (2007)
	Escherichia coli (Gram−)		No	
	Micrococcus luteus (Gram+)		No	
	Beavueria bassiana (Fungus)		No	
	Salmonella typhimurium (Gram−)		No	
	Listeria monocytogenes (Gram+)		No	
	Mycobacterium marinum (Gram+)		No	
	Pseudomonas aeruginosa	Survival, phagocytic activity, bacterial clearance	Yes	Christofi and Apidianakis (2013)
	Drosophila C Virus	Survival	No	Longdon et al. (2013)
Chlamys farreri (Bivalvia, Ostreoida)	*Listonella anguillarum*	Survival, phagocytic activities, and acid phosphatase activity	Yes	Cong et al. (2008)

(continued)

Table 1 (continued)

Study subject	Pathogen	Measured response	Evidence of priming	References
Tribolium castaneum (Insecta, Coleoptera)	*Bacillus thurigiensis* (Gram+)	Survival	Yes	Roth et al. (2009)
	Escherichia coli (Gram−)		No	
	Bacillus subtilis (Gram+)		Yes	
	Bacillus thurigiensis (*Gram+*)	Survival	Yes	Milutinović et al. (2014) Futo et al. (2016) Greenwood et al. (2017) Kennedy et al. (2017) Khan et al. (2016) Khan et al. (2017) Thomas and Rudolf 2012
Anopheles gambiae (Insecta, Diptera)	*Plasmodium berghei*	Intensity of infection Hemocyte differentiation	Yes	Rodrigues et al. (2010)
	Plasmodium	Lipoxin/Lipocalin complex	Yes	Ramírez et al. (2015)
Plodia interpunctella (Insecta, Lepidoptera)	*Plodia interpunctella Granulosis Virus*	Proportion of infection	Yes	Tidbury et al. (2011)
Litopenaeus vannamei (Malacostraca, Decapoda)	*Vibrio alginolyticus* (Gram−)	Hemocyte levels PO activity, SOD lysozyme activity Phagocytic activiy RBs Clearance	Yes	Lin et al. (2013)
	Vibrio alginolyticus (Gram−)	Survival	Yes	Valdez et al. (2014)
	Vibrio harveyi (Gram−)	Phagocytic activity Antibacterial activity	Yes	Pope et al. (2011)
Formica selysi (Insecta, Hymenoptera)	*Beauveria bassiana* (Fungus)	Surivival	No	Reber and Chapuisat (2012)
Camponotus pennsyilvanicus (Insecta, Hymenoptera)	*Serratia marscecens* (Gram−)	Survival	Yes	Rosengaus et al. (2013)

(continued)

Table 1 (continued)

Study subject	Pathogen	Measured response	Evidence of priming	References
Biomphalaria glabrata (Gastropoda)	*Schistosoma mansoni* (Trematoda)	Prevalence	Yes	Portela et al. (2013)
Mnemiopsis leidyi (Tentaculata, Lobata)	*Listonella anguillarum* (Gram−)	Gene expression	Yes	Bolte et al. (2013)
	Planococcus citreus (Gram+)		Yes	
Helicoverpa armigera (Insecta, Lepidoptera)	*Photorhabdus luminescens*	Gene expression	Yes	Zhao et al. (2013)
Cherax quadricarinatus (Malacostraca, Crustacea)	White spot syndrome virus	Dscam expression	Yes	Ng et al. (2014)
Rhynchophorus ferrugineus (Insecta, Coleoptera)	*Escherichia coli* (Gram−)	PO activity Antibacterial activity	Yes	Shi et al. (2014)
Bombyx mori (Insecta, Lepidoptera)	Gram negative PepG	Survival Gene expression	Yes	Miyashita et al. (2014)
Lasius niger (Insecta, Hymenoptera)	*Beauveria bassiana* (fungus)	Survival	Yes	Gálvez and Chapuisat (2014)
Crassostrea gigas (Bivalvia, Ostreoida)	*Vibrio splendidus*	Total hemocyte counts Phagocytic activity Gene expression	Yes	Zhang et al. (2014) Lafont et al. (2017)
Anopheles albimanus (Insecta, Diptera)	*Plasmodium berghei* (Apicomplexa)	Intensity of infection Survival	Yes	Contreras-Garduño et al. (2014)
		Intensity of infection, attacin, cecropin, and gambicin	Yes	Contreras-Garduño et al. (2015)
Galleria mellonella (Insecta, Lepidoptera)	*Photorhabdus luminescens*	Survival, antibacterial activity, hemocyte density, phagocytosis, and encapsulation	Yes	Wu et al. (2014)
	Bacillus thuringiensis		Yes	Wu et al. (2016)
	LPS of *P. luminescens*	Different immune response effectors	Yes	
Parasemia plantaginis (Insecta, Lepidoptera)	*Serratia marcescens* (Gram−) *Escherichia coli* (Gram−)	ROS activity Survival	Yes No	Wu et al. (2015) Mikonranta et al. (2014)

(continued)

Table 1 (continued)

Study subject	Pathogen	Measured response	Evidence of priming	References
Daphnia magna (Branchiopoda, Cladocera)	*Pasteuria ramosa*	Proportion of population infected	Yes	Garbutt et al. (2014)
	Pasteuria ramosa	Proportion of population infected	Yes	McTaggart et al. (2012)
Aedes aegypti (Insecta, Diptera)	*Escherichia coli* (Gram−)	PO activity Nitric oxide production Antimicrobial activity Antimicrobial peptide transcript response	Yes	Moreno-García et al. (2015) Vargas et al. (2016)

transcriptional, translational, or posttranslational levels. Second, to account for observed specificity, they suggested that some of the molecules would be susceptible, revealing diversity. Third, some kind of selection process is required to take into account specificity in immune response. Considering the enormous number of insect species and their extraordinary ability to adapt to different environmental conditions, it is possible to venture the guess that the molecular mechanism of immune memory may vary among different groups and that this may apply also in general to invertebrates and even, within insects (Kurtz, pers. commun.).

Possible Mechanisms Involved in Immune Memory in Insects

One of the main criticisms that the field of innate memory has received in invertebrates and particularly in insects is the lack of molecular mechanisms involved in immune memory (Hauton and Smith 2007). Recently, advances have appeared that can help accept the notion of new mechanisms in insects.

It is important to recognize that the mechanism(s) underlying insect immune memory must be able to retain information from previous contacts with pathogens or antigens. Also, it is of fundamental importance not to confuse the effector mechanisms of memory with its generating mechanism. Vertebrates base their immune memory on clonal selection theory and their ability to proliferate after contact with the specific antigen for each clone. These clones were previously generated, and the receptors emerged by genetic recombination mechanisms based on the transposons RAG1 and RAG 2. These mechanisms have not been found in invertebrates, and therefore we may not expect similar mechanisms and regulation behind immune memory in invertebrates and insects.

In recent years, interesting advances have been made particularly in *Drosophila* and mosquitoes. David Schneider's group found that an injection of a sublethal dose of *S. pneumoniae* in Drosophila protects against a second lethal challenge of *S.*

Table 2 Studies that tested immune priming across generations

Study subject	Pathogen	References
Daphnia magna (Branchiopoda, Cladocera)	*Pasteuria ramosa* (*Gram+*)	Little et al. (2003) Duneau et al. (2016)
Tenebio molitor (Insecta, Coleoptera)	LPS from *E. coli* (Gram−) Serratia marcescens	Moret (2006) Dubuffet et al. (2015) Dhinaut et al. (2018a) Dhinaut et al. (2018b); Castro-Vargas et al. (2017); Contreras-Garduño et al. (2018).
Tribolium castaneum (Insecta, Coleptera)	*Bacillus thurigiensis* (*Gram+*) *Escherichia coli* (Gram−)	Roth et al. (2010)
	Bacillus thurigiensis (Gram+)	Eggert et al. (2014)
		Eggert et al. (2015) Kurtz and Armitage (2017) Tate et al. (2017)
Plodia interpuctella (*Insecta, Lepidoptera*)	*Plodia interpuctella Granulosis Virus*	Tidbury et al. (2011)
Spodoptera exigua (Insecta, Lepidoptera)	*Bacillus thurigiensis* (*Gram+*)	Hernández-Martínez et al. (2010)
Myzus persicae (Insecta, Homoptera)	*Diaeretiella rapae* (*Parasitoid*)	Vorburger et al. (2008)
Bombus terrestris (Insecta, Hymenoptera)	*Arthrobacter globiformis*	Sadd and Schmid-Hempel (2009b)
	Crithidia bombi (*Parasitoid*)	Barribeau et al. (2016)
Chlamys farreri (Bivalvia, Ostreoida)	*Vibro anguillarum* (Gram−)	Yue et al. (2013)
Teleogryllus oceanicus (Insecta, Orthoptera)	*Serratia marcescens* (Gram−)	McNamara et al. (2014)
Apis melliferas (Insecta, Hymenoptera)	*Paenibacillus larvae* (Gram+)	Hernández-López et al. (2014)
Galleria mellonella (Insecya, Lepitoptera)	*Beauveria bassiana*	Dubovskiy et al. (2013)
	Pseudomonas entomophila Serratia entomophila	Freitak et al. (2014)
Anopheles coluzzii (Insecta, Diptera)	*Plasmodium falsiparum*	Vantaux et al. (2014)
Rhynchophorus ferrugineus (Insecta, Coleoptera)	*Escherichia coli* (Gram−)	Shi et al. (2014)
Spodoptera littoralis (Insecta, Lepidoptera)	*nucleopolyhedrovirus*	Wilson and Graham (2015)
Artemia (Branquiopoda, Anostraca)	*Vibrio campebellii* (Gram−)	Norouzitallab et al. (2014)
Tribolium (Insecta, Coleptera)	*Bacillus thuringiensis* (*Gram+*)	Tate and Graham (2015) Tate et al. (2017)
Anoplophora glabripennis (Coleoptera)	*Metarhizium brunneum* (Fungus) *Metarhizium anisopliae* (Fungus) *Serratia marcescens* (Gram−)	Fisher and Hajek (2015)
Trichoplusia ni	*Autographa californica* multiple nucleopolyhedrovirus (AcMNPV)	Shikano et al. (2016)
Crassostrea gigas	Ostreid herpes virus	Green et al. (2016)

pneumoniae (Pham et al. 2007). Survival correlates with lower bacterial load, demonstrating that the immune response is activated to kill bacteria faster. This protective effect can be observed from day 1 and persists for 14 days post priming. Four microbes were tested for their ability to elicit a priming response, including *Listeria monocytogenes*, *Salmonella typhimurium*, and the entomopathogenic fungi *Beauveria bassiana* and *Mycobacterium marinum*. *S. pneumoniae* and *B. bassiana* were able to elicit a highly specific protective effect. Only one dose of *S. pneumoniae* is able to protect against a lethal dose of *S. pneumoniae*, and only a priming dose of *B. bassiana* confers protection against *B. bassiana* infections. There was no cross-protection between these two microorganisms. In addition, Pham et al. (2007) studied the involvement of melanization (prophenoloxidase system) and antimicrobial peptides (AMPs). No differences were observed in melanization between *Drosophila* control and priming and the induction kinetics of AMPs after a second challenge that was identical between priming insects and controls.

Regarding some probable signaling pathway known in the insect immune response and its relation to memory, Pham et al. (2007) observed that while the Imd pathway was dispensable for priming, the Toll pathway seems necessary but not sufficient to trigger memory. Toll mutants cannot generate priming, but activation of Toll using a mixture of inducers was not enough to protect flies. Pham et al. (2007) proposed a model in which the Toll pathway may be necessary for the detection of microbes, and Toll activation becomes another critical path for the priming-specific response. To determine the involvement of hemocytes, Pham et al. (2007) used an inhibition assay for phagocytosis by inoculating polystyrene beads into the *Drosophila* hemocele (Elrod-Erickson et al. 2000). These polystyrene beads are not encapsulated by hemocytes, and the numbers of hemocytes are not altered. The authors inhibited phagocytosis and then treated them with a lethal dose of *S. pneumoniae* 1 week later, at which time phagocytosis was still inhibited. Priming flies died at the same time as the control flies and therefore were not protected by a priming dose with *S. pneumoniae*. *Drosophila* phagocytes appear to be an important effector in the priming response. Recently, Tassetto et al. (2017) described a novel mechanism of RNAi amplification and dissemination in which hemocytes take up dsRNA from infected cells and, using a transposon reverse trancriptase, produce virus-derived complementary DNAs (vDNA). These vDNAs provide a template of de novo synthesis of secondary viral siRNA (vsRNA), which are secreted in exosome-like vesicles, conferring passive protection against virus challenge in naive animals.

As regards mosquitoes, contradictory results have been obtained. Rodrigues et al. (2010) observed that *Anopheles gambie* mosquitoes can generate a memory against *Plasmodium berghei*, but this memory is induced by the bacteria of the middle intestine that are introduced into the hemocoel during infection. Invasion of the midgut of the mosquito by *Plasmodium* ookinetes alters the barriers that normally prevent the intestinal microbiota from coming into direct contact with the epithelial cells. This triggers a long-term response characterized by increased abundance of granulocytes, a subpopulation of hemocytes circulating in insect hemocele, and increased immunity to bacteria, which indirectly reduces the survival of

Plasmodium parasites upon reinfection. In mosquitoes, differentiation of hemocytes was necessary and sufficient to confer the response against *Plasmodium.* The ookinetes pass through the peritrophic matrix (PM) and invade the epithelial cells of the midgut. During this process, parasites alter gut barriers and allow microbiota bacteria to come into direct contact with damaged cells of the midgut. That is, bacteria are responsible for the generation of immune memory and indirectly affect the development of *Plasmodium*. Immune priming triggers production of a soluble factor in the hemolymph, and transfer of cell-free hemolymph from challenged females also triggers the priming response in naïve recipient mosquitoes (Rodrigues et al. 2010; Ramirez et al. 2014). This soluble factor was called hemocyte differentiation factor (HDF). HDF is synthesized after parasite infection and persists for the rest of the life of challenged mosquitoes. Biochemical studies showed that HDF is a lipid/protein complex (Ramirez et al. 2015). Later this compound was renamed Evokin/Lipoxin A4, which enhances hemocyte differentiation, leading to an increase in granulocyte proportions with antiplasmodial immunity. The pathway involved in production of Evokin/Lipoxin A4 is not well established, nor is its role in specific immune memory. It has also been observed that this compound is produced constitutively, which represents a challenge for mosquito physiology and a probable tradeoff. More studies are required to define its role in mosquito immune memory.

On the other hand, Contreras-Garduño et al. (2015) determined that the induction of memory is specific against *P. berghei* and independent of midgut bacteria. To test immune priming, *An. albimanus* mosquitoes were fed gametocytes of *P. berghei*. The mosquitoes were kept at 20–21 °C, since this temperature favors parasite development. Also, the mosquitoes were treated with a mixture of antibiotics to kill bacteria. The mosquitoes were subsequently transferred to a 27 °C environment, since the development of parasites is interrupted at this temperature. Using this strategy, memory was induced in the mosquitoes without a true infection. After the fifth day, the mosquitoes were fed *P. berghei* ookinetes, and infection was evaluated. This experiment lasted 25 days. The number of oocysts and the percentage of infected mosquitoes were recorded and compared among treatments in both experiments. In both cases, primed mosquitoes showed less significant numbers of oocysts and a lower percentage of infection. By contrast, the offspring of mosquitoes with priming were more resistant to infection with *Plasmodium*, which suggests an intergenerational response (Contreras-Garduño et al. unpublished). Bacteria of normal flora of the mosquito *An. albimanus* have been isolated to determine participation of the intestinal bacteria in the memory response. *Enterobacter durans* were abundant in *An. albimanus* inoculated in the hemocele before priming with gametocytes of *P. berghei*; an effect of priming was not observed against the parasite. The absence of bacteria by antibiotic treatment and injection of bacteria of normal flora from mosquitoes into the hemocele indicate that responses are specific against the parasite. In addition, Contreras-Garduño et al. (2015) analyzed the transcripts of three responses related to antimicrobial peptide anti-*Plasmodium* (atacine, cecropin, and gambicine) and observed a biphasic pattern rather than a sustained response. These results indicate that immune memory in mosquitoes can be established without bacteria with a specific response against *Plasmodium*.

We observed that the induction of the immune response in *An. albimanus* was accompanied by an intensive DNA synthesis and polytene chromosome formation (Hernandez-Martinez et al. 2006). In the same way, during the induction of immune memory in *Anopheles albimanus*, we observed intensive DNA synthesis in the midgut and other tissues after priming with *P. berghei.* DNA synthesis is higher following a challenge with large amounts of parasites (Contreras-Garduño et al. 2015). It is likely that cells of different tissues begin a process known as endoreplication, in which multiple copies of the genome or amplicons can be made unless the cell enters mitosis or proliferation (Edgar et al. 2014). On the other hand, during priming in *An. albimanus* we observed the overexpression of the hindsight gene (*hnt*), which is involved in changes of the cell cycle to endoreplication in *Drosophila* (Sun and Deng 2007). DNA synthesis and formation of polytene chromosomes could be a mechanism to increase the activity of genes in order to synthesize large amounts of immune defense proteins (Contreras-Garduño et al. 2015). We have observed similar results with *Aedes aegypti* against dengue virus (DV), where intensive DNA synthesis, activation of the Notch pathway, and overexpression of Delta and Notch (ligand and receptor of Notch pathway) and hnt (Serrato-Salas et al. 2018) occurred. It is also interesting that blocking DNA synthesis with Cisplatin, which reduces the global transcriptional machinery through DNA abduct formation, eliminates the memory effect in both species of mosquitoes, indicating that DNA synthesis and endoreplication are part of the memory mechanisms (Serrato-Salas et al. 2018, b). The production of immune molecules could occur through amplification of genes, which would lead to an increase in the number of templates available for transcription. By amplifying the number of copies of genes, mosquito epithelial cells can efficiently produce the RNA and proteins necessary for parasite or pathogen elimination. This mechanism also avoids the cost of cell proliferation shown in vertebrate immune memory (Fig. 1).

Finally, another possible mechanism is related to epigenesis because this mechanism is involved in the insect immune response (Mukherjee et al. 2017; Heitmueller et al. 2017). Castro-Vargas et al. (2017) observed differences in RNA methylation in *Tenebrio molito*r larvae, where primed adults and larvae showed lower levels of methylation than the control groups, and it is important to note, that no differences were found taking into account DNA methylation and that RNA methylation was not related with immune priming across generations. This observation requires confirmation in other models to determine the role of epigenetics in immune priming and more epigenetic mechanisms should be taken into account (Castro-Vargas et al. 2017).

Finaly, parasites or pathogens activate the Delta ligand. Delta is recognized by Notch, which in turn activates the translocation of hnt to the nucleus. DNA synthesis and endoreplication are activated by multiple copies of immune genes. These genes can rapidly activate during a second encounter with the same parasite or pathogen. Insects are capable of mounting effective immune memory responses following an immune challenge. The memory response can be nonspecific to highly specific. The mechanism(s) is not well established, and, considering the high insect variability and capacity to adapt to different environments, there could be different

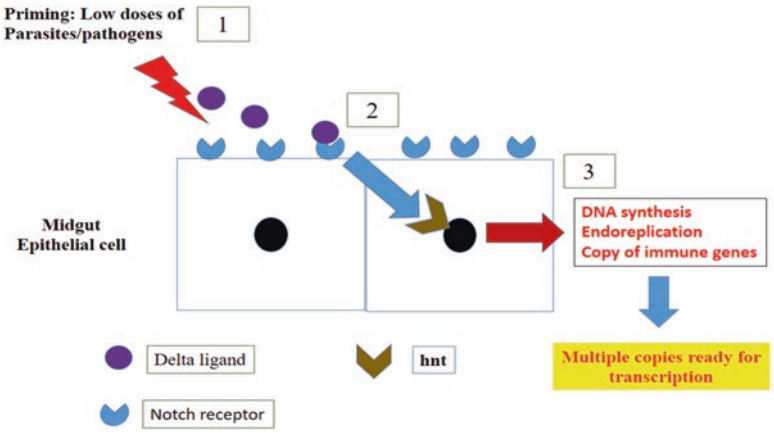

Fig. 1 Immune priming in *Anopheles albimanus*

mechanisms. More studies are required to confirm these observations and determine which of them are general in insects or species-specific.

We would like to conclude by mentioning that an important part of our knowledge of invertebrate immune systems derives from studies in insects, and recent advances have been made in insect immune memory. However, it is always worth remembering that knowledge obtained in insects is not easily generalized. This chapter presented the current understanding of the molecular mechanisms that mediate immune memory in insects, and in particular mosquitoes, in response to different pathogens. From the point of view of comparative immunology, it is essential to understand the properties, mechanisms, and characteristics of insects' immune memory, which will provide valuable information for understanding the relation of this mechanism to the adaptive response of vertebrates and of the molecular basis of immune memory in invertebrates, and it will also allow us to manipulate or modify immune responses to prevent the transmission of diseases (such as malaria or dengue) and will also promote the prevention of invertebrate diseases of commercial interest, such as crustaceans (shrimp, prawns, and oysters). In key examples, Jorge Olmos, working with shrimp (Valdez et al. 2014), and Caroline Montagni, working with oysters (Lafont et al. 2017), have shown that a prior "vaccination" protects these invertebrates from viruses, which could make it possible to reduce economic losses to this industry. Apart from the mechanisms and ecological and evolutionary implications of innate immunity (Milutinovic and Kurtz 2016; Contreras-Garduño et al. 2016), more efforts should be devoted by scientists to the study of immune memory to better deal with biological regulation and insect diseases.

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Glossary: Key Definitions Regarding Immune Priming Theory

Priming: A challenge that activates the immune response and that may favor host molecule recognition.

Immune priming: Host-improved protection in terms of immune response, parasite elimination, and survival after been able to respond to a parasite, pathogen, or immune challenge following a first specific exposure; recognized within and across generations.

Immune enhancement or enhanced protection: A condition where an immune response is activated by artificial methods such as adding probiotics or exposure to nonharming immune-stimulant molecules, rendering an immune response over the physiological levels or keeping pathogens at bay (microbiota effect on many pathogens) but without exhibiting specificity and memory. This may occur within and across generations, and the protection against a second challenge after a first challenge could be due to a sustained immune response or an unspecific biphasic response.

Specificity: In invertebrate biology, it is difficult to determine specificity against an epitope of a given antigen. However, many molecules recognize molecular patterns such as Scavenger receptors, Toll-like receptors, and Nod-like receptors (NLRs), which bind and transduce specific signals to molecules present in pathogens without exhibiting high specificity as vertebrate immunoglobulins. At a functional level, immune protection should occur, for example, in homologous (similar) challenges with the same parasite or pathogen species or strains rather than in heterologous (dissimilar) challenges. This means that the secondary response should only be elicited by homologous challenges or should be stronger and faster than heterologous challenges.

Nonspecific immune response: Humoral and cellular responses not directly linked to a given pathogen's structure. For example, a first challenge with a fungus may protect against Gram-positive bacteria, nematodes, or yeasts.

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Arthropoda: Pattern Recognition Proteins in Crustacean Immunity

Lage Cerenius and Kenneth Söderhäll

Introduction

Invertebrates comprise a diverse collection of animals whose immune reactions are equally diverse. They lack immunoglobulin-based antibodies, and invertebrate immune reactions, in general, are triggered by the binding of pattern recognition proteins (PRPs)/receptors to molecules characteristic of potential pathogens. Common examples of such molecular patterns, which in many cases are capable of eliciting a strong immune response, are β-1,3-glucans, peptidoglycans, and lipopolysaccharides (LPSs). Small quantities of such compounds of microbial origin have long been known to trigger a robust immune response if introduced into the hemolymph. Recent research has added further layers of complexity to this picture; microorganisms may be tolerated in certain parts of the animal, such as the alimentary canal. Bioinformatics data show an expansion of the number of potential PRP genes in some lineages and the absence of some PRP classes in other phyla. In this chapter, we aim to provide a historic perspective on the nature of pattern recognition in crustaceans in light of recent advances in biochemical and genetic characterization of these PRPs.

β-1,3-Glucan Pattern Recognition

Much of the early research in the field of immunology was directed towards β-1,3-glucan as it is a known elicitor of defense reactions throughout the animal kingdom. β-1,3-Glucan is an important component of the cell walls of fungi and oomycetes and thus a signature characteristic of important pathogens. Despite their obvious role in the immune system, the major mammalian PRP for these sugars, the C-type

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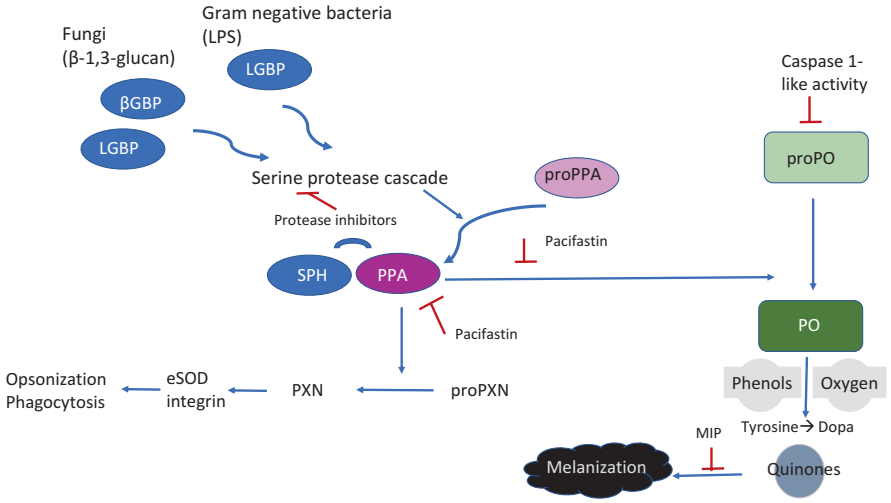

Fig. 1 Schematic representation of the crustacean prophenoloxidase-activating system. Microbial products will, via pattern recognition proteins such as βGBP, LGBP, and many others, trigger activation of the prophenoloxidase system, a complement-like system of different activities such as cytotoxic and opsonic reactions (for details see, for example, Cerenius et al. 2008). Activation and release of prophenoloxidase from hemocytes is modulated intracellularly by a caspase 1-like activity (Jearaphunt et al. 2014). (Figure courtesy of Gül Gizem Korkut). *eSOD* extracellular superoxide dismutase, *LGBP* lipopolysaccharide and glucan-binding protein, *LPS* lipopolysaccharide, *MIP* melanization inhibitory protein, *ppA* prophenoloxidase activating enzyme (a trypsin-like serine proteinase), *PXN* peroxinectin, *PO* phenoloxidase, *ROS/RNS* reactive oxygen/nitrogen species, *SPH* serine proteinase homologues, βGBP β-1,3-glucan-binding protein)

lectin dectin-1, was only found to constitute a β-1,3-glucan PRP relatively recently (Brown and Gordon 2001). In the crayfish *Astacus astacus*, minute amounts of β-1,3-glucans specifically activated prophenoloxidase (proPO) to produce melanin (Fig. 1) (Unestam and Söderhäll 1977). Detailed investigations in this species demonstrated that several linear β-1,3-glucans in concentrations far smaller than 0.1 μg/ml are potent proPO activators and that a linear 1,3-linked pentaose would suffice to trigger activation (Söderhäll and Unestam 1979), findings that indicate the presence of specific recognition proteins in the hemolymph. A 100-kDa plasma protein has been purified from the crayfish *Pacifastacus leniusculus* and biochemically and functionally characterized (Duvic and Söderhäll 1990). This β-1,3-glucan-binding protein (βGBP) could, after binding the glucans, enhance the peptidase activity of the proPO-activating enzyme (ppA). Active ppA proteolytically processes proPO into an active melanin-producing phenoloxidase (PO), and the latter attaches to foreign surfaces and thereby spatially restricts the immune reaction (Söderhäll 1981). The glucan (laminaran)-reacted βGBP (βGBP-L) binds to a heterodimeric 350-kDa membrane receptor on the hemocytes (Duvic and Söderhäll 1992). The detailed intracellular signaling events that may follow upon receptor binding have not been established but the binding will result in hemocyte spreading and degranulation (Figs. 2 and 3) (Barracco et al. 1991; Duvic and Söderhäll 1992)

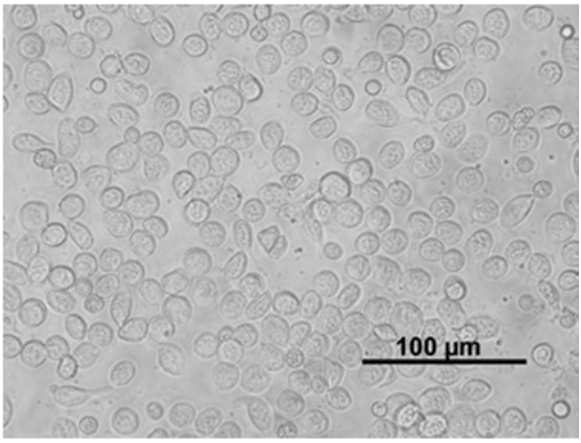

Fig. 2 Naïve hematopoietic cells before reacting to microbial compounds. Most cells are round. (Figure courtesy of Kingkamon Junkulo)

and βGBP-L will function as an opsonin, as demonstrated in the crab *Carcinus maenas* and in crayfish (Thörnqvist et al. 1994; Cerenius et al. 1994), possibly mediated through the above-mentioned membrane receptor. The ability of the βGBP-L complex to agglutinate yeast cells (as shown in two shrimp species) is likely to facilitate their melanization as well as their phagocytosis (Goncalves et al. 2012). This protein is present in high concentration in the plasma (Duvic and Söderhäll 1990), synthesized in hepatopancreas (Cerenius et al. 1994), and is involved in other physiological processes such as lipid transport and also clotting (Hall et al. 1995; Yepiz-Plascencia et al. 1998).

However, as is discussed further, the major mediator of β-1,3-glucan-induced immunity in crustaceans is the LPS- and β-1,3-glucan-binding protein (LGBP) (sometimes called Gram-negative bacteria-binding protein or glucanase-related protein), a protein with a much broader phylogenetic distribution than βGBP, the latter seemingly restricted to crustaceans. As its name implies, LGBP will bind cell wall components from Gram-negative bacteria and fungi. The LGBP primary amino acid sequence was first determined in insects (Lee et al. 1996; Dimopoulos et al. 1997) and then in the first crustacean, the crayfish *P. leniusculus* (Lee et al. 2000). Its sequence contains glucanase-like motifs (i.e., glycoside hydrolase family) and it is often assumed that LGBPs have evolved from dietary glucanases. In both insects and crustaceans LGBP will mediate proPO activation by binding microbial polysaccharides (Lee et al. 2000; Amparyup et al. 2012), and *LGBP* gene knockdown in the shrimp *Penaeus monodon* will reduce hemolymph PO activities (Amparyup et al. 2012). Genomic data show that *LGBP*-like genes are widespread in different crustacean phyla and that there may be seven to eight *LGBP*-like genes in several crab and crayfish species. It was therefore speculated that the LGBP family has expanded in the crustaceans, perhaps to compensate for the lack of peptidoglycan recognition receptors in these animals (Lai and Aboobaker 2017). There are no data yet to show whether these genes give rise to proteins with any differences in activities, tissue specificity, and so on. Perhaps the somewhat conflicting reports (e.g., Roux et al. 2002; Sritunyalucksana et al. 2002; Cheng et al. 2005; Amparyup et al. 2012;

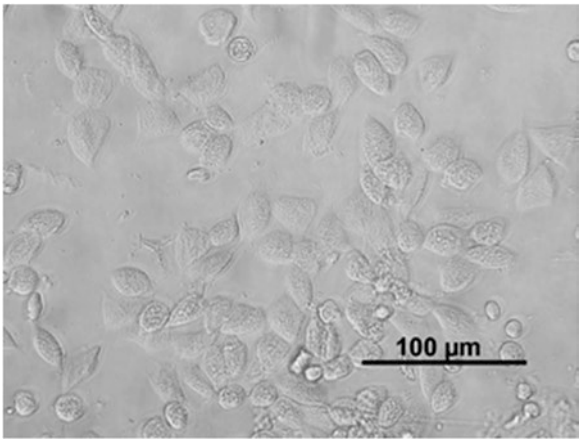

Fig. 3 Hematopoietic cells after incubation with β-1,3-glucans. Both hematopoietic cells and hemocytes will become flattened as the result of the presence of microbial products. (Figure courtesy of Kingkamon Junkulo)

Chaosomboon et al. 2017) on different shrimp and crayfish species as to whether LGBP is mainly produced in hemocytes or by the hepatopancreas can be attributed to this apparent diversity of *LGBP* genes.

Interestingly, in crustaceans there are additional PRPs, which, despite lacking sequence similarities to known glucan-binding proteins, still function as avid binders of β-1,3-glucans (and LPS) and mediators of immune reactions. In *P. leniusculus* a masquerade (mas)-like protein produced by hemocytes (Huang et al. 2000) has both β-1,3-glucan-binding and LPS-binding activities (Lee and Söderhäll 2001). Upon binding to microbial polysaccharides, this protein is processed proteolytically and an opsonic activity is produced. The crayfish mas-like protein is a large heterodimeric protein composed of two subunits of 134 and 129 kDa (Lee and Söderhäll 2001). The subunits give rise to four fragments each upon reaction with microbial cell walls, including one with a mass of 33 kDa that possesses cell-adhesion activity and is thus likely to be required for opsonic activity. It is apparent from the literature on arthropod immunity that serine protease homologs (SPHs) have regularly been shown to be used as components of immune systems. For example, in addition to acting as PRRs, several insect (Yu et al. 2003) and crustacean SPHs (Liu et al. 2011; Jearaphunt et al. 2015) are components of the molecular complexes that trigger proPO activation.

In summary, three well-characterized proteins, namely βGBP, LGBP, and the mas-like protein, have been demonstrated to act as PRRs for β-1,3-glucans in crustaceans. The details of how these three, and possibly additional, PRRs act to mediate β-1,3-glucan-induced immune reactions remains to be established. Several possible scenarios can be suggested, such as that several PRRs are needed to cooperate for a full response, that they are back-ups for each other, or that they specialize on different β-1,3-glucans with different biochemical characteristics such as the nature of their side chains. Further research is needed to discriminate among these and other possibilities.

Lipopolysaccharide Pattern Recognition

LPS, which is characteristic of the outer cell-layers of Gram-negative bacteria, has been shown early on to induce the melanization reaction in crayfish; the minimal concentration needed is about 10^{-10} g/ml (Häll and Söderhäll 1984). As discussed in section "β-1,3-Glucan Pattern Recognition", LGBP and the mas-like protein can both bind LPS, but other LPS-binding PRRs are likely to exist in crustaceans as well. Obvious candidates for such a role are the numerous C-type lectins characterized from crustaceans (see, for example, Wang and Wang 2013; Pees et al. 2016), several of which bind LPS and/or agglutinate bacteria. The C-type lectins PcLec1–4 from *Procambarus clarkii* were shown to bind LPS (as well as lipoteichoic acid [LTA] and peptidoglycan) in an enzyme-linked immunosorbent assay (ELISA) using recombinant lectin (Zhang et al. 2011, 2013a, b, 2016). A *Marsupenaeus japonicus* lectin (MjGCTL) that exhibits proPO-activating and hemocyte encapsulation-promoting activities was shown to bind to different bacteria, and these binding activities were to varying degrees prevented in the presence of LPS and peptidoglycan (Alenton et al. 2017). There are several additional lectins (C-type lectins, L-type lectins, and galectins) described from different crustacean species whose binding activities may be affected by LPS, such as the C-type lectins PmLec (Luo et al. 2006), MrLec (Feng et al. 2016), MrCTL (Huang et al. 2016), and LvCTL3 (Li et al. 2014), the L-type lectin MjLTL1 (Xu et al. 2014), and the crab galectin EsGal (Wang et al. 2016).

In an analysis of transcripts from the shrimp *Litopenaeus vannamei* 65 different C-type lectins were identified; other crustaceans also appear to encode significant numbers of such lectins (Lai and Aboobaker 2017). Recently, shrimp C-type lectins have been implicated in binding to hemocyte β-integrins and thereby in promoting cytoskeletal reorganization and phagocytosis by FcLec4 (Wang et al. 2014a) and stimulating expression of genes coding for antimicrobial peptides (Wang et al. 2014b). In one example of the *LvCTL3* gene mentioned above, a nuclear factor (NF)-κB binding motif has been found in its promotor region, indicating that the lectin gene might be regulated together with other immune-related genes (Li et al. 2014). Clearly, this high number of crustacean lectins will require extensive functional analyses to determine their role in immunity, although it should be kept in mind lectins have important endogenous roles not directly connected to immunity as well, such as in promoting glycoprotein folding in the endoplasmic reticulum and in binding to endogenous glycoproteins and to the extracellular matrix. Of note, one such protein-folding lectin, calnexin, binds bacteria and promotes phagocytosis in the shrimp *M. japonicus* (Zhang et al. 2014a). One phylogenetically widespread group of lectin-like proteins is the fibrinogen-like proteins (FREPs) to which, among many others, the ficolins and tachylectins belong. Other members of the group are the snail FREPs (reviewed in Loker et al. 2004), proteins that possess highly variable immunoglobulin-like and fibrinogen domains. In the crayfish *P. leniusculus*, two ficolin-like proteins (FLPs) are able to agglutinate and clear gram-negative bacteria have been described (Wu et al. 2011). Similar proteins have been found in *L. vannamei*

(Coelho et al. 2016), *M. japonicus* (Chai et al. 2012; Sun et al. 2014) and *Macrobrachium rosenbergii* (Zhang et al. 2014b), and in *P. monodon*, a melanization inhibiting protein belongs to this group (Angthong et al. 2010).

As discussed in section "β-1,3-Glucan Pattern Recognition" regarding the mas-like protein, SPHs may, in addition to other related roles such as participation in the proPO-activating molecular complexes, directly act as a PPR. A *P. monodon* 52 kDa SPH will bind LPS to mediate hemocyte adhesion and bacterial clearance (Jitvaropas et al. 2009). The LPS-binding activity has been shown by the use of recombinant polypetides corresponding to different parts of the protein to reside in the carboxy-terminal region that contains the serine proteinase domain (Jitvaropas et al. 2009). Other types of proteins may potentially act as PRRs for LPS or other bacterial compounds such as scavenger receptors (SRs). This structurally diverse group of proteins bind and internalize oxidized or acetylated low-density lipoproteins and other endogenous damaged lipid-containing compounds on, for example, apoptotic cells. They have traditionally been divided into three main classes—SR class A, B and C—although more complex divisions now exist (Canton et al. 2013). Vertebrate SRs have been demonstrated to bind bacterial compounds such as LPS and LTA and thus act as a PRR (Dunne et al. 1994). In shrimps and crabs, several class B SRs have been cloned (Hou et al. 2017; Yang et al. 2016; Bi et al. 2015; Mekata et al. 2011). Some of the crustacean SRs might mainly or entirely be involved in endogenous tasks such as tissue remodeling, but for some of them a role as a PRR seems evident. In the shrimp *M. japonicus*, an SR, MjSR-B1, has been found to be engaged in binding bacteria and to promote their phagocytosis (Bi et al. 2015). The recombinant MjSR-B1 is capable of agglutinating both Gram-positive and Gram-negative bacteria as well as binding to LPS and LTA but not to peptidoglycan. Knocking down the *MjSR-B1* gene resulted in reduced phagocytosis and increased bacterial titers in the hemolymph (Bi et al. 2015). A C-type SR, MjSRC, from *M. japonicus* was demonstrated to mediate white spot virus endocytosis into hemocytes via binding to one of the virus envelope proteins, VP19 (Yang et al. 2016).

Peptidoglycan Pattern Recognition

Throughout metazoans, peptidoglycans—cell wall constituents present in practically all bacterial groups—are recognized by a conserved group of proteins: peptidoglycan-binding proteins (PGRPs). However, with a few exceptions (Lai and Aboobaker 2017), they are in general conspicuously absent in crustacean genomes (Lai and Aboobaker 2017; Kao et al. 2016; McTaggart et al. 2009). This begs the question of how peptidoglycan pattern recognition occurs in crustaceans. In *P. leniusculus* a complex of two SPHs and the LGBP will bind lysine-type peptidoglycans and this complex will induce proPO activation (Liu et al. 2011). All three proteins seem to be necessary for the proPO activation, as knocking down any of them in a cell culture will interfere with peptidoglycan-induced activation of proPO (Liu et al.

2011). Similar results on peptidoglycan-induced PO activation have been recently obtained in the shrimp *P. monodon* (Jearaphunt et al. 2015). Two SPHs were identified that were both found to bind peptidoglycans with dissociation constants around 6 nM. To achieve maximal PO activation both SPHs were required, although only one of them, PmSPH1, was found to interact directly with the ppA (Jearaphunt et al. 2015). No role for a LGBP was reported in this system. The PmSPH1 was earlier shown to bind LPS as well (Jitvaropas et al. 2009). A mud crab (*Scylla paramamosain*) SPH has also been shown to bind both LPS and peptidoglycan and to mediate proPO activation by the use of the recombinant protein (Zhang et al. 2013a, b). It is obvious that in many crustaceans routes exist for peptidoglycans to activate immune reactions without the involvement of a classic PGRP. In *P. monodon* a QM protein has been suggested to constitute part of a protein complex mediating PGN recognition, as based on a pull-down assay with PGN (Udompetcharaporn et al. 2014). Recombinant PmQM used to coat agarose beads will mediate melanization of these beads.

Other Pattern Recognition Events in Crustaceans

Much less is known about recognition of microbial/pathogenic signature patterns in crustaceans besides the three compounds discussed in this chapter. They are likely to exist, however. A number of other proteins (for a discussion, see, for example, Wang and Wang 2013) still without a well-defined target among microbial compounds have been suggested as putative PRRs in crustaceans, such as thioester-containing proteins (TEPs) based on their function in pattern recognition in other animal groups (e.g., insects). There is, although beyond the scope of this review, evidence for the recognition of double-stranded RNA in crustaceans such as the trans-activation response RNA-binding protein in the shrimp *Marsupenaus japonicus* that will trigger an antiviral response (Wang et al. 2012). One possible candidate group for pattern recognition in crustaceans, the TEPs, have been isolated and characterized from many sources (e.g., Hall and Söderhäll 1994; Gollas-Galvan et al. 2003; Rattanachai et al. 2004; Ho et al. 2009; Ma et al. 2010; Perazzolo et al. 2011; Wu et al. 2012; Li et al. 2017) and shown to regulate the immune response (Ponprateep et al. 2017; Aspán et al. 1990). Still, however, there are no data in a crustacean for a direct role as a PRR, as there are for mosquito TEP1 (Levashina et al. 2001) or fruit fly macroglobulin complement-related (Mcr) (Stroschein-Stevenson et al. 2006). TEP1 forms a thioester linkage when the protein, after being proteolytically processed, binds to bacterial cell walls, whereas Mcr binding to fungal cell walls appears to involve neither a thioester linkage nor proteolytic processing of the TEP. Another obvious candidate is the highly variable immunoglobulin-related protein Dscam, which is widely present in all investigated crustacean phyla and which, potentially by alternative splicing, may appear in thousands of different variants (Lai and Aboobaker 2017). We are, however, still far from having a clear picture of an immunological role for this protein in crustaceans or in any invertebrate.

Concluding Remarks

Pattern recognition has been in the forefront of crustacean (and other) immunology for a long period and we now have a relatively detailed picture of how β1,3-glucans, LPS, and peptidoglycans are dealt with by the immune system. A combination of evolutionary highly conserved proteins such as LGBP with a wide presence in several phyla acting side-by-side with each other in pattern recognition is possibly unique to crustaceans or even groups of crustaceans. It is likely that a combination of an urgent need to be able to efficiently bind pathogen-derived molecules and the relative ease with which different protein motifs or parts thereof can be co-opted for the use of pattern recognition has been instrumental in creating the wide variety of crustaceans PRPs seen today. A challenge for the future is to link them into functional units within the different arms of the crustacean immune system.

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Immunity in Molluscs: Recognition and Effector Mechanisms, with a Focus on Bivalvia

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An Introduction to Bivalve Molluscs

Evolution and Life Cycle

The phylum Mollusca includes eight taxonomic classes comprising more than 85,000 living species, and 60,000 additional species documented by fossil records (Fig. 1). This ranks molluscs as the second most abundant phylum of animals after arthropods and before chordates (Ponder and Lindberg 2008). Molluscs are successful invertebrates characterized by a broad morphological and physiological diversity. They are extraordinarily well adapted to adverse environmental conditions and, starting from the early radiation that occurred in the Late Cambrian era, they have colonized almost

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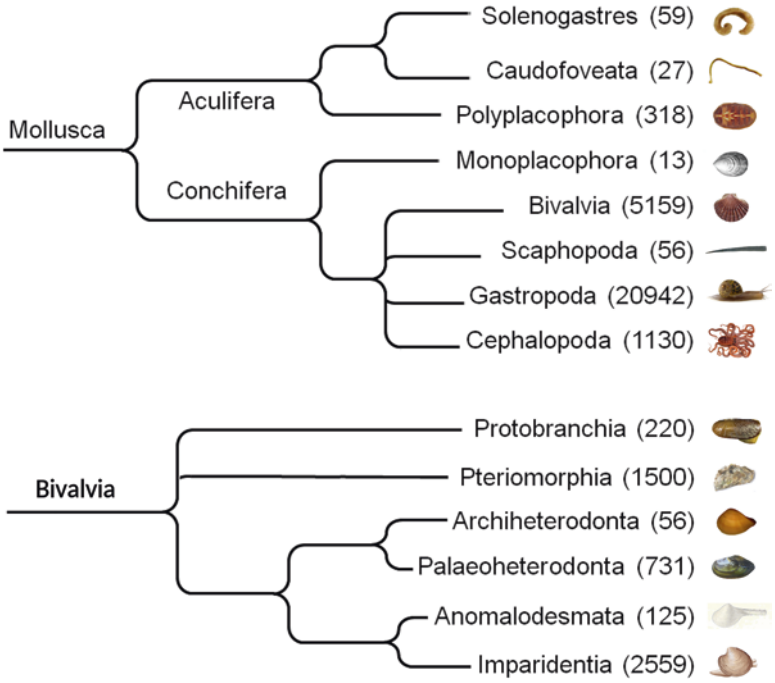

Fig. 1 Simplified tree of life of molluscs (above) and bivalves (below), based on Bieler et al. (2014) and the Tree of Life web project (http://tolweb.org/Mollusca/2488). The number of species currently registered in the NCBI Taxonomy database for each taxon (data retrieved in December 2017) is displayed between brackets

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all ecological niches: from terrestrial habitats over 3000 meters above sea level to deepsea hydrothermal vents, coping with extreme levels of heavy metals, pH, temperature, CO_2, methane, and sulfide (Plazzi and Passamonti 2010).

Bivalvia represent the second largest class within the phylum Mollusca, with over 5000 recognized species, mostly adapted to marine environments. Although the phylogenetic relationship among the different groups of bivalves and, more generally, of all molluscs have been the subject of debate for decades (Kocot et al. 2011; Smith et al. 2011; Sigwart and Lindberg 2015), recent studies tried to reorganize the bivalve tree of life into six major lineages, as shown in Fig. 1 (Bieler et al. 2014). Briefly, the authors recognized the primitive and relatively small group of Protobranchia, the large groups of Pteriomorphia (comprising oysters, mussels, and scallops, among others), Palaeoheterodonta (mostly freshwater clams and mussels), Imparidentia (the largest and most diverse group of bivalves, comprising over 2500 clam species), and two additional small groups with peculiar morphological features, i.e., Archiheterodonta and Anomalodesmata.

Bivalves can be protandric hermaphrodites (oysters in the genus *Crassostrea*), simultaneous hermaphrodites (scallops in the genus *Pecten*), and rhythmical consecutive hermaphrodites (oysters in the genus *Ostrea*). As exemplified in Fig. 2, the life history of the majority of molluscan bivalve species starts during the main spawning season when adult animals with mature gonads release oocytes and spermatozoa in the water column and external fertilization occurs (Pechenik 2010). Bivalve larvae are planktonic (free-living) and remain in the water column for days to weeks, depending on the species and the environmental conditions. During larval development, the molluscan embryo becomes a planktonic trochophore larva. The late trochophore is the phylotypic stage, defined as the ontogenetic stage, characterized by maximum similarity among the species within a phylum (Xu et al. 2016a). After a few days, the primordium of the shell appears and the bands of cilia used by

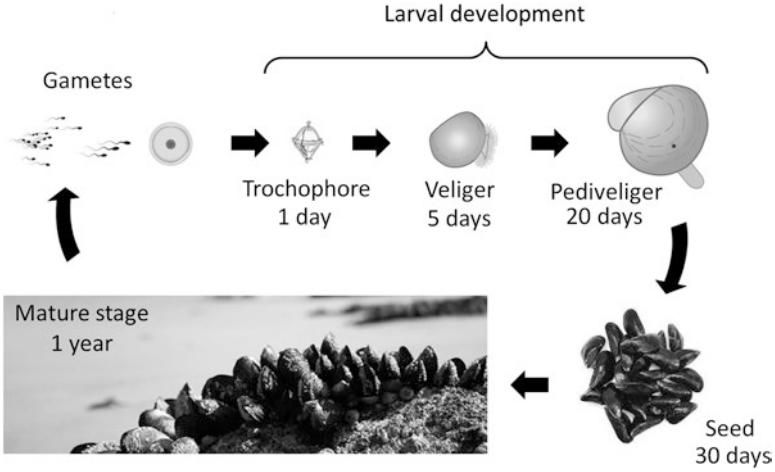


Fig. 2 Life cycle of a bivalve, as exemplified for the Mediterranean mussel, *Mytilus galloprovincialis*

larvae to feed and swim develop into the velum, a characteristic organ of the veliger stage. Then, larvae develop a foot, characteristic of the pediveliger stage, and undergo metamorphosis. Once metamorphosis is complete, their body plan and physiological aspects resemble those of the adult form and the larvae will settle out of the water column where, depending on the species, they might attach to a substrate, lie on a substrate and swim, or bury themselves in sediments (Balseiro et al. 2013). When adults become mature, gametogenesis occurs, with modalities that depend on the species, geographic region, water depth, and season (Shumway and Parsons 2006).

Anatomy and Physiology of Bivalves

Although the adult anatomy of molluscs can greatly differ from one taxon to another, they share a general basic plan derived from a hypothetical shared ancestor (Fig. 3). This includes a soft oval body with bilateral symmetry, a muscular foot, a mantle—which secretes the shell (absent or internalized in some groups) or the spicules—and a feeding organ formed by chitinous sharp structures, called radula (absent in bivalves).

Overall, this shared body plan results in a great morphological diversity of bivalve groups adapted to different ecological niches, as shown in Fig. 4 (Ruppert et al. 2004). Bivalve shells consist of two, sometimes symmetric, hinged valves. The shell is produced by secretory cells in the epithelium of the mantle or pallium, with contributions from the hemocytes (blood cells) (Mount et al. 2004). Bivalve shells are formed mainly of conchiolin, which is composed of protein-hardened calcium

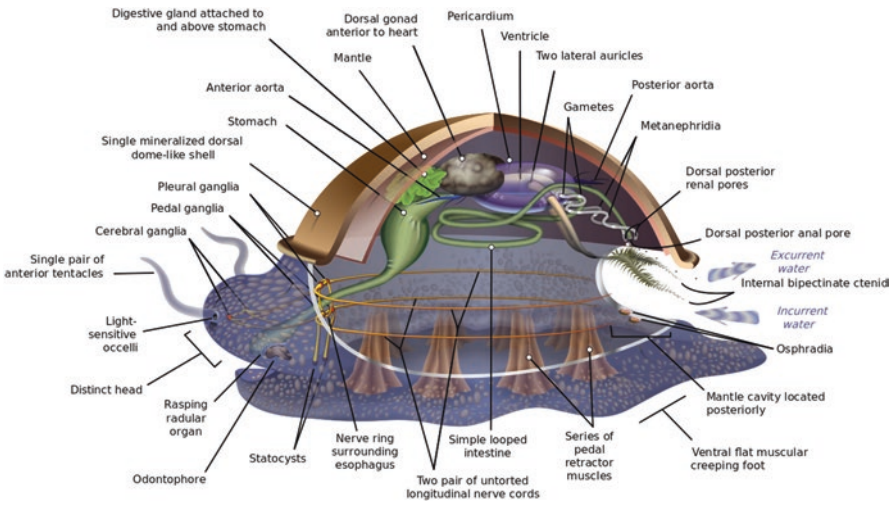


Fig. 3 Anatomy of the hypothetical common ancestor of all molluscs. (Author: KD Schroeder—Archimollusc-en.svg from Wikimedia Commons—License: CC-BY-SA 3.0)

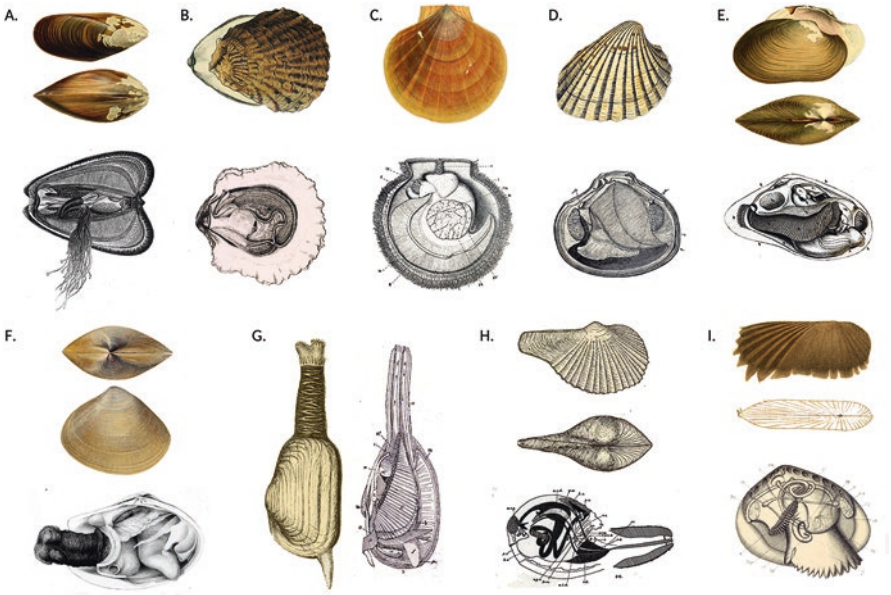


Fig. 4 Examples of diversity in the basic anatomy of different bivalve lineages. (**a**) Anatomy of Mytiloida (Pteriomorphia): *Mytilus unguiculatus* (external) and *Mytilus galloprovincialis* (internal). (**b**) Anatomy of Ostreoida (Pteriomorphia): *Ostrea edulis*. (**c**) Anatomy of Pectinoida (Pteriomorphia): *Placopecten magellanicus*. (**d**) Anatomy of Archiheterodonta: *Cardites floridanus* (external) and *Astarte borealis* (internal). (**e**) Anatomy of Palaeoheterodonta: *Anodonta cygnaea*. (**f**) Anatomy of Mactroidea (Imparidentia): *Mactra antiquata* (external) and *Tresus capax* (internal). (**g**) Anatomy of Myida (Imparidentia): *Mya arenaria*. (**h**) Anatomy of Anomalodesmata: *Cardiomya reticulata* (external) and *Laternula elliptica* (internal). (**i**) Anatomy of Protobranchia: *Solemya velum* (external) and *Ennucula delphinodonta* (internal). To better show anatomic internal details, in most cases one of the valves and the mantle have been removed. (The anatomic tables have been taken from multiple sources, kindly provided by the Biodiversity Heritage Library)

carbonate (aragonite or calcite) and has three layers: the outer layer (periostracum), a middle layer, and the inner layer, which is often nacreous and in some cases has exceptional economic value. The mantle encloses a chamber surrounding the bivalve body called the mantle or pallial cavity, which is in direct contact with the environment when the shell is open. Organs that have direct contact with the pallial cavity include the gills (or ctenidia), the osphradia (chemical sensors), and the openings of the nephridia, gonads, and digestive system. The space between the mantle and the shell constitutes the extrapallial cavity (Ruppert et al. 2004).

The movement of shell valves is controlled by one, two, or (rarely) three adductor muscles that control shell closure and keep it tightly shut when needed, and by an elastic ligament that acts as a spring, allowing the shell to open when muscles are relaxed. Some bivalves also possess a pair of siphons (inhalant and exhalant) used in the exchange of water. These systems ensure the flow of water into the pallial cavity for feeding and respiration.

The gills divide the mantle cavity into distinct chambers and their cells possess cilia, which produce a laminar flow of water that facilitates feeding and enhances respiratory gas diffusion and exchange. Gills also exhibit osmoregulatory, ion transport, homeostasis, and sensorial functions (Moreira et al. 2015). Gas exchange occurs mainly in the center of the gill filament, where the hemocytes circulate through hemolymph vessels. Most bivalves absorb oxygen directly from water through their tissues and oxygen-carrying molecules such as hemocyanin have been identified in only a few genera. As coelomates, bivalves have another characteristic cavity, the coelom, a small pericardial cavity enclosing the heart. Hemolymph is pumped throughout the body by the heart, which receives oxygenated blood from the gills and pumps it into the main blood vessel, a short artery that opens directly into the hemocoel. Bivalve molluscs have an open circulatory system, with the hemolymph reaching all of the organs by passive diffusion aided by the pumping effect of the heart, which also has excretory functions. A pair of nephridia connected to the coelom extracts any reusable materials from the coelomic cavity, dumps additional unwanted products into it, and then excretes all of the materials into the mantle cavity. In bivalves, gonads are located within the connective tissue at the edge of the mantle, with spawning occurring directly in the mantle cavity (Ruppert et al. 2004).

Depending on the species, bivalves feed on suspended particles in the water column, using an inhalant opening or siphon and ctenidia (e.g., *Crassostrea* spp. oysters); on deposits or particles on top of sediments, using an inhalant siphon and ctenidia (e.g., *Macoma* spp. clams); or on deposits in the sediments, using proboscides (e.g., *Yoldia* spp. clams). Many bivalves are able to pump large volumes of water while feeding. In bivalve species that use the ctenidia to feed, food particles (mainly phytoplankton) are selectively trapped in a thick layer of mucus covering the gills, transported with the aid of the cilia, sorted, and directed to the outer labial palps, where particles are further sorted on the basis of size and other physical and chemical characteristics. Some particles are then transferred to the mouth by the inner palps, while other particles are rejected in pseudofeces released into the pallial space. Mucus and cilia facilitate particle movement toward the stomach, where there is further sorting and selection of particles (Ward and Shumway 2004), leading to the prostyle, a mass of food and mucus. The prostyle is extracellularly digested by the action of the enzymes produced by the digestive gland. In most bivalve species, phagocytic cells have been evidenced in the tubules of the digestive diverticula, where they contribute to intracellular digestion of the selected particles reaching this organ. The remaining particles are excreted via the nephridia or via the gut and finally reach the mantle cavity through the anus (Ruppert et al. 2004).

Although mostly a sedentary group in their adult life stages, some bivalve species are able to move. Most bivalves rely on the foot, a muscular organ with sensorial abilities achieved through balance receptors, the statocysts (Williamson 1993). Larval pediveligers use the foot to sense and locate appropriate substrate for settlement. In burrowing species such as clams, the foot is used by adults to burrow into the sediments. In mussels, the foot is linked to the production of byssus, an extremely

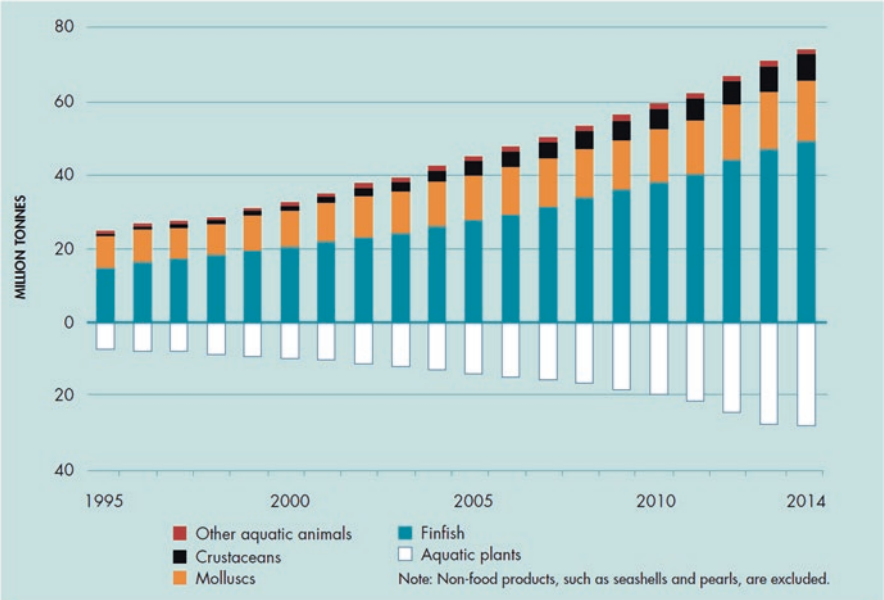
resistant extracellular protein used to attach to the substrate (Carrington et al. 2015). Some species of bivalves (e.g., scallops) are also able to swim by rapidly opening and closing the two valves of the shell (Ruppert et al. 2004).

The nervous system of bivalve molluscs has a simple structure, organized in paired ganglia connected by nerve commissures within them and nerve cords along them in a "rope ladder structure." The visceral cords innervate the internal organs and the pedal cords innervate the foot. The ganglia are divided in two groups: (1) cerebral, pleural (absent in bivalves), and visceral above the esophagus; and (2) the pedal ganglia below. These two differentiated parts are connected by the collar nerve, which surrounds the esophagus (Ruppert et al. 2004).

Ecological and Economical Roles

Bivalve molluscs cover multiple important roles, from both ecological and socioeconomic points of view. Ecologically, bivalves have a key role in the environmental energy flux, in the maintenance of water quality by filter feeding and, for reef-building species such as oysters, in providing substrates and habitats for other species (Zu Ermgassen et al. 2012). Several bivalve species, and mussels in particular, have been used worldwide as sentinels for environmental pollution because of their sedentary and cosmopolitan nature in coastal waters, ease of sampling, ability as filter feeders to concentrate pollutants, and commercial use as an important food staple (Campos et al. 2012; Farrington et al. 2016; Burgos-Aceves and Faggio 2017). Bivalves can also concentrate pathogens and marine toxins, reaching harmful levels for consumers (Visciano et al. 2016). Moreover, as exemplified in Fig. 5, bivalves constitute a major sector of world fishery and aquaculture production, with more than 16 million metric tons with a value of almost US$18 million produced in 2015, representing 15% of total aquaculture production (FAO 2016).

The main purpose of the molluscan aquaculture industry is to produce food, although this industry also has other applications such as ecosystem restoration, extraction of pharmaceutical and industrial products, and ornamentation (aquaria, nacre, pearls). The most important cultured species of molluscs are bivalves such as oysters, mussels, clams, cockles, and scallops, hence the focus of this chapter on these species. The culture process generally starts with the "conditioning" of broodstock in hatcheries by feeding them nutrient-rich cultured microalgae. Spawning is initiated by manipulation of environmental conditions (i.e., temperature, food availability) or, in some cases, gametes are surgically harvested. Fertilization is achieved by mixing of sperm and eggs. Larvae are kept in the hatchery while being fed cultured microalgae until they undergo metamorphosis and settle, and the small juveniles (also called spat) are moved out of the hatchery to a nursery and/or grow-out facility in open water to take advantage of the natural food supply. Grow-out culture technology varies depending on the species and location but can include the use of rope culture (mussels), cages/bags (oysters), and planting in natural beds (clams). Feeding relies on natural phytoplankton production at the site, and most of the labor involves predator and biofouling control.



		INLAND AQUACULTURE	MARINE AND COASTAL AQUACULTURE	TOTAL
			(Tonnes)	
World	Finfish	43 559 260	6 302 631	49 861 891
	Molluscs	277 744	15 835 450	16 113 194
	Crustaceans	2 744 537	4 170 536	6 915 073
	Other animals	520 850	372 718	893 568
	TOTAL WORLD	47 102 391	26 681 334	73 783 725

Fig. 5 World aquaculture production from 1995 until the present day. (FAO 2016)

Major Infectious Diseases Affecting Bivalve Molluscs

The commercial importance of many bivalve molluscs and efforts to manage diseases that severely impact the aquaculture industry have driven much of the research in the immunology of these species. Bivalve aquaculture has been severely impacted in recent years by infectious diseases and toxins from harmful algal blooms causing morbidity and mortality, as well as closures of the industry due to the accumulation of toxins and pathogens affecting the health of human consumers (GLOBEFISH 2017). The relevance of these diseases is highlighted by the fact that the World Organization for Animal Health (most commonly known as the OIE) lists six diseases affecting bivalve molluscs among those with major relevance for animal protection (OIE 2017). While pathologies caused by viruses, bacteria, and parasites have been documented in nearly all major molluscan classes, in this chapter we will present an overview of the pathological agents that have so far been relevant causes

of concern for marine aquaculture activities and most commonly used as models in the study of bivalve immunity, leaving a discussion of infectious agents targeting other molluscs to the section "An Overview of Infectious Agents with Which Molluscs Must Contend" in Chap. 12.

Many diseases affecting bivalves result from an accidental side effect derived from the transfer of aquaculture species, leading to naïve hosts (indigenous or introduced) being exposed to new pathogens. Disease dynamics are heavily influenced by environmental factors, mainly temperature and salinity (Carella et al. 2015; Lafferty and Hofmann 2016; Stentiford et al. 2017), which are remarkably influenced by human activities, as thoroughly discussed in the section "Challenges for Molluscs in the Anthropocene Epoch" in Chap. 12. The study of bivalve immunology has benefited from many decades of research on host–pathogen interactions, the identification of species displaying natural resistance to diseases, the development of disease-resistant strains through selective breeding, and the recent application of -omic tools to bivalve research (Allam and Raftos 2015; Gómez-Chiarri et al. 2015). Most of the research has been focused on pathogens that can be cultured (Fernández Robledo et al. 2014).

Major Viral Diseases of Marine Bivalves

Although the characterization of viral diseases in bivalves has been hampered by the lack of cell lines from marine molluscs, recent advances in sequencing and the development of challenge models and disease-resistant strains have resulted in a better understanding of viral pathogenesis and immunity in several commercially important marine molluscs (Arzul et al. 2017). The best-characterized viral disease of bivalves is caused by oyster herpesvirus 1 (OsHV-1) and its variants (OsHV-1 Var and several microvariants, µVar). Massive mortalities of bivalve larvae and/or juveniles due to OsHV-1 infection have seriously impacted the oyster industry in Europe, but also in Mexico, the USA, Australia, New Zealand, China, Japan, and Korea. These infections are recurrent in Pacific oysters (*Crassostrea gigas*), but other species of oysters, clams, mussels, and scallops are affected as well (Arzul et al. 2017). As shown for other diseases, some strains and species of bivalves appear to be resistant to or tolerant of the disease, such as the Sydney rock oyster, the eastern oyster, and mussels (Masood et al. 2016). Susceptibility to the disease also varies with age, size, and genetics within a species, and several selectively bred lines of Pacific oysters with increased resistance have been developed (Dégremont et al. 2015). In contrast to herpesviruses infecting vertebrates, both inter- and intra-species horizontal transmission of OsHV-1 have been shown, with more tolerant individuals or species acting as disease carriers and reservoirs (Arzul et al. 2017).

Morphological and genomic characterization has led to the classification of this large enveloped virus as a member of the *Malacoherpesviridae* (Mushegian et al. 2018). The function of most of the 124 ORFs found in the OsHV-1 viral genome is unknown, mostly because of lack of homology with sequences with known function (He et al. 2015; Arzul et al. 2017). Infection of oysters with OsHV-1 causes reduced

feeding and swimming in larvae. High levels of viral replication are observed mainly in connective tissues, leading to changes in tissue and cellular architecture, including dilation of the digestive tubules, nuclear chromatin margination and pycnosis, and damage to the cytoskeleton and organelles. The disease is also characterized by massive infiltration of hemocytes. High levels of mortality occur within 48 h post-infection in susceptible animals (He et al. 2015; Young et al. 2017).

Exposure of oysters to the virus through experimental challenges indicates that viral particles infect the host through the digestive gland and/or other mucosal surfaces, probably exploiting hemocytes to reach target tissues (Segarra et al. 2016; Morga et al. 2017). The virus is able to rapidly (within 1 h) infect and initiate replication in hemocytes. The formation of viral particles has not been observed in hemocytes, however, suggesting that these cells impede completion of the viral cycle, as observed in vertebrate macrophages infected with other herpesviruses (Morga et al. 2017). Viral infection leads to activation of the integrin pathway in the host cells, followed by activation of the actin pathway, indicating that the virus exploits these pathways to enter the cell and eventually deliver the viral genome into the nucleosome. Proteomic and metabolomic studies in challenged oysters show that OsHV-1 causes substantial alterations in central carbon metabolism and glycolysis (Warburg effect) in the host, as well as alterations in lipid metabolism and a characteristic fatty acid signature indicative of lipolysis. These metabolic alterations increase the availability of substrates for virion synthesis and assembly. They can also lead to increased inflammation and pathology through the activation of immune-responsive gene 1 protein/*cis*-aconitic acid decarboxylase (IRG1/CAD), a protein linking cellular metabolism with immunity, activation of the respiratory burst, increased permeabilization of the mitochondrial membrane, and reduced ATP production (Corporeau et al. 2014; Young et al. 2017).

Major Bacterial Diseases of Marine Bivalves

With a few exceptions (detailed below), mass mortalities caused by bacterial pathogens in bivalves are observed in larvae and, less often, in juveniles in hatcheries and nurseries (Travers et al. 2015). Experimental challenges with bacterial pathogens, however, are commonly used to study immune responses in bivalves because of the ability to perform culturing and ease of isolation and characterization (Gómez-Chiarri et al. 2015). A wide variety of *Vibrio* spp., including several belonging to the *V. splendidus, V. harveyi*, and *V. tubiashii/coralliilyticus* clades, have been isolated from outbreaks in bivalve hatcheries. In general, early signs of infection of bivalve larvae by pathogenic vibrios include decreased feeding and damage to the velum, followed by widespread necrosis of tissues and rapid mortality (Travers et al. 2015). Strains of *V. aestuarianus, V. splendidus, V. crassostreae*, and others are often detected during summer mortality events in juvenile and adult Pacific oysters, also associated with infection with OsHV-1. Mass mortalities are, in general, seen during the spawning season and other conditions of stress (De Decker et al. 2011). The genomes of many of these pathogenic vibrios have been sequenced, facilitating the

identification of mechanisms of virulence (Travers et al. 2015; Gómez-Chiarri et al. 2015). Examples of virulence factors involved in vibriosis include a variety of metalloproteases, hydrolases, cytotoxins, siderophores, secretion systems, and the OmpU from *V. tasmaniensis* LGP32, which is involved in internalization of the bacteria into *C. gigas* hemocytes (Travers et al. 2015; Le Roux et al. 2016).

Two bacterial pathogens of bivalves—*Aliiroseovarius crassostreae* and *Vibrio tapetis*—are notable for their ability to colonize the periostracal lamina of the inner side of bivalve shells. These pathogens cause Roseovarius Oyster Disease (ROD, also called Juvenile Oyster Disease) in the eastern oyster *Crassostrea virginica* and Brown Ring Disease in *Ruditapes* spp. clams, respectively. Susceptible bivalves respond to the presence of the pathogen in the inner side of the shell and the pallial cavity by producing conchiolin mixed with melanin and other quinones with antimicrobial action, resulting in pathognomonic brown deposits that surround the edge of the mantle (Travers et al. 2015). Little is known about mechanisms of virulence in ROD, but it is likely that formations of polar fimbriae and biofilm on the shell of oysters by *A. crassostreae* are involved in the disease (Boardman et al. 2008). Virulence factors identified in the genome of *A. crassostreae* include a hemolysin/cytotoxin and a putative type IVA secretion system (T4ASS) (Kessner et al. 2016). The metabolic demand of the chronic infections derived from an unsuccessful immune response in susceptible animals may contribute to mortality (Paillard et al. 2014; McDowell et al. 2014).

A few selected bacterial pathogens have been associated with sporadic episodes of mortality in adult bivalves, most notably *Nocardia crassostreae* and several intracellular Rickettsia-like organisms (RLOs). Little is known, however, about mechanisms of virulence and host immunity in these diseases (Travers et al. 2015; Zannella et al. 2017).

Major Parasitic Diseases of Marine Bivalves

Haplosporidian Parasites

Protistan parasites constitute the largest cause of adult bivalve morbidity and mortality. Among the most devastating groups of protozoan parasites of bivalve molluscs are several parasites belonging to the phylum Haplosporidia (Arzul and Carnegie 2015). In particular, the haplosporidians *Bonamia ostreae, B. exitiosa*, and *Haplosporidium nelsoni* have been well known for decades for causing significant economic and ecological losses, mainly in Europe and the USA. The growth of the bivalve aquaculture industry has led to the recent identification of many other haplosporidian parasites affecting a variety of bivalves. Most of the outbreaks caused by the best-known representatives of this phylum, *B. ostreae* and *H. nelsoni*, have been observed in adult oysters. While species from the genus *Bonamia* are only known to affect oysters, have a direct mode of transmission, and are mostly intracellular, other haplosporidian taxa have representatives affecting a wide variety of bivalve hosts, are transmitted through intermediate hosts, and are typically extracellular. Many aspects of the life cycle of these parasites are unknown, as they cannot

be maintained in culture. However, it is presumed that infective stages of *H. nelsoni* enter the host through the epithelial lining of the gill, developing into multinucleated plasmodia, which are seen in all tissues in heavily infected oysters. Depending on the haplosporidian species, sporulation occurs in the epithelium of the digestive diverticula or in connective tissues of the host, leading to the development of sporocysts, which are thought to eventually burst upon death of the host, releasing spores into the environment. Sporulation of *H. nelsoni* has rarely been observed in *C. virginica*, indicating that this oyster may be an atypical host. Oysters that have survived outbreaks of *H. nelsoni* and *B. ostreae* show increased resistance to these diseases, a fact that has been exploited in the development of selectively bred disease-resistant strains (Arzul and Carnegie 2015; Morga et al. 2017).

Cercozoan Parasites

Several *Marteilia* spp. (Cercozoa, Paramyxida) have been responsible for flat and Sydney rock oyster epizootics in Europe and Australia. These parasites affect a diversity of molluscan hosts, including oysters, clams, and mussels, and disease pathogenesis varies depending on the *Marteilia* spp. and the host. Clinical signs of the disease may include nodules (a gross manifestation of an encapsulation response) and, in many of the species, necrotic damage to the digestive gland. As other Paramyxean parasites, *Marteilia* spp. show a characteristic cell-within-cell development by budding. Therefore, most aspects of their complex life cycle, pathogenesis, mechanisms of virulence, and modes of transmission remain a mystery, since efforts to culture these parasites or transmit the disease using cohabitation challenges have been unsuccessful (Carrasco et al. 2015).

Perkinsozoan Parasites

Perkinsosis is caused by a variety of species belonging to the genus *Perkinsus* (phylum Perkinsozoa, superphylum Alveolata). The first *Perkinsus* spp. to be characterized, *Perkinsus marinus*, was identified in the 1940s as the cause of mass mortalities of eastern oysters in the Gulf of Mexico. As is the case for haplosporidian parasites, many other species have been described with the growth of the bivalve aquaculture industry, including *P. olseni, P. chesapeaki, P. mediterraneus, P. beihaiensis, P. honshuensis*, and *P. qugwadi*. While the geographic range of *P. marinus* seems to be limited mainly to that of *C. virginica* in North America, other *Perkinsus* spp., such as *P. olseni*, have a wider geographic and host range. Therefore, *Perkinsus* spp. affect oysters, clams, scallops, cockles, and mussel species in Australia, New Zealand, Asia, America, and Europe (Reece et al. 2017). These parasites have a direct life cycle with four described life stages: trophozoites, hypnospores (or prezoosporangia), zoosporangia, and biflagellated spores (Soudant et al. 2013). The disease is transmitted horizontally, infecting the host through the epithelia of the digestive tract and mantle after the parasites are brought into the pallial cavity and ingested through feeding. Although *Perkinsus* spp. can cause relatively rapid mortality with few clinical signs in the most susceptible individuals within a population, it is most frequently manifested as a chronic disease in adult bivalves. Signs of disease are characterized by severe hemocytic infiltration of tissues, a decrease in

gametogenesis and the condition index and, in some individuals, death by occlusion of vascular sinuses, tissue necrosis, and/or emaciation. In some host species, such as *Ruditapes* spp. clams infected by *P. olseni*, the chronic response is characterized by granuloma-like formations, which can be visibly detected as nodules at the base of gills. Parasites are transmitted to other hosts after being released to the water through diapedesis, in feces, or at the death of the host (Soudant et al. 2013; Ruano et al. 2015). Clonal cultures of most *Perkinsus* spp. are available, allowing for the characterization of putative virulence factors through genetic, genomic, and proteomic studies (Gómez-Chiarri et al. 2015; Hasanuzzaman et al. 2016; Fernández-Boo et al. 2016). Some interesting examples of mechanisms of virulence potentially contributing to the ability of *P. marinus* to survive within the hemocytes of the eastern oyster (Alavi et al. 2009) include antioxidant enzymes, such as superoxide dismutases (Schott and Vasta 2003; Schott et al. 2003; Asojo et al. 2006; Fernández-Robledo et al. 2008) and ascorbate-dependent peroxidases (Schott et al. 2003), and a natural resistance–associated macrophage protein (NRAMP) (Lin et al. 2011). Exposure of *P. marinus* to oyster tissue homogenates or pallial fluid in vitro modulates the production of serine proteases and the expression of genes coding for anti-apoptotic proteins, heat shock proteins, and proteinase inhibitors (Soudant et al. 2013; Pales Espinosa et al. 2014). Another interesting feature of *Perkinsus* spp. may be the presence of a relic plastid with no photosynthetic capabilities (Fernández Robledo et al. 2011) and the ability to secrete several fatty acids, including arachidonic acid (Soudant et al. 2013). Differences in resistance to or tolerance of infection by *Perkinsus* spp. have been documented within and between bivalve species, and selectively bred lines with moderate resistance to or tolerance of *P. marinus* are available (Proestou et al. 2016).

Quahog Parasite Unknown

The protist Quahog Parasite Unknown (Labyrinthulomycetes, Stramenopiles), better known as QPX, causes an opportunistic disease in the quahog *Mercenaria mercenaria* in the northeast and mid-Atlantic regions of the USA (Burge et al. 2013). The disease caused by QPX is characterized by the presence of areas of massive focal inflammation, visibly manifested as nodules commonly observed at the edge of the mantle or the base of the siphon. Differences in susceptibility to QPX infection have been observed between clam populations from different geographic locations (with clams originating south of Virginia being more susceptible than northern clams) and lines of clams derived from survivors of disease outbreaks. Resistance is probably due to a combination of factors, including adaptation to local conditions, as well as selection for molecules involved in more effective immune responses against the parasite (Wang et al. 2016b). QPX is a saprophyte that secretes a thick mucus layer while in tissues of the clam that appears to protect the parasite from the immune response of the host. Putative virulence factors include a variety of hydrolytic enzymes and proteases, antioxidants, polysaccharide production, and factors involved in recognition, such as lectins. The expression of many of these putative virulence factors—in particular, genes that may be involved in the formation of the protective mucus layer—are significantly regulated by temperature (Rubin et al. 2017).

Metazoan Parasites

Some metazoan parasites have been documented in molluscs, including the copepod *Mytilicola intestinalis* (a parasite of mussels) and the trematode *Schistosoma mansoni* (a parasite of humans that also infects snails). Trematode infections are common in molluscs, which act as intermediate hosts. This complex host–parasite interplay is modulated by pattern recognition and effector molecules, as thoroughly reviewed by other authors (Zhang and Loker 2004; Adema et al. 2010; Pila et al. 2017) and discussed in detail in the section "Disease-Transmitting Snails" in Chap. 12.

A General Overview of Bivalve Immunity

Feeding: An Aspect Not to Be Overlooked

Invertebrates, including molluscs, lack the acquired response in a narrow sense (Criscitiello and de Figueiredo 2013), but they possess a potent and efficient cellular and humoral innate immune system, physical barriers such as the shell and the mucus, and behavioral avoidance. This innate response involves, as its major players, circulating hemocytes and a broad range of diverse molecular effectors. A general overview of immune defenses in bivalves is depicted in Fig. 6. One of the first lines of defense of bivalves against pathogens derives from their ability to sense the environment and sort particles during feeding (Ben-Horin et al. 2015). As described in the section "Anatomy and Physiology of Bivalves", bivalves are filter feeders, and the surfaces of the mantle and the gills are exposed to large volumes of water containing microbes and plankton. Bivalves are able to distinguish non-nutritious or potentially harmful particles on the basis of size, physical, and chemical cues, and reject (expel) these particles using mucociliary mechanisms. Bivalves are also able to shut down feeding and keep the valves tightly closed under unfavorable environmental conditions (e.g., low oxygen or blooms of an undesirable phytoplankton species). Although the specific roles of sensing and behavioral responses in disease resistance and immunity have not been well studied, some recent evidence indicates that these may be an interesting avenue for further study. For example, it is thought that oysters accumulate relatively less domoic acid (a toxin produced by the harmful algae *Pseudo-nitzschia* spp.) than mussels, in part because oysters ingest fewer algal cells (Mafra et al. 2010). There is also evidence that feeding behavior contributes to resistance to the parasite *P. marinus*, as observed in some selectively bred families of eastern oysters, with oysters from resistant families removing (filtering) fewer algal cells from the water when mixed with *P. marinus* than susceptible oysters (Ben-Horin et al. in press).

Mucosal Immunity: An Important Yet Understudied Topic

Mucosal immunity constitutes the next barrier to infection on those tissue surfaces in contact with the external environment, while maintaining tolerance of nonharmful commensal microbes and innocuous substances. Mucosal immunity represents

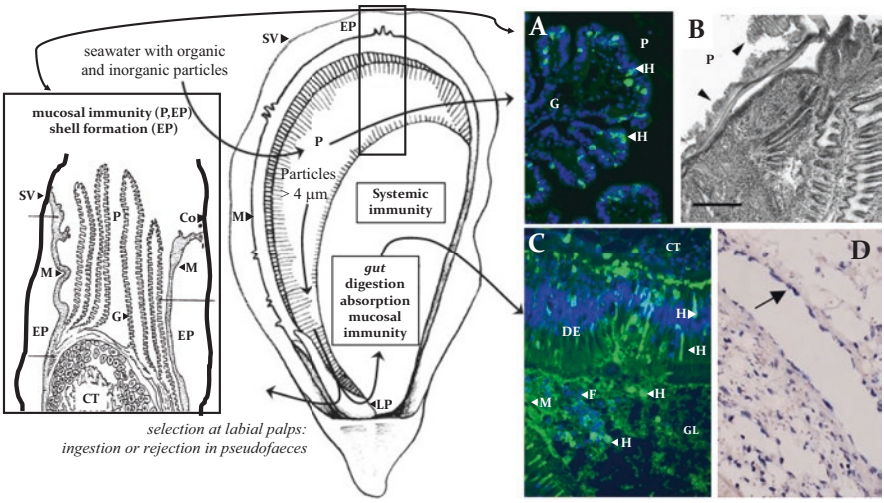


Fig. 6 Overview of immune responses in a representative bivalve (an oyster). *Center:* View of oyster tissues on top of one of the valves, illustrating the flow of water and particles during feeding (Troost 2010). Left: Lateral view of the ventral side of an oyster, showing the pallial (P) and extrapallial (EP) cavities. Right: Micrographs illustrating examples of cellular responses in different immune compartments. (**a**–**c**) Examples of mucosal immune responses. (**d**) Example of a systemic immune response. When the two shell valves (SV) characteristic of bivalves open to allow for feeding, water is pumped through the gills (G) and particles are selected to be either rejected or brought into the gut (central panel). Cells in the mucosal epithelium of the gills and mantle (M) secrete mucus and other effectors. The mantle is also responsible for sealing the edge of the shell valve from the environment (left panel) and producing conchiolin (Co, in the drawing on the right and the arrowhead in (**b**). Hemocytes (H) can migrate into the pallial and extrapallial cavities (**a** and **b**), the gut (**c**), and the blood sinuses (**d**) to recognize, capture, and digest particles and pathogens. (**a**) Immunofluorescence image of a section of oyster gill (G) tissue, showing hemocytes labeled in green (H). Shown in blue are cell nuclei stained with Hoestch. (**b**) H&E-stained sections of a challenged oyster showing degeneration and erosion of the mantle associated with hemocytic infiltration (arrows) and the presence of conchiolin (arrowheads) (scale bar = 100 µm) (Gomez-Leon et al. 2008). (**c**) Immunofluorescence image of a section of oyster gut showing the digestive epithelium (DE), with hemocytes labeled in green (H). The presence of mucus (M) and algal food (F) can be observed in the gut lumen (GL). Shown in blue are cell nuclei stained with Hoestch. (**d**) Big-defensin labeling in hemocytes (arrow) at the edge of a blood vessel in Pacific oysters challenged with *V. anguillarum* (Rosa et al. 2011)

an important, but understudied, first line of immune defense, extending the defensive role of mucus beyond that of a simple physical barrier (Allam and Pales Espinosa 2016) in all molluscs, as detailed in the section "Molluscan Immunity Begins at the Mucosal Surface, an Immunologically Active Site That Remains Understudied" in Chap. 12. This aspect seems to be of primary importance in bivalves, as their life is tightly linked to aquatic environments. Indeed, bivalves can overcome an experimental pathogen challenge by bath exposure but cannot overcome experimental challenge with smaller amounts of the same pathogen if exposed by injection. Pathogens able to bypass these initial barriers to infection (either by surviving inside phagocytic cells or by directly migrating through epithelial

junctions) then trigger a systemic immune response. In general, for both mucosal and systemic immunity, the recognition of nonself (in the form of microbe-associated molecular patterns (MAMPs)) by lectins and other pattern recognition receptors (PRRs) and opsonins in hemolymph (see section "Recognition, Agglutination, and Opsonization"), and by sentinel cells (most probably hemocytes), present in the tissues, triggers signaling transduction cascades and the release of cytokines (see section "Signaling and Regulatory Pathways"), leading to humoral immune responses (see section "Humoral Immune Effectors") and cellular immune responses (see section "Cellular Immune Responses") that vary according to the nature and location of the immune stimuli. A fine regulation of the immune response is achieved through the neuroendocrine immunomodulation (NEI) regulatory network (see section "Connections with the Neuroendocrine System"), a cross talk between the nervous, endocrine, and immune systems that maintains homeostasis and tunes innate immune response in all animals.

In particular, mucosal immune responses include (a) the production of humoral defense factors secreted into the mucus covering the epithelium of tissues in either the pallial or the extrapallial space; (b) chemotaxis and the transepithelial migration of hemocytes into the pallial and extrapallial spaces, followed by phagocytosis and intracellular killing; (c) phagocytosis and intracellular digestion by cells in the digestive epithelium; and, if needed, (d) an encapsulation response in the extrapallial cavity characterized by the secretion of conchiolin and antimicrobial products and activation of the prophenoloxidase cascade (see section "The Phenoloxidase Cascade") (Allam and Raftos 2015; Allam and Pales Espinosa 2016; Zannella et al. 2017). Systemic immune defenses include (a) recognition, opsonization, phagocytosis, and intracellular killing by circulating hemocytes and other, yet to be identified, phagocytic cells within tissues; (b) killing in plasma through secretion of humoral effectors and activation of an ancient complement system and the phenoloxidase system; and, if needed, (c) an encapsulation response that leads to granuloma-like formations, grossly visible as nodules in extreme cases.

Hemocytes: Key Cellular Players in Bivalve Immune Response

Hemocytes are a key component of the bivalve immune system. These cells are present in all cavities of bivalves, circulating in the hemolymph (which bathes all tissues) and migrating into the pallial and extrapallial spaces. Different types of hemocytes have been described in molluscs on the basis of morphological characteristics (see section "A Short Journey in the 'Immune System' of Cephalopods" for a brief comparative overview between bivalve and cephalopod hemocytes and the section "Hemocytes Play a Central Role in Molluscan Immune Responses: Some Basics Regarding Their Morphology and Origins" in Chap. 12 for a broader discussion), and their roles in both physiological processes (e.g., digestion and shell formation) and immune functions (e.g., phagocytosis, synthesis of immune effectors, and modulation of immune responses) are well known (Cheng 1984; Ordás et al. 2000; Goedken and De Guise 2004; Costa et al. 2009b; Wang et al. 2017c; Ivanina et al. 2017).

The lack of specific cell markers, however, has so far prevented detailed characterization of the functionality and mechanism of action of specific cell populations; thus, recent efforts dedicated to the development of these markers are particularly exciting (Donaghy et al. 2009; Sekine et al. 2016; Allam and Pales Espinosa 2016). Moreover, the location of the hematopoietic organ and the process of hematopoiesis and maturation into distinct hemocyte populations are still controversial topics (Pila et al. 2016; Dyachuk 2016). While the hematopoietic organ in gastropods is the amoebocyte-producing organ (Jeong et al. 1983) and that in cephalopods is the white gland (Cowden and Curtis 1973), a variety of tissues in different species and developmental stages have been proposed as hematopoietic organs in bivalves. These include an irregularly folded structure in the gills (Jemaà et al. 2014) and unspecified locations within the mantle and gills (Song et al. 2016) of adult oysters, the mantle edge of mussel larvae (Balseiro et al. 2013), the connective tissues and gill epithelium of recently settled larvae from the flat oyster *Ostrea edulis* (Xue and Renault 2001), and a ring structure around the dorsal side of the embryo in oyster trochophore larvae (Song et al. 2016).

Expansion and Molecular Diversification: The Bivalve Immune System Is Not as "Simple" as We Thought

Exploration of molluscan genomes has revealed massive expansion and functional divergence of gene families involved in immune recognition and opsonization (detailed in section "Recognition, Agglutination, and Opsonization"), adhesion (syndecan, protocadherin), acute phase responses (hsp70), signal transduction (see section "Signaling and Regulatory Pathways"), cytokine production (see section "Production of Cytokines"), apoptosis (see section "Apoptosis and Autophagy"), or oxidation and antioxidation (cytochrome p450, superoxide dismutase) (Zhang et al. 2012a; Simakov et al. 2013; Albertin et al. 2015; Murgarella et al. 2016; Sun et al. 2017; da Silva et al. 2017; Mun et al. 2017; Du et al. 2017). Many of these immune gene family expansions are lineage (bivalve) specific (Zhang et al. 2015; McDowell et al. 2016). The mechanisms (i.e., gene duplications, rearrangements, polymorphism, etc.) and functional relevance of these gene expansions and divergence are still being studied, but there are indications that gene diversity may be responsible for a certain level of species specificity in bivalve immune responses (see Chap. 12, section "Expansion and Diversification of Innate Immune Gene Families" for a comparative overview of a few specific cases).

Evidence of "Immunological Memory" in Bivalves

The plasticity of bivalve immune responses is also evidenced by indications that the immune system can be primed, leading to short-term memory. For example, scallops and oysters showed enhanced pathogen-specific phagocytosis upon a secondary challenge and upregulation of expression of genes involved in phagocytosis and hematopoiesis (Zhang et al. 2014d; Wang et al. 2015b; Green et al. 2015; Pinaud

et al. 2016; Wang et al. 2017a). Recent experiments have further indicated that experimentally infected juvenile oysters can mount a long-lasting antiviral immune memory, persisting for at least 5 months, which protects them from subsequent viral infections (Lafont et al. 2017). Furthermore, transgenerational immune priming has been demonstrated in bivalves (Green et al. 2016). The specific mechanisms involved in these two types of priming are still unclear, but the switch from cellular to humoral response and epigenetic regulation are believed to play crucial roles. An in-depth discussion of the relevance of this poorly understood phenomenon in molluscs is provided in the section "Immune Priming" in Chap. 12. The role of maternal transfer has been also studied as a part of the innate immune response in molluscan larvae, making transgenerational immune priming possible. Bivalve oocytes possess significant antibacterial, lysozyme, and agglutinating activities against pathogens, and several immune factors have been identified in embryos (Wang et al. 2015b; Moreira et al. 2018).

How Do Environmental Factors Affect the Bivalve Immune Response?

Bivalves are poikilotherm species living in highly diverse and variable environments. Consequently, immune responses are heavily affected by environmental conditions, such as temperature, salinity, dissolved oxygen, pH, and pollution. Therefore, an extensive body of knowledge has been built about the potential effect of environmental stress and pollution on immune parameters in these organisms and other molluscan groups—in particular, in connection with human activities, as discussed in detail in the section "Challenges for Molluscs in the Anthropocene Epoch" in Chap. 12. For example, exposure of bivalves to environmental toxins of natural origin, like those derived from harmful algal blooms or toxic cyanobacteria, has been shown to affect the phagocytic responses of bivalves, generally leading to immunosuppression (Hégaret et al. 2011; Soudant et al. 2013; Queiroga et al. 2017). Exposure of oyster hemocytes to pollutants such as TBT in vitro and in vivo reduces their production of ROS and phagocytic activity (Soudant et al. 2013), and exposure of bivalve hemocytes in vitro to nanomaterials leads, in general, to decreased phagocytic activity, increased antioxidant levels, and increased apoptosis, indicating immunotoxicity (Rocha et al. 2015). The effects of environmental stressors on bivalve immunity, however, depend on the evolutionary history of the bivalve species and the history of exposure to different environmental conditions between populations within a species.

Recognition, Agglutination, and Opsonization

The Role of Lectins in Immune Recognition

A critical step of innate immune responses against an infectious challenge is the immediate recognition of the "nonself" carbohydrate moieties on the surface of potential pathogens and parasites, such as viral envelope glycoproteins, bacterial

lipopolysaccharides and exopolysaccharides, and various surface glycans on eukaryotic parasites (Boehm 2012). These surface structures encode vast information that is "decoded" by the hosts' carbohydrate-binding proteins (lectins) (Vasta and Ahmed 2008) which, upon binding to the recognized ligand, can immobilize the infectious agents and activate downstream signaling pathways, leading to their uptake and intracellular killing by phagocytic cells. Furthermore, lectin-mediated activation of the complement system can also promote phagocytosis and killing of potential pathogens (Fujita et al. 2004; Vasta et al. 2007) (see section "Evidence of an Ancient Complement System in Bivalves?"). Thus, lectins are critical components of innate immune mechanisms as both recognition and effector factors—functions that are facilitated by the oligomerization of lectin peptide subunits, leading to increased avidity for the multivalent glycan ligands typically found on the microbial surface (Taylor and Drickamer 2003; Vasta et al. 2007). On the basis of the identification of unique amino acid sequence motifs and the structural fold of the carbohydrate recognition domain (CRD), and the requirement of divalent cations or a reducing environment for ligand binding, lectins have been classified into several major families. These include C-type lectins (CTLs), FTLs, RTLs, HTLs, PTLs, XTLs, I-type lectins, pentraxins, galectins (formerly S-type lectins), ficolins, and others (Vasta et al. 2007). Members of several lectin families such as CTLs, RTLs, FTLs, peptidoglycan-binding proteins, ficolins, pentraxins, and galectins have been implicated in immune surveillance and homeostasis (Vasta and Ahmed 2008) (Fig. 7).

Unlike immunoglobulins (Igs) and Ig superfamily members such as DSCAM (Yue et al. 2016) and FREPs (Zhang et al. 2004), which generate recognition diversity by genetic mechanisms, lectins are typically described as "hard wired" in the germline (Vasta et al. 2007). Therefore, given the great diversity of potential infectious agents present in the aquatic or terrestrial environments that molluscs inhabit, how their innate immune systems are able to cope with these infectious challenges is an outstanding question that remains to be fully addressed (Harvell et al. 1999). However, the complexity of the lectin repertoires in organisms that lack the typical Ig-mediated adaptive immunity, such as molluscs, strongly suggests that a wide variety of molecular topologies can be effectively recognized in surface carbohydrate moieties common to diverse microbial pathogens, leading to activation of effector mechanisms that can kill and eliminate them for successful innate immune protection (Vasta et al. 2007, 2012a; Vasta and Ahmed 2008). A discussion of the best-characterized lectin families identified in molluscs follows below.

C-Type Lectins

Together with the S-type lectins (currently known as galectins; see section "Galectins") C-type lectins (CTLs) were the first two families to be rigorously defined by the presence of unique sequence motifs in their CRDs (Drickamer 1988). CTLs are characterized by the CTL-like domain (CTLD) of the unique structural fold and the requirement of Ca^{2+} for ligand binding. The CTLD can be structurally diversified and associated with a variety of lectin and nonlectin domains constituting "mosaic" or "chimeric" proteins endowed with multiple functional properties (Zelensky and Gready 2005; Pees et al. 2016). In mammals, this highly

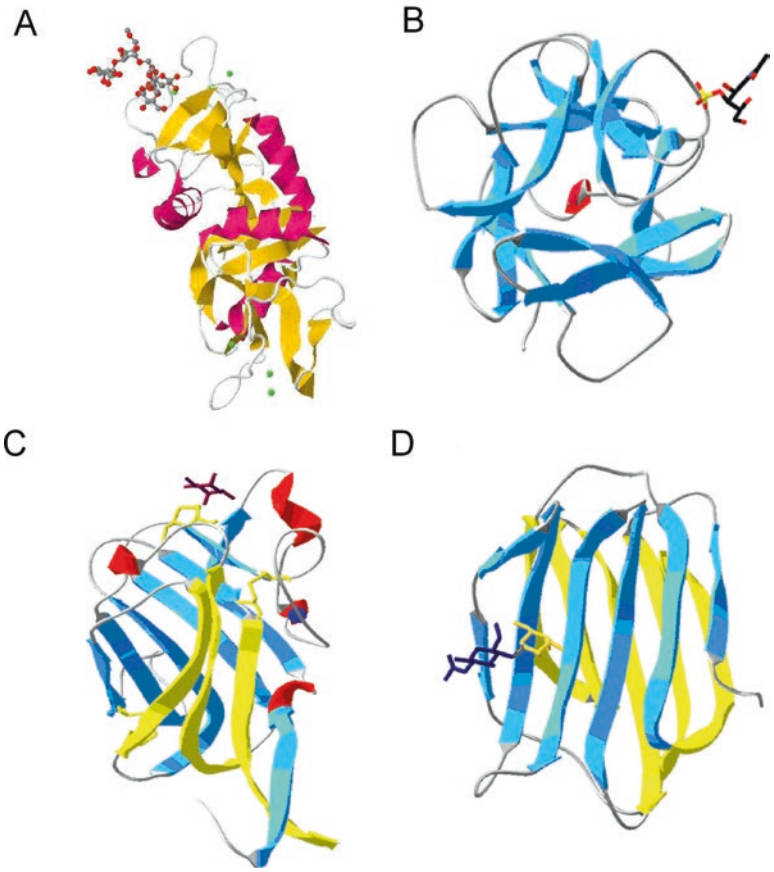


Fig. 7 Typical structural fold of four of the most important lectin families with functions in immune recognition in bivalve molluscs. (**a**) C-type lectin with bound carbohydrate ligand (PDB accession ID: 2MSB). (**b**) R-type lectin with bound 4-sulfated GalNAc (PDB accession ID: 1DQ0). (**c**) F-type lectin with bound fucose (PDB accession ID: 1K12). (**d**) Galectin with bound LacNAc (PDB accession ID: 1KJL)

heterogeneous lectin family is currently subdivided into 17 groups based on their domain organization (Zelensky and Gready 2005; Vasta and Ahmed 2008; Pees et al. 2016). CTLs participate not only in the initial step of pathogen recognition via the CRD but also in various antimicrobial effector functions, including pathogen recognition, opsonization, and activation of the complement cascade (Vasta et al. 2007). In invertebrate taxa, CTLs are also key factors in carbohydrate-mediated recognition of the infectious challenge, but also in effector roles such as immobilization, phagocytosis, clearance, and encapsulation of the infectious agent. Furthermore, they have also been implicated in nodule formation, in the activation of the prophenoloxidase/melanization cascade, and in other functions, including direct antimicrobial activity and regulation of antimicrobial peptide (AMP)

expression (Vasta et al. 2007; Vasta and Ahmed 2008; Wang et al. 2014b; Pees et al. 2016; Zhao et al. 2016b). Numerous studies have been conducted in various mollusc species, aimed at investigating the potential role of CTLs in immune defense, and their roles in recognition, agglutination/immobilization, and opsonization of bacterial pathogens have been firmly established (Zheng et al. 2008; Zhu et al. 2008; Jing et al. 2011; Huang et al. 2013a; Zhang et al. 2014b; Mu et al. 2014; Martins et al. 2014; Chovar-Vera et al. 2015; Huang et al. 2015b; Yang et al. 2015). In general, the CTL repertoire in any single species appears to be highly diversified and complex, and the temporospatial expression and localization of CTLs includes hemocytes, plasma, and pallial mucus, as well as organs and tissues relevant to immune responses such as the mantle, gills and gut. Additionally, infectious challenge experiments have revealed that in most cases their expression is modulated by exposure to potential pathogens (Zhu et al. 2008; Mu et al. 2014; Martins et al. 2014; Chovar-Vera et al. 2015). The report that molluscs can express components of the complement system (see section "Evidence of an Ancient Complement System in Bivalves?") (Li et al. 2015a; Wang et al. 2017b) has suggested that CTLs may function not only as pathogen agglutinins and opsonins but also in activating the complement cascade with further antimicrobial activity.

R-Type Lectins

The R-type lectins (RTLs) are lectins characterized by a CRD of unique structure, consisting of three lobes arranged around a threefold axis CRD (β-trefoil), in which each lobe may contain a carbohydrate-binding site (Cummings and Schnaar 2017). This structure is found in RTLs from higher plants as well as in hydrolases from prokaryotes, mammalian glycosyltransferases, and macrophage mannose receptors (Cummings and Schnaar 2017). RTLs with binding preference for α-D-galactose/GalNAc moieties and a very similar amino acid sequence have been isolated from the mussels *Crenomytilus grayanus* (CGL) (Jakób et al. 2015; Chernikov et al. 2017a, b), *Mytilus galloprovincialis* (MytiLec-1) (Hasan et al. 2016; Terada et al. 2016), *Mytilus trossulus* (MTL) (Chikalovets et al. 2016), and *Mytilus californianus* (García-Maldonado et al. 2017). The RTL known as MytiLec-1 displays the typical β-trefoil structure (Terada et al. 2016), whereas two additional isoforms (MytiLec-2 and -3) identified in the same mussel species contain an additional pore-forming aerolysin-like domain (Hasan et al. 2016; Terada et al. 2016). The structure of CGL was resolved recently and shows a similar β-trefoil structure (Jakób et al. 2015). RTLs from mussels can recognize and agglutinate both Gram-positive and Gram-negative bacteria in a carbohydrate-dependent manner, display bacteriostatic activity, and also show antifungal activity by binding to and inhibiting hyphal growth (Jakób et al. 2015; Hasan et al. 2016; Terada et al. 2016; Chernikov et al. 2017a, b). It is noteworthy that mytilectins and CGL also show immunomodulatory activity for mammalian macrophages, and proapoptotic/antitumoral activity by binding to globotriose [Gb3; Galα(1,4)Galβ(1,4)Glcα1] on the cell surface glycolipids such as globotriaosyl ceramide (Chernikov et al. 2017a, b)—properties that have revealed their promise as effective diagnostic and therapeutic agents and have already led to the computational design of an artificial lectin named Mitsuba-1 (Terada et al. 2017).

F-Type Lectins

F-type lectins (FTLs) are the most recent lectin family to be identified (Odom and Vasta 2006), and they are characterized by a fucose recognition domain (F-type lectin domain; FTLD) that displays a novel β-barrel jellyroll fold ("F-type" fold), and unique carbohydrate- and calcium-binding sequence motifs (Bianchet et al. 2002). FTLs may exhibit single, double, or greater multiples of the FTLD and are widely distributed in nature (Bianchet et al. 2002; Odom and Vasta 2006; Bianchet et al. 2010). Like the CTLs, FTLs may display FTLDs combined with other structurally and functionally distinct domains, yielding lectin subunits of pleiotropic properties even within a single species (Bianchet et al. 2002; Odom and Vasta 2006; Bianchet et al. 2010; Vasta et al. 2012a). Although the F-type fold is distinctive for FTLs, it is not unique to these lectins, as other proteins with various functions also display the FTLD fold (Bianchet et al. 2002). Interestingly, although a phylogenetic analysis of FTLD sequences from viruses to mammals has revealed consistency with the taxonomy of extant species, the surprisingly discontinuous distribution of FTLDs within each taxonomic category suggests not only an extensive structural/functional diversification of FTLs along evolutionary lineages but also that they have been subject to frequent gene duplication, secondary loss, lateral transfer, and functional co-option (Bianchet et al. 2002; Bishnoi et al. 2015).

In addition, FTLs are unique in the extraordinary sequence variability (isoforms) that can be expressed in a single individual as a result of genetic mechanisms of diversification in ligand recognition, characterized in detail in the so-called bindins, proteins involved in gamete recognition in the Pacific oyster, *C. gigas* (Springer et al. 2008; Moy et al. 2008; Moy and Vacquier 2008). In addition to their roles in gamete recognition, oyster FTLs also mediate microbial recognition in innate immune responses. FTLs can display single or tandemly arrayed CRDs of distinct specificity in a single subunit (Odom and Vasta 2006; Bianchet et al. 2010), and can potentially cross-link the recognized pathogens to the endogenous glycans on the surface of the host's phagocytic cells (Odom and Vasta 2006). In this regard, the expression of CvFBL4 in *C. virginica* hemocytes is dramatically upregulated upon LPS challenge, suggesting that FTLs may function in pathogen recognition in the oyster's innate immune response (Saito and Vasta unpublished data). Moreover, PmF-lectin from the pearl oyster (*Pinctada fucata martensii*) is an FTL highly expressed in the hemocytes and gill that is significantly upregulated by experimental challenge with *Vibrio* sp. (Wang et al. 2011a). The identification of FTLs in both the shell matrix and mantle tissue proteins of the blunt-gaper clam, *Mya truncata*, has led to the proposal that during the shell biomineralization process, FTLs secreted by the mantle may carry out immune defense functions and are later incorporated into the shell matrix (Arivalagan et al. 2016). It is noteworthy that the highly diversified FTL repertoire found in the common periwinkle (*Littorina littorea*), a gastropod, has been rationalized as an immune defense system (Gorbushin and Borisova 2015). However, in contrast to other expanded lectin and lectin-like gene families, this connection has not been hypothesized yet in bivalves.

H-Type Lectins

H-type lectins (HTLs) are lectins initially identified in gastropods such as the Roman snail *Helix pomatia* as abundant proteins in the albumin gland secretion that coats the fertilized oocytes before the eggs are laid underground (Uhlenbruck and Prokop 1966). This unique localization as perivitelline active factors, their presence in the snail's hemolymph, and their strong binding to several streptococci strains and other potentially pathogenic bacteria led to the proposal that their role was to protect the snail eggs and adults from infection, as part of the innate immune defense (Uhlenbruck and Prokop 1966). Their shared specificity for N-acetylgalactosamine (GalNAc) and the human blood group A led to their use as typing reagents (Uhlenbruck and Prokop 1966). Recent structural studies revealed that HTLs are characterized by hexameric organization of peptide subunits that display a β-sandwich fold. Although other snail species from the genus *Helix* and the garden snail *Cepaea hortensis* also produce similar lectins (Sanchez et al. 2006), to date, no functional information has been collected yet about HTLs in bivalves, other than the fact that they do not represent an expanded gene family (Gerdol 2017).

Galectins

Galectins are β-galactosyl-binding lectins that require a reducing environment for binding activity but, unlike CTLs and some FTLs, do not require Ca^{2+} (Vasta and Ahmed 2008; Vasta et al. 2012b). Although galectins are structurally conserved and taxonomically widely distributed, they display a remarkable functional diversity by participating in developmental processes, cell adhesion and motility, regulation of immune homeostasis, and recognition of glycans on the surfaces of viruses, bacteria, and protozoan parasites (Vasta 2009). On the basis of their primary structure and subunit organization, mammalian galectins are classified as "proto," "chimera," and "tandem-repeat" types (Vasta and Ahmed 2008; Vasta 2009; Vasta et al. 2012b). Prototype galectins contain one CRD per subunit and are usually homodimers of noncovalently linked subunits. The chimera-type galectins have a single C-terminal CRD, like the prototype, and a non-CRD N-terminal domain that mediates the formation of trimers and pentamers. In contrast, the tandem-repeat galectins, in which two CRDs are joined by a linker peptide, are monomeric.

Molluscan galectins are less diversified that those in mammals but also show different domain organizations, carbohydrate specificity for blood group oligosaccharides, and upregulation of expression by infectious challenge, a feature that supports their proposed role in innate immune responses (Tasumi and Vasta 2007; Feng et al. 2013, 2015; Kurz et al. 2013; Vasta et al. 2015). In contrast to vertebrates, the identification and characterization of galectins in aquatic molluscs has been relatively recent, with most of the studies being aimed at the identification of their transcripts or proteins in diverse tissues and cell types, including hemocytes, and the assessment of their expression upon environmental or infectious challenge (Yamaura et al. 2008; Yoshino et al. 2008; Song et al. 2010, 2011; Zhang et al. 2011a; Bao et al. 2013; Dheilly et al. 2015; Bai et al. 2016). In the eastern oyster, *C. virginica*, however, the galectins CvGal1 and

CvGal2 have been characterized in their detailed molecular, structural, and functional aspects (Tasumi and Vasta 2007; Feng et al. 2013, 2015; Kurz et al. 2013). As a result, unique features of the galectin repertoire of aquatic molluscs have become apparent, such as their domain organizations, as well as structural and functional aspects (Vasta et al. 2015). CvGal1 and CvGal2 carry four canonical galectin CRDs (Tasumi and Vasta 2007; Feng et al. 2013, 2015), a domain organization that does not conform to any of the galectin types described in vertebrates (Vasta and Ahmed 2008; Vasta et al. 2012b). Since then, galectins have been identified in an increasing number of aquatic mollusc species, including both bivalves and gastropods, and can be classified, in the vast majority of cases, into the 2-CRD and 4-CRD types (Vasta et al. 2015). As revealed by a phylogenetic analysis, these galectin types are ancient, as they were already present in the most recent common ancestor of both bivalves and gastropods (Vasta et al. 2015). From the functional standpoint, CvGal1 can recognize microbial pathogens and parasites and promote their phagocytosis, but it can also selectively bind to phytoplankton components, suggesting its participation in uptake of microalgae (Tasumi and Vasta 2007). Furthermore, recent studies suggest that the protozoan parasite *P. marinus* has adapted to subvert the oyster's innate immune/feeding recognition mechanisms to gain entry into the host cells by being preferentially recognized by CvGal1 and CvGal2 over algal food or bacterial pathogens (Tasumi and Vasta 2007; Feng et al. 2013, 2015; Kurz et al. 2013; Vasta et al. 2015).

Fibrinogen-Related Domain–Containing Proteins

A class of proteins containing a C-terminal fibrinogen-related domain (FReD), and similar to vertebrate ficolins, has gained a significant amount of attention in molluscs. Because of their important role in the resistance of the snail *B. glabrata* to trematode infection, together with their somatic sequence diversification (Adema et al. 1997; Adema 2015; Gordy et al. 2015), a subclass of FReD-containing proteins (which also contain one or two immunoglobulin-like domains), named fibrinogen-related proteins (FREPs), have been studied as one of the first examples in support of immune memory in invertebrates (Milutinović and Kurtz 2016). Unlike fibrinogen chains, these lectin-like molecules are primarily involved in immune recognition and are not linked to coagulation (Hanington and Zhang 2011). While these immune properties have been extensively documented in snails since the 1990s (as reported in detail in the section "Expansion and Diversification of Innate Immune Gene Families" in Chap. 12), the first studies of FReD-containing proteins in bivalve molluscs are quite recent.

The first indications pointing toward an involvement of bivalve FReD-containing proteins in immune recognition came from the upregulation of AiFREP in the scallop *Argopecten irradians* in response to *V. anguillarum* but not to *Micrococcus luteus* infections. The recombinant protein could agglutinate Gram-negative and Gram-positive bacterial cells, confirming AiFREP as a reasonable

soluble PRR candidate (Zhang et al. 2009b). Years later, AiFREP-2 was functionally characterized in the same species, confirming and to some extent even extending the marked recognition properties of these two scallop proteins (Yang et al. 2014). Very similar results were obtained in *Crassostrea hongkongensis*, where the recombinant protein ChFCN could selectively bind different bacterial species, agglutinate *Escherichia coli* cells, and enhance hemocyte phagocytosis in vitro (Xiang et al. 2014b). Purified *M. galloprovincialis* transcripts encoding FReD-containing proteins were upregulated in mussels by multiple challenges and could similarly improve the phagocytic rate of hemocytes (Romero et al. 2011). Indirect indications supporting the immune involvement of FReD-containing proteins have been also collected from transciptomic studies in QPX-infected *M. mercenaria* (Wang et al. 2016b) and *V. splendidus*–infected *Mytilus edulis* hemocytes (Tanguy et al. 2013).

Early sequence database mining approaches revealed that FReD-containing proteins are part of a large multigene family in *Mytilus* spp. (Gorbushin and Iakovleva 2011), and it is now well recognized that the genome of several bivalve species encodes more than 100 such genes, which are, for the most part, expressed in the hemocytes, gills, and digestive gland (Zhang et al. 2015; Huang et al. 2015a; Gerdol and Venier 2015). Bivalve FReD-containing proteins are characterized by a simpler domain organization than snail FREPs, as they lack N-terminal immunoglobulin domains, which are thought to play a fundamental role in somatic mutation (Gerdol 2017). Comparative genomics analyses have further revealed that the Ig–FReD domain combination is exclusively found in heterobranch gastropods (Gorbushin et al. 2010). In most cases, bivalve proteins contain a single FReD associated with a coiled coil region, which probably allows oligomerization (Skazina and Gorbushin 2016). In addition, while the process of somatic mutation in snail FREPs is supported by experimental evidence, no data have been provided yet to sustain a similar mechanism in bivalve FReD-containing proteins, which are however characterized by a relevant sequence diversity. This topic has been investigated in detail in *C. gigas*, where the occurrence of polymorphisms in five of these transcripts was originally attributed to allelic recombination or somatic diversification (Zhang et al. 2012b). However, the large number of FReD genes in bivalves suggest that some of these variants might be the result of recent duplications or interindividual sequence variability, mirroring the evolutionary patterns observed for C1q domain–containing (C1qDC) proteins and other expanded PRR families (Huang et al. 2015a).

The remarkable immune properties of FReD-containing proteins, together with their remote sequence similarity with vertebrate ficolins, suggest that these secreted PRRs are somehow involved in the lectin pathway of the bivalve complement system (see section "Evidence of an Ancient Complement System in Bivalves?") (Gerdol and Venier 2015; Wang et al. 2017b). However, definitive proof in support of this hypothesis remain to be collected, in particular for what concerns the identification of mannose-binding protein-associated serine proteases (MASPs)—essential mediators of the complement system, which have not been identified yet in molluscs.

C1q Domain–Containing Proteins

Some Insights into the Massive Gene Family Expansion of C1q Domain–Containing Proteins

Although the outstanding binding potential of the C1q domain allows high functional versatility in the recognition of different ligands, no metazoan taxa seem to have exploited these properties to the same extent as bivalve molluscs. The genomes of these animals encode several hundred secreted proteins containing this conserved domain at their C-terminal end, collectively known as C1q domain–containing (C1qDC) proteins. The immune properties of the C1q domain, whose structural fold is exemplified in Fig. 8, have been well documented from the study of the vertebrate complement system, where it is the major structural unit in the three chains of the C1q complex. However, the first indications pointing toward a similar role in molluscs only surfaced in 2004, with the isolation of a sialic acid–binding lectin from the garden snail *Cepaea hortensis* (Gerlach et al. 2004).

In bivalves, C1qDC proteins were first tentatively linked to pathogen recognition because of their high sequence diversity, exemplified by the identification of 168 different transcripts in *M. galloprovincialis* which, for the most part, strikingly displayed hemocyte specificity (Gestal et al. 2010; Gerdol et al. 2011), and the presence of over 300 genes in the Pacific oyster genome (Gerdol et al. 2015b).

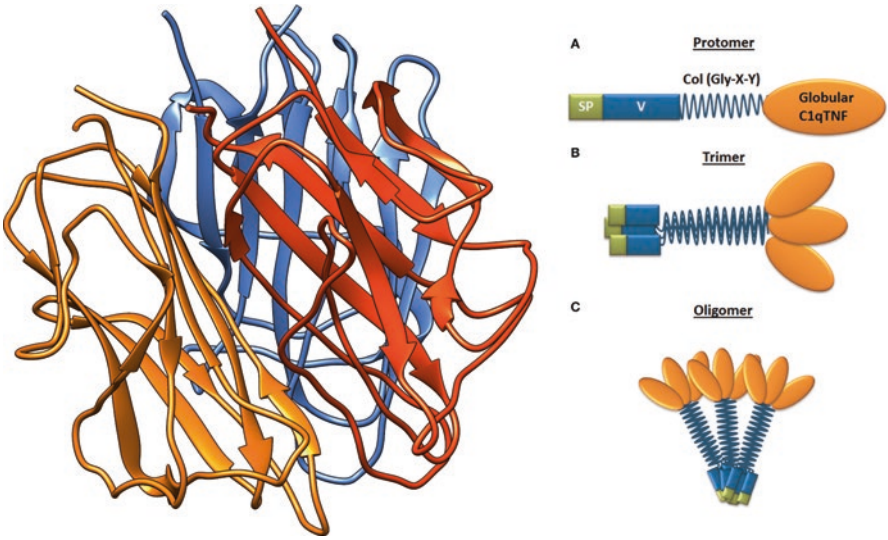


Fig. 8 Left: Three-dimensional structure of the three chains of the human C1q globular head (PDB accession ID: 2WNU; C1qa, C1qb, and C1qc chains are colored in orange, red, and blue, respectively). Right: Prototypical organization of vertebrate C1qDC proteins: **a** single protomer, comprising a signal peptide (SP), followed by a variable region (V, which might be absent in bivalve molluscs), a collagen region (usually replaced by a coiled coil domain in bivalve molluscs), and the globular C-terminal C1q domain. Protomers can assemble into trimers (**b**) and form higher-order bouquet-like structures (**c**). (Source: Thanasupawat et al. 2015)

While most vertebrate C1qDC proteins, including those involved in the complement system, contain a central collagen region required for oligomerization (Fig. 8), about half of the oyster C1qDC proteins contain a coiled coil region, possibly exerting a function homologous to that of collagen. A relevant number of the other members of this gene family, however, lack oligomerization motifs and contain only an N-terminal signal for secretion followed by a globular head C1q domain, identifying the sgC1q subfamily. Surprisingly, just a few gene products have shown an association with additional domains; among these, the most notable example is provided by proteins containing multiple consecutive C1q domains (Gerdol et al. 2015b).

Another interesting finding was that such a massive expansion and diversification event occurred in Pteriomorphia and Heterodonta but not in the two other major subclasses, Palaeoheterodonta and Protobranchia, which possess only a few C1qDC genes, like most other protostomes (including nonbivalve molluscs). This lineage-restricted expansion event might have had important biological implications in mussels, clams, oysters, and scallops, providing these marine organisms with an unparalleled array of recognition molecules to be potentially used in microbe-associated molecular pattern (MAMP) recognition (Gerdol et al. 2015b). Another key piece in the puzzle of the evolution of bivalve C1qDC proteins was provided by the genome of the Manila clam, *Ruditapes philippinarum*. Indeed, most of the 1589 C1qDC genes found in this clam appear to be unrelated to those found in oyster, thereby suggesting that the astounding molecular diversity in the two species derives from independent evolution (Mun et al. 2017).

Functional Studies Are Progressively Revealing the Immune Functions of C1q Domain–Containing Proteins

Genomic investigations are, however, insufficient in the absence of a functional characterization to link this expansion event to improved immune functions. Confirmations, in this sense, have been provided by different experimental approaches, i.e., gene expression studies that have evidenced the upregulation of oyster C1qDC transcripts in response to Rickettsia-like organisms and revealed their implication in the response to Brown Ring Disease, *P. olseni*, and QPX infections in clams (Xu et al. 2012; Leite et al. 2013; Allam et al. 2014; Wang et al. 2016b). Experimental challenges have further demonstrated that many bivalve C1qDC genes are induced by infection with various Gram-positive and Gram-negative bacteria, as well as by fungi (Kong et al. 2010; Gestal et al. 2010; Li et al. 2011a; Gerdol et al. 2011; Jiang et al. 2015), but also by direct stimulation with LPS, PGN, β-glucan, and polyI:C (Wang et al. 2012a, b, 2015a; Yang et al. 2012), altogether reinforcing their role as PRRs. The indications collected from gene expression studies were later confirmed by the binding properties of C1qDC recombinant proteins toward LPS, PGN, polyI:C, mannan, β-1,3-glucan, and yeast glucan (Wang et al. 2012a, 2015a; Jiang et al. 2015) as well as toward live bacteria (Wang et al. 2015a; Zhao et al. 2016a; Huang et al. 2016).

From a functional point of view, an oyster recombinant C1qDC protein was capable of significantly inhibiting the growth of Gram-positive and Gram-negative

bacteria (He et al. 2011), and others displayed strong agglutinating activity toward Gram-positive bacteria, Gram−negative bacteria, and fungi, with a certain degree of selectivity (Kong et al. 2010; Wang et al. 2012a). Some studies have tried to better elucidate the mode of action of bivalve C1qDC proteins and their connection with other molecular components of the immune system. For example, the bactericidal properties of mussel hemolymph appear to be mediated by a C1qDC serum opsonin that binds bacterial D-mannose, promoting the phagocytic action of hemocytes (Pezzati et al. 2015). Similarly, a protein isolated from the scallop *Azumapecten farreri* is capable of enhancing the phagocytosis of invading *E. coli* cells (Wang et al. 2012b), and an oyster LPS-binding C1qDC protein could sensibly boost this activity toward *E. coli* and *V. splendidus* (Jiang et al. 2015). Furthermore, other recombinant proteins are able to interact with heat-aggregated human IgGs and IgMs (Wang et al. 2015a), providing novel and stimulating insights into the possible involvement of these components in the activation of the prototypical complement system of bivalve molluscs (see section "Evidence of an Ancient Complement System in Bivalves?").

Although bivalve C1qDC proteins were initially considered as hemocyte-specific products, it is now clear that they are broadly expressed in all main tissues, with a particular prevalence in the gills or in the digestive gland (Gerdol et al. 2015b), leaving some open questions concerning their involvement in functions other than immune recognition. In fact, the extreme diversification and binding properties of these proteins would, in principle, allow additional physiological functions, which are progressively starting to emerge.

Evidence of an Ancient Complement System in Bivalves?

A Brief Description of the Complement System

Despite the highly divergent evolutionary strategies adopted by metazoans to develop an efficient immune system in highly diverse life environments, a complex molecular machinery of the utmost importance in pathogen recognition and clearance is surprisingly conserved in nearly all animals. This protein complex, able to enhance recognition and removal of microbial cells by recruiting the main players of the vertebrate immune system (phagocytic cells and immunoglobulins), has been named the "complement" system.

The complement system can be potentially activated by different biochemical pathways, which involve components of both innate and adaptive immunity, and has thereby been defined as a functional link between these two major branches of the immune system (Dunkelberger and Song 2009). In vertebrates, the different routes that can lead to complement activation involve either the binding of C1q to antigen-complexed M or G immunoglobulins (the classical pathway), the recognition of MAMPs by mannan-binding lectins (MBLs) and ficolins (the lectin pathway), or the direct recognition of MAMPs by C3b following spontaneous C3 hydrolysis (the alternative pathway) (Fig. 9). Overall, complement activation triggers, through a proteolytic cascade, the opsonization of invading microbes, their lysis by the action

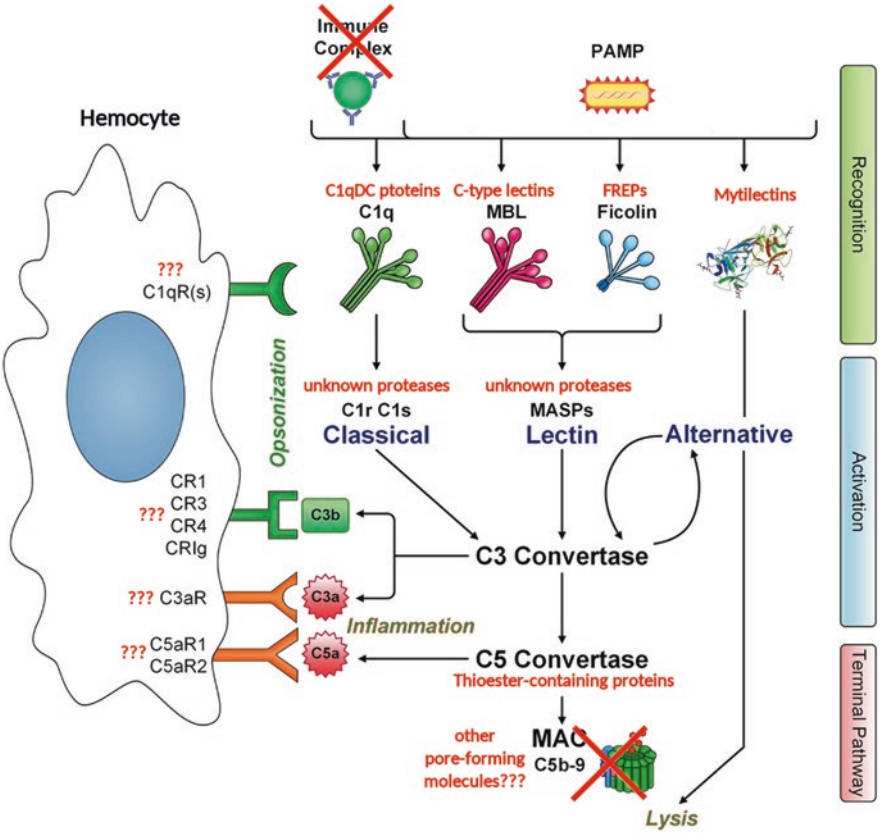


Fig. 9 Overview of the complement system in bivalves and comparison with vertebrates. The vertebrate molecular players are shown in black and the bivalve homologous components are indicated in red, whenever needed. Components that are absent in bivalves (namely, the membrane attack complex and antigen-complexed immunoglobulins) are struck through. (Edited from Bohlson et al. (2014))

of the membrane attack complex (MAC), and the recruitment of phagocytic cells for their final elimination.

The Conserved "Core Components": C2 and C3

With the exception of Ecdysozoa, the near universal conservation of two core molecular components of the complement system—C3 and C2/factor B—suggest that a prototypical complement system was present in the common ancestor of all metazoans (Smith et al. 1999; Pinto et al. 2007). Accordingly, genes encoding these two highly conserved elements are also readily identifiable in most bivalve genomes and transcriptomes (Moreira et al. 2012a; Zhang et al. 2014c; Gerdol and Venier 2015). Their first formal description was provided in the grooved carpet shell, *Ruditapes decussatus* (Prado-Alvarez et al. 2009). The C3 component of the razor

clam *Sinonovacula constricta* was strongly upregulated in hemocytes and digestive gland upon bacterial challenges. In addition, the serum of *S. constricta* was activated by LPS and bacteria, confirming that the function of the bivalve protein was highly homologous to vertebrates (Peng et al. 2016). Further confirmation was recently provided by the use of polyclonal antibodies directed toward three distinct fragments of the Pacific oyster C3 protein, homologous to the α, β, and γ chains obtained in vertebrates from the proteolytic cleavage of the C3 precursor. The observation of a single band recognizable in serum under non-reducing conditions, as opposed to the presence of three distinct bands of 110, 60, and 30 KDa under reducing conditions, pointed out that bivalve C3 molecules are processed by serum proteases in a similar fashion to what happens in animals with a canonical complement system (Wang et al. 2017b).

The bivalve complement system might also involve thioester-containing proteins (TEPs), accessory complement proteins that share a high degree of similarity with C3/C4/C5 and promote opsonization of invading microbes and their elimination by phagocytosis in other invertebrates (Blandin et al. 2008; Bou Aoun et al. 2010). TEPs have been functionally characterized only in the scallop *A. farreri*, where they possess a highly variable central region produced by the alternative splicing of six mutually exclusive exons. This sequence variation appears to cover a key role in the specificity of the immune response to be triggered, as the amount of the isoforms produced largely varies on the basis of the type of challenge and the sex of the specimens (Zhang et al. 2009c). A very recent study went into the subject in depth, evidencing that like C3, scallop CfTEP undergoes fragmentation due to the action of endogenous serum proteases (Xue et al. 2017b).

Present Uncertainties and Future Directions

The absence of immunoglobulins rules out the existence of the classical pathway of the complement system in animals lacking an adaptive immune system, which include bivalve molluscs. At the same time, the remote homology between vertebrate C1q, ficolins, and MBLs, and similar sequences in invertebrate organisms, further complicates the interpretation of the functional overlap between the lectin pathway of the complement system between vertebrates and invertebrates. However, the high diversification of C1qDC proteins might potentially provide a very broad potential of recognition toward MAMPs, even in absence of immunoglobulins. At the same time, while no bona fide sequence that is homologous to vertebrate MBLs or ficolins is present in molluscs, both C-type lectins and FReD-containing proteins (see sections "The Role of Lectins in Immune Recognition" and "Fibrinogen-Related Domain (FReD)-Containing Proteins") underwent massive expansion and diversification events similar to C1qDC proteins. This further reinforces the idea that bivalves possess an astoundingly complex arsenal of soluble PRRs, which are possibly part of a complement lectin pathway. However, it is presently unclear how their recognition signals would converge to C3, as no clear homologs to the serine proteases MASP-1, MASP-2, C1r, and C1s, required for downstream activation of C3 in vertebrates, are present in bivalves (Gerdol and Venier 2015).

Altogether, these reports support the existence of a prototypical complement system in bivalve molluscs, therefore expanding the taxonomic distribution of this ancient immune defense system to Lophotrochozoa, in addition to echinoderms, horseshoe crabs, tunicates, and amphioxus. However, many uncertainties remain about the modes of activation of this system, and some of the hypothetical molecular players that are expected to be involved still remain to be identified. The mechanism of regulation of the complement system in oysters in response to LPS has been hypothesized in a recent study. The authors suggested that 12 serine protease domain–containing proteins might somehow play a key role in complement activation, and they further identified some possible C3 receptors containing integrin α/β domains and similar to ascidian C3 receptors (Wang et al. 2017b).

Finally, it is presently difficult to assess whether the final outcome of this process is simply the opsonization of pathogenic cells, which would facilitate their elimination by the recruitment of phagocytic cells, or whether it also involves lytic components functionally homologous to the membrane attack complex. As will be discussed in section "Lysozymes, BPIs and Other Pore-Forming Molecules", while the constituents of the terminal pathway of the complement system appear to have been specifically developed in the vertebrate lineage, it is possible that other divergent pore-forming molecules function in a similar manner, sometimes combining MAMP-sensing and pore-forming properties within the same protein precursor.

Toll-Like Receptors

Structure and Function of Toll-Like Receptors

Toll-like receptors (TLRs) are metazoan immune receptors, which have found major evolutionary success. Because of their ability to recognize a broad range of ligands, TLRs are important players of the innate immune system of both vertebrate and invertebrate animals, functioning as MAMP sensors either on the plasma membrane or in endosomal compartments. The recognition properties of TLRs are provided by several extracellular leucine-rich repeats (LRRs), which can be organized either in a single cysteine cluster (scc) or in a multiple cysteine cluster (mcc) configuration, whereas the transduction of the immune signal occurs thanks to an intracellular TIR (Toll–interleukin receptor) domain (Fig. 10). This conserved signaling module is separated from the extracellular LRRs by a short transmembrane α-helical domain, which anchors TLRs to cell membranes.

The prototypical Toll protein of the fruit fly *Drosophila melanogaster*, after which all TLRs are named, is a multifunctional protein, acting both as a primary determinant of embryonic dorsal–ventral polarity and as the receptor for the proinflammatory cytokine Spätzle. However, most of the TLRs described so far in vertebrates function exclusively as immune receptors by directly recognizing LPS, PGN, foreign nucleic acids and other MAMPs, without the mediation of cytokine-like molecules. While the organization of TLRs has long been considered to be similar to that of *Drosophila*, genomic studies have progressively unearthed some important peculiarities that strikingly differentiate arthropods from all other animals. In

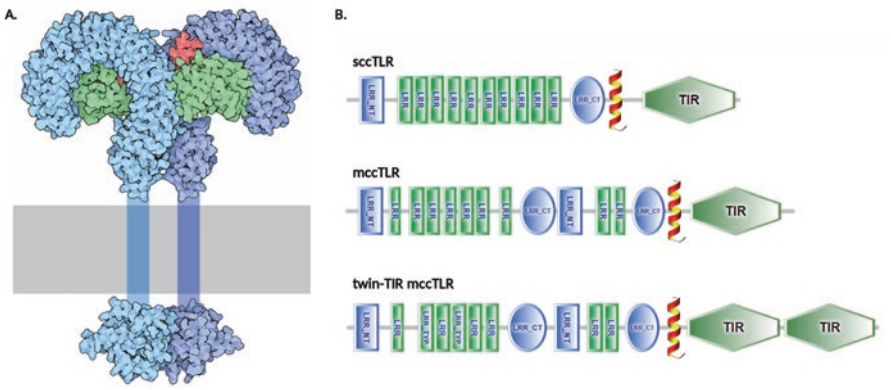


Fig. 10 (**a**) Structure of the human Toll-like receptor 4 dimer (blue) bound to bacterial lipopolysaccharide (red) through its extracellular LRR domains. The transmembrane region is shown schematically. (Image courtesy of RCSB PDB, http://pdb101.rcsb.org/motm/143). The intracellular TIR domain is shown on the inner side of the cell membrane. (**b**) Schematic domain organization of single cysteine cluster (scc), multiple cysteine cluster (mcc), and twin-TIR mcc Toll-like receptors found in bivalve molluscs

particular, echinoderms have independently developed an expanded arsenal of immune receptors that are potentially capable of recognizing a very broad range of invading microorganisms (Buckley and Rast 2012).

The Emerging Role of Toll-Like Receptors in Bivalve Molluscs

Besides echinoderms, the massive expansion of the TLR repertoire by gene duplication involved other phyla, including molluscs (Gerdol et al. 2017), as most notably evidenced by the identification of 83 TLR genes in the genome of the Pacific oyster (Zhang et al. 2015). However, the genomic expansion of the bivalve TLR gene family occurred independently from that of sea urchins, as it mostly targeted a group of phylogenetically distinct genes. Because of the high molecular diversification of bivalve TLR sequences, a novel uniform nomenclature has been recently suggested to avoid confusion in the discussion of the functional properties of these receptors (Zhang et al. 2015; Gerdol et al. 2017). Thus, it has been suggested that bivalve receptors should be categorized as P-type, sPP-type, or twin-type (in the case of mccTLRs), or as V-type or sP-type (in the case of sccTLRs) (Fig. 10). V-type TLRs, present in hundreds of members in the sea urchin genome, include only a few sequences in bivalve molluscs, where most TLRs are ascribable to the sP-type expanded group (Gerdol et al. 2017).

CfToll-1 was the first TLR to ever be described in bivalve molluscs, providing the first pieces of evidence in support of the possible involvement of these receptors in bivalve immune recognition. Indeed this TLR, identified in the scallop *A. farreri* and pertaining to the P-type subfamily, is mildly upregulated by LPS challenges, pointing out a role in the detection of Gram-negative bacteria (Qiu et al. 2007). Following this initial report, several gene expression studies have implicated TLRs

in the immune response to different types of microbes and associated pathologies. For example, a single TLR was strongly modulated in QPX-infected *M. mercenaria* (Perrigault et al. 2009) and in *P. marinus*–infected *C. virginica* oysters (Tanguy et al. 2004). Finally, TLRs have been also reported to be upregulated in response to *V. alginolyticus* challenges in different marine clam and mussel species (Moreira et al. 2012b; Martins et al. 2014).

These observations encouraged the design of targeted functional experiments aimed at identifying the microorganisms recognized by bivalve TLRs and their possible ligands. The most significant studies have been carried out in (1) *C. gigas*, where a TLR was found to be strongly induced by *V. anguillarum* challenges (Zhang et al. 2011c) and a second one (CgTLR6) displayed binding ability toward Gram-positive and Gram-negative bacteria, further revealing affinity to LPS and PGN but not to mannan (Wang et al. 2016b); (2) *Hyriopsis cumingii*, where three different TLRs, responsive to distinct microbial challenges, have been identified, pointing out a remarkable functional specialization (Ren et al. 2013, 2014; Zhang et al. 2017); (3) the noble scallop, *Mimachlamys nobilis*, where an sccTLR responded to *V. parahaemolyticus*, LPS, and PolyI:C challenges in hemocytes (Lu et al. 2016); and (4) *M. galloprovincialis*, where the upregulation of the P-type TLR MgTLR-i could be observed in response to *Vibrio* spp. and *M. luteus* but not to *Fusarium oxysporum* injection (Toubiana et al. 2013). The high selectivity of TLRs, in terms of both transcriptional responsiveness and binding potential, has been further confirmed by the transcriptional analysis of the entire complement of oyster TLR genes, which often responded to just a single pathogenic challenge in a highly specific manner (Zhang et al. 2015).

One of the most praiseworthy studies aimed at clarifying the placement of these receptors in the molecular networks of immune signaling targeted four different sccTLRs in *C. gigas* and permitted the demonstration of their participation in the activation of nuclear factor kappa B (NF-κB). The finding that oyster sccTLRs are localized both on the plasma membrane and in late endosomal vesicles was equally important, as it revealed a possible role of TLRs also in the modulation of immune response upon phagocytosis of invading microbes (Zhang et al. 2013a). Although only little effort has so far been put into the identification of the effector molecules whose production is controlled by TLRs, preliminary results clearly point toward a key role of TLR signaling in the regulation of AMP and lysozyme production through a MyD88-dependent pathway (see section "Canonical TLR Signaling").

The experimental data collected so far confirm that the fundamental role of TLRs in the bivalve immune response to invading microorganism appears to be supported by overwhelming evidence. However, one might wonder whether this large family of receptors has acquired additional physiological roles due to neofunctionalization, as has been suggested for other bivalve recognition protein families. While evidence in support of this hypothesis still remains scarce, some reports hint that TLRs might be modulated by other stimuli, i.e., biotoxins (Detree et al. 2016b), abiotic stress (Zhang et al. 2015), and variations of pH (Xing et al. 2017).

Other Membrane-Bound Immune Receptors

Peptidoglycan Recognition Proteins

Peptidoglycan recognition proteins (PGRPs) are a class of well-characterized PGN-binding molecules that, in the fruit fly *D. melanogaster*, comprises both membrane-bound and secreted members. Membrane-bound PGRPs are directly involved in MAMP recognition during infections by Gram-negative bacteria and activate the Immune deficiency (IMD) signaling cascade (Royet and Dziarski 2007). On the other hand, secreted PGRPs cooperate with Gram-negative Binding Proteins (GNBPs) in the extracellular environment, triggering the prophenoloxidase cascade, which leads to the activation of Toll signaling (see section "Canonical TLR Signaling") and melanization (see section "The Phenoloxidase Cascade"). While PGRPs are also present in vertebrates, they are not anchored to the plasma membrane and they mostly exert bactericidal/bacteriostatic activity in the extracellular environment (Montaño et al. 2011).

PGRPs have been functionally characterized in detail in arthropods and vertebrates, but nearly no information is available for the other major animal phyla. In bivalve molluscs, genome and transcriptome screenings show the presence of both membrane-bound and secreted PGRPs, even though large margins of uncertainty remain about their functional overlap with arthropods and vertebrates. First, there is no evidence in support of an extracellular pathway homologous to that of the *Drosophila* prophenoloxydase proteolytic cascade, and the absence of Spätzle-like proteins make it highly doubtful that secreted PGRPs participate in TLR activation in bivalves (see section "The Phenoloxidase Cascade"). Second, the high sequence divergence between bivalve PGRPs and those from other organisms does not allow similarity-based functional inference (Gerdol and Venier 2015).

The first report of PGRPs in bivalve molluscs, in the form of a short secreted protein, dates back to 2007, when an inducible gene product was identified in the scallop *A. farreri* following Gram-positive and Gram-negative bacterial challenges (Su et al. 2007). This finding was later confirmed in *M. galloprovincialis*, *Bathymodiolus azoricus* (Martins et al. 2014), and *H. cumingii*, where broad-spectrum antibacterial activity and lytic activity toward both Lys-PGN and DAP-PGN were demonstrated (Yang et al. 2013c). Furthermore, another study reported the modulation of the expression of two secreted short PGRPs in *Solen grandis*, in particular, in response to PGN but not LPS (Wei et al. 2012), confirming previous results concerning PGN specificity obtained in the bay scallop (Ni et al. 2007). Finally, another secreted PGRP molecule from *C. gigas* displays a unique domain architecture, as it combines the PGN-binding domain with a G-type lysozyme domain, which could potentially enable the coexistence of bacterial recognition and killing properties in the same molecule (Itoh and Takahashi 2009) (see section "Lysozymes, BPIs and Other Pore-Forming Molecules"). Overall, the vast majority of the studies that have targeted bivalve secreted PGRPs so far are seemingly concordant in attributing to them functional properties more similar to those of vertebrate PGRPs than to those of arthropods. Their cooperation with GNBPs and their involvement in the activation of TLRs seem unlikely at this point.

Interestingly, while no membrane-bound PGRP has been functionally characterized yet in bivalves, at least two proteins of this type are present in the Mediterranean mussel transcriptome. Together with the contemporary identification of some conserved intracellular mediators, this prompted researchers to hypothesize the possible existence of an IMD-like pathway (see section "Other Immune Signaling Pathways") (Gerdol and Venier 2015). While this hypothesis still awaits experimental confirmation, a recent study carried out in *B. azoricus* identified five paralogous PGRP genes, which were connected to the regulation of bacterial endosymbiosis in gills (Détrée et al. 2017).

Recently Discovered Receptors

Besides TLRs and PGRPs, only a very few other cases of PRRs anchored to the extracellular surface of bivalve immune cells have been studied so far. The most relevant are the Nimrod-like receptor (CgNimC) and LRR and Ig domain–containing proteins (LRRIGs), both identified in *C. gigas*. The former receptor has been implicated in the recognition of Gram-negative bacteria because of its relevant upregulation in response to *Vibrio* spp. challenges and LPS binding. Further functional assays established that CgNimC plays a fundamental role in regulating the phagocytic rate of hemocytes toward invasive Gram-negative bacteria (Wang et al. 2015d). On the other hand, the two LRRIgs genes identified in the genome of *C. gigas* encode large proteins bearing extracellular LRRs (like TLRs), coupled with an immunoglobulin-like domain, a transmembrane domain, and a short uncharacterized cytosolic C-terminal domain. Immunoglobulin-like domains are abundant in bivalve genomes, and their marked immunological properties have been well defined in vertebrates and, partly, also in invertebrates (e.g., gastropod FREPs; see Chap. 12, section "Defense-Associated Humoral Components"). LRRIGs can bind a broad range of MAMPs and are upregulated in hemocytes in response to various types of challenges. Furthermore, they can modulate the expression of cytokine-like factors (i.e., TNF and IL-17) and promote hemocytic phagocytosis of *Vibrio* cells, thereby reinforcing their position as key regulators of immune response in oysters (Wang et al. 2017a; Huang et al. 2018).

Cytosolic Pattern Recognition Receptors

In comparison with the impressive amount of literature produced about soluble and membrane-bound PRRs, it is perhaps surprising that only a handful of studies have so far taken into account the possible involvement of cytosolic receptors in the immune system of bivalves. Most of the molecular players described below have been identified just at the sequence level and therefore emerge as interesting targets for future functional investigations.

Different intracellular PRRs are potentially capable of recognizing MAMPs present in the cytosol. These receptors have a dual function in: (1) directly detecting the presence of pathogens (e.g., viruses) in the cellular space; and (2) indirectly detecting microbes in the extracellular environment from their degradation products

(e.g., peptidoglycan components). In summary, this system works in a synergistic manner with membrane-bound PRRs, thereby reinforcing the immune response through the combination of converging signaling routes derived from the intracellular and extracellular environments.

NACHT–Leucine-Rich Repeat Proteins and Bacterial Sensing

NACHT–leucine-rich repeat (NACHT-LRR) proteins (NLRs) act as sensors of the two major peptidoglycan-derived bacterial components, muramyl dipeptide (MDP) and γ-D-Glu-meso-diaminopimelic acid (iE-DAP) in the cytosol (Fritz et al. 2006). These MAMPs can be translocated inside the cytoplasm whenever bacteria present in the extracellular environment are attacked by antimicrobial effectors, or they can be released as a consequence of the digestion of phagocytosed bacterial cells. Activated NLRs oligomerize, recruiting adaptor molecules that can modulate immune response, cell death, or survival. Vertebrate NLRs are also responsible for the assembly or inflammasomes—large macromolecular complexes involved in the modulation of inflammation—which are however unlikely to exist in invertebrate animals (Latz et al. 2013).

In spite of the great expansion of NLRs in many metazoans, no such receptor has ever been functionally characterized in molluscs. The typical tripartite domain architecture of NLRs comprises C-terminal leucine-rich repeats required for ligand binding, a central NACHT domain, which regulates oligomerization, and an N-terminal death fold domain (DFD), whose type (DEATH, DED, CARD, or PYD) determines the recruitment of specific downstream signaling adaptors. Although the single NLR-like protein identified in *M. galloprovincialis* displays a CARD/NACHT/LRR domain combination, it bears limited sequence homology with bona fine vertebrate NLRs, leaving its possible involvement in immunity a matter of speculation (Gerdol and Venier 2015).

RIG-Like Receptors: Fundamental Receptors of Viral Infection

While NLRs are mainly employed in bacterial sensing, a series of other receptors collectively known as RIG-like receptors (RLRs) cover an analogous function in the sensing of viruses. Upon activation, these helicase-like molecules trigger the antiviral response through the their N-terminal caspase recruitment domain (CARD) (Yoneyama and Fujita 2007). RLRs are capable of recognizing a broad range of dsDNA viruses, thanks to the mediation of DNA-dependent RNA polymerase III, which uses viral DNA as a template for the generation of 5′ triphosphate single-stranded RNAs, which are efficiently recognized by the helicase domain of RLRs.

Consistently with the expected rapid evolution of antiviral defense mechanisms in the continuous race to arms between the host and the pathogen, this molecular machinery diverged significantly among animal groups (Paro et al. 2015). Bona fide RLRs were long thought to be exclusively present in vertebrates. However, following early reports of RLR-like genes in the genomes of cnidarians (Zou et al. 2009), a RLR highly responsive to poly(I:C) stimulation was also identified in *C. gigas* (Zhang et al. 2014e). Definitive proof about the involvement of RLRs in antiviral immunity was provided in a study demonstrating that the RLR CgRIG-I-1 was

upregulated in response to OsHV-1 infection in Pacific oyster larvae, and that it could directly bind poly(I:C). The identification of the key adaptor protein IPS-1/MAVS (see section "Other Immune Signaling Pathways"), brought convincing evidence in support of the existence of an RLR-mediated signaling pathway activated in response to dsDNA viruses, closely matching that of vertebrates.

Another important aspect in the context of viral sensing is the possible involvement of Dicer, the main antiviral molecule in the cytosol of insect cells, which lack RLRs. In particular, only one out of the two Dicer gene copies present in the genome of *Drosophila* (Dicer-2) can process dsRNAs to produce siRNA (Lee et al. 2004), whereas the single mammalian Dicer gene is mostly involved in the production of miRNAs and only in some cell types can it generate siRNAs (Maillard et al. 2013). While the preferential substrates of this catalytic helicase in bivalves are presently unknown, all molluscs bear a single-copy Dicer gene (Rosani et al. 2016).

Stimulator of Interferon Genes: A Major Hub for Microbial Sensing in the Cytosol

The third major intracellular sensor of microbial infections is the Stimulator of Interferon Genes (STING). Unlike NLRs and RLRs, STING is a multifunctional protein, which can act either as a direct MAMP sensor or as a signaling adapter collecting infection signals derived from several pathogenic agents (Burdette and Vance 2013). This broad spectrum of recognition is guaranteed by the interaction with different cytosolic cofactors, whose presence in molluscs is mostly unconfirmed and sometimes even unlikely due to lineage-specific gene losses and high sequence divergence (Gerdol and Venier 2015).

In vertebrates, the dimerization and migration of STING from the endoplasmic reticulum membrane to the perinuclear region is a fundamental step for the subsequent activation of interferon response and inflammation (Ishikawa et al. 2009) (see section "Lysozymes, BPIs and Other Pore-Forming Molecules"). Although only a few reports have documented the existence of STING in bivalve molluscs (Gerdol and Venier 2015; He et al. 2015), the peculiar domain architecture of this molecule suggests a different subcellular localization and mode of action. Indeed, all lophotrochozoan STING molecules lack transmembrane domains and present a duplicated STING globular domain associated with a TIR domain; this structure could potentially enable self-dimerization upon ligand binding and the activation of downstream immune signaling through TIR–TIR heterotypic interactions. At the same time, it might imply important functional differences in comparison with vertebrates, including the interaction with different and presently unknown alternative MAMP cosensors.

In any case, the main functional property of the STING globular domain, i.e., the ability to bind cyclic dinucleotides in the cytosol, is expected to be retained. The most relevant ligands of STING are cyclic diguanylate (c-di-GMP) and cyclic guanosine monophosphate–adenosine monophosphate (cGAMP). While the former is a second messenger directly produced by bacteria, the latter is synthetized by cyclic GMP–AMP synthases (cGAS) whenever foreign DNA is detected in the cytoplasm, playing a fundamental role in the detection of both bacterial and viral nucleic acids

(Ablasser et al. 2013). Although the importance of the cGAS/STING complex in activating the antiviral response has been only recently uncovered, it is certainly noteworthy that bivalve genomes display a significant expansion of cGAS-like genes in comparison with gastropods, which would suggest improved competence for viral detection (Gerdol 2017).

Signaling and Regulatory Pathways

MAMPs of various natures, such as glycoproteins, components of cell walls and membranes, and exogenous nucleic acids can be recognized by the broad array of bivalve PRRs described in the previous sections, activating a cascade of intracellular events that eventually result in cell response to the perceived stimulus. Multiple signal transduction pathways, mostly based on protein–protein interactions and modifications (e.g., kinase-mediated phosphorylation), regulate the timing and intensity of the immune response, as well the cellular fate (death or survival).

Canonical Toll-Like Receptor Signaling

The Essential Role of MyD88 in Immune Signal Transduction

The main signal transduction pathway reported to mediate the immune responses of bivalve species is TLR/NF-κB signaling (Fig. 11), which is deeply intertwined with other accessory networks that will be described in the section "Other Immune Signaling Pathways." The recognition of ligands by the extracellular LRR domains of TLRs leads to their dimerization, which in turn activates key transcription factors, enabling the production of AMPs, lysozymes, interleukins (ILs), and other immune effectors against bacterial, fungal, and viral pathogens. The first essential step of TLR-mediated signal transduction involves the recruitment of TIR-DC adaptor proteins, which in vertebrates are primarily the myeloid differentiation primary response protein 88 (MYD88) and the TIR-domain-containing adapter-inducing interferon-β (TRIF) (O'Neill and Bowie 2007).

Because of the lack of a TRIF homolog, the TLR signaling in bivalves is essentially a MyD88-dependent pathway, even though the possible involvement of alternative evolutionarily conserved TIR-DC adapters cannot be excluded (Gerdol et al. 2017). The fundamental signaling mediator MyD88 is characterized by an N-terminal death domain, required for perpetrating signal transduction, and by a C-terminal TIR domain that interacts upstream with the cytosolic TIR domain of TLRs. The upregulation of MyD88 transcripts has been documented in different bivalve species in response to various bacterial MAMPs (Toubiana et al. 2013; Ren et al. 2016; Xin et al. 2016a) and OsHV-1 infection in oysters (Renault et al. 2011; Du et al. 2013). The multiple MyD88 genes identified in the genomes of *C. gigas* and *M. yessoensis* indicate an expanded gene family (Zhang et al. 2015; Ning et al. 2015), possibly linked with the diversification of TLRs (see section "Toll-Like Receptors"). Some MyD88-like proteins lack the N-terminal death

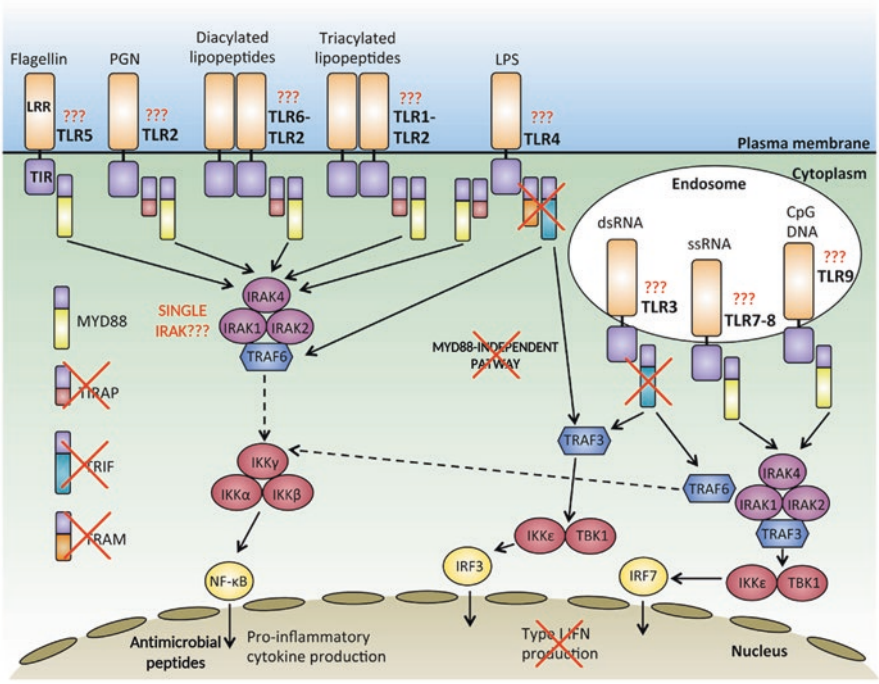


Fig. 11 Vertebrate canonical Toll-like receptor (TLR) signaling and comparison with that of bivalve molluscs. Unidentified components in bivalves are struck through and elements whose presence is uncertain are indicated by question marks. In particular, the low similarity between vertebrate and molluscan TLRs leaves the binding specificity of bivalve TLRs, for the most part, unknown. The homo- or heterodimerization of TLRs following ligand binding, either in the extracellular environment or in the endosomal compartment, recruits adaptor proteins, which propagate immune signals. Only MyD88, among the vertebrate adaptors, has been identified so far in bivalves. The recruitment and activation of IRAK kinases and the IKK complex results in the migration of the NF-κB and possibly IRF transcription factors to the nucleus, where they regulate the production of proinflammatory cytokines and antimicrobial peptides. (Edited from Wang et al. 2014c)

domain and are therefore thought to function as negative regulators (Xu et al. 2015b), together with the sterile alpha and armadillo motif containing protein (SARM), an evolutionarily conserved negative regulator of TLR signaling, as well as an intermediary of apoptosis and antiviral innate response (Belinda et al. 2008; Panneerselvam and Ding 2015).

Toll-Like Receptor–Mediated Signal Transduction: From the Cell Membrane to the Nucleus

All of the expected elements of canonical MyD88-dependent TLR signaling have been identified in the transcriptomes of *C. gigas* (Zhang et al. 2011c), *M. galloprovincialis* (Toubiana et al. 2014), and *Saccostrea glomerata* (Ertl et al. 2016), and even physically mapped to *A. farreri* bacterial artificial chromosomes by

fluorescence in situ hybridization (Wang et al. 2011b; Zhao et al. 2015). These approaches highlighted a remarkable similarity with the immune signaling system of deuterostomes and a less significant overlap with arthropods. The immune role of such molecules has been confirmed by the assessment of their upregulation following immune stimulation trials and a detailed functional characterization in several bivalve species. While many accessory factors take part in this elaborate signaling network, either as positive or negative regulators, or as molecular switches to activate connected pathways, we will discuss below only the main molecular players (Fig. 11).

The second intracellular step of the MyD88-dependent TLR signaling involves the interaction between MyD88 and the *Interleukin-1 receptor-associated kinases* (IRAK)-1/-4 complex, with the subsequent recruitment of the *TNF receptor-associated factor 6* (TRAF6). The two IRAK proteins identified in mussels (both homologous to IRAK-4) were strongly overexpressed in hemocytes following bacterial challenges (Toubiana et al. 2014), similarly to the soft shell clam *Mya arenaria* IRAK-4-like transcript, responsive to *V. splendidus* challenges (Mateo et al. 2010). The turnover of IRAK kinases is regulated by the Toll interacting protein TOLLIP, characterized as an acute phase protein in *M. yessoensis* (Zhang et al. 2015) but present with steady expression levels in *M. galloprovincialis* (Toubiana et al. 2014). TRAF6 is one of the key components of the pathway, as it regulates the activation of the IKK complex together with the Transforming growth factor activated kinase-1 (TAK1). TRAF6 responds to Gram-positive and Gram-negative bacteria, as well as to LPS challenges in the scallop *A. farreri* and in the mussel *M. galloprovincialis* (Wang et al. 2011b; Toubiana et al. 2014). Very limited functional information has been collected so far about TAK1, the associated proteins TAB1/2, and the components of the *Inhibitor of kappa-B kinase* (IKK) complex, in bivalves. Most notably, an IKK-like sequence has been characterized in oyster and connected to the activation of NF-κB (Escoubas et al. 1999). As a major difference with vertebrates, only a single IKKα/β homolog is present in *M. galloprovincialis*. The IKK complex finally phosphorylates the *Inhibitor of nuclear factor kappa-B* (IKβ), which is then ubiquitinated and targeted for proteasomal degradation. This process allows the entering of the NF-κB or Rel transcription factors in the nucleus, ultimately enabling the transcription of the target effector genes.

After the initial characterization of an IKβ homolog in *C. gigas* (Montagnani et al. 2008), three paralogous genes were identified in this species. All of them were positively regulated by MAMP and heat-killed bacteria stimulation (Zhang et al. 2011e; Xu et al. 2015a). Similarly, *M. galloprovincialis* possesses at least two IKβ genes, which both experienced moderate to strong upregulation in response to bacterial challenges (Toubiana et al. 2014). IKβ homologs were also found to be responsive to various types of challenges in *A. farreri*, *Cyclina sinensis*, *Meretrix meretrix*, *P. fucata*, *R. philippinarum*, *S. glomerata*, and *S. grandis* (Zhang et al. 2009a; Green and Barnes 2009; Wang et al. 2011b; Yang et al. 2011b; Moreira et al. 2012a; Lee et al. 2013; Liu et al. 2014; Gao et al. 2016). In this respect, a contrasting result was obtained in *A. irradians*, as IKβ was downregulated following *V. anguillarum* challenges (Mu et al. 2010). The consensus of

studies further seems to indicate widespread expression of these inhibitors in all adult tissues, even though most experimental studies have been focused on expression dynamics in hemocytes.

Nuclear Factor Kappa B: A Key Regulator of Immune Response

Nuclear factor kappa B (NF-κB) family members, sharing a domain architecture similar to human p100/p105 or to p65, have been identified in multiple bivalve species, where they are present as single-copy genes (Li et al. 2015b). The first functional confirmation of the involvement of bivalve NF-κB homologs in immune response came from the observation that the overexpression of the oyster gene in *Drosophila* cell lines was able to induce the expression of a NF-κB reporter gene (Montagnani et al. 2004). This molecule could be further placed within the TLR-mediated MyD88-dependent circuitry thanks to RNAi studies in *C. sinensis* (Gao et al. 2016). Furthermore, the *A. farreri* homolog controls the expression of AMPs, providing direct evidence in support of its involvement in the production of effector molecules (Oyanedel et al. 2016). Overall, compelling evidence demonstrates the MyD88-dependent inducibility of NF-κB in the acute phase of response to various bacterial and viral MAMPs in bivalves, supporting the role of these transcription factors in regulating the expression of proinflammatory factors, effector molecules, and cytokines involved in fundamental aspects of bivalve immunity (Wang et al. 2011b; Huang et al. 2012; Toubiana et al. 2014; Li et al. 2015b; Gao et al. 2016). However, significant differences in the magnitude of this response exist among species which might, to some extent, even explain the different interspecies susceptibility to disease, as evidenced by the comparative analysis of shallow-water and deepsea mussels (Martins et al. 2014).

Other Immune Signaling Pathways

Role of the Mitogen-Activated Protein Kinase Cascade in Immune Signaling

While the processes outlined above cover the main signaling pathway from MAMP sensing to the activation of nuclear factors, some components of the TLR/NF-κB signaling found in vertebrates and invertebrates alike represent a bridge to other signaling pathways (O'Neill and Bowie 2007; Brown et al. 2011). Most notably, TRAF6 can interact with MEKK1 thanks to mediation by the *Evolutionarily conserved signaling intermediate in Toll pathways* adapter (ECSIT), which is also found in bivalves (Toubiana et al. 2014; Lin et al. 2017), activating the mitogen-activated protein kinase (MAPK) cascade. In essence, the MAPK signaling is a phosphorylation cascade activated by many immune and nonimmune signals (e.g., growth factors, cytokines, bacteria, viruses, oxidative stress), which modulates various cell processes. This important signaling cascade activates classical MAP kinases (ERK, p28, JNK), whose concerted action can determine alternative cellular fates, including cell survival and proliferation, differentiation, or death. The successful use of commercial antibodies targeting MAPK components evidenced the

remarkable conservation of this pathway in all animals (Canesi et al. 2002; Bettencourt et al. 2009). Sequences denoting MAPK proteins have been identified in different mussel and oyster species (Martins et al. 2014; Zou et al. 2015; Gerdol and Venier 2015; Wang et al. 2017a) and p38, JNK, and ERK kinases in particular have been specifically linked to bivalve immune response (Sun et al. 2016; Qu et al. 2016, 2017a). Ultimately, MAPK signaling results in the activation of AP-1, a heterodimeric transcription factor composed of Jun and Fos subunits. The immune role of bivalve AP-1 has been so far mostly inferred from gene expression data collected in *C. hongkongensis* and *R. philippinarum* (Xiang et al. 2014a; Wu et al. 2015; Qu et al. 2015a). Regardless of the alternative activation of the IKK complex or of the MAPK cascade downstream of MyD88, the two signaling branches extensively communicate with each other, as TAK1 can phosphorylate (and activate) MAPKs, and MEKK1 can phosphorylate (and activate) the IKK complex (Moustakas and Heldin 2003).

Interferon-Responsive Factors

Another alternative signaling route potentially activated upon the interaction between TLRs and intracellular adaptors would lead to the activation of *Interferon-Responsive Factors* (IRFs), a class of transcription factors that enable the expression of interferons and other proinflammatory cytokines. However, this typical vertebrate pathway implies the mediation of TRIF (instead of MyD88) and RIP kinase 1 (instead of IRAKs) which both lack convincing homologs in bivalves (Meylan et al. 2004). Bivalve IRFs have been linked to resistance to infections in *H. cumingii* (Wang et al. 2013a) and to the transcriptional activation of genes with ISRE elements in the pearl oyster *P. fucata* and the mussels *Bathymodiolus platifrons* and *Modiolus modiolus* (Huang et al. 2013b, 2017a). However, since the existence of MyD88-independent TLR signaling seems unlikely in bivalves, these IRF-like molecules are probably related to other signaling routes originated from cytosolic PRRs, which will be described in detail in section "Signaling Pathways Activated in Response to Microbial Sensing in the Cytosol".

Is an Immune Deficiency–Like Pathway Present in Bivalve Molluscs?

The possible presence of a bivalve immune deficiency (IMD)–like pathway involved in the recognition of Gram-negative bacteria and homologous to that found in *Drosophila* (Lemaitre and Hoffmann 2007) has been long hypothesized. In this case, the immune signals would originate from membrane-bound PGRPs and be transduced in the cytosol by signaling molecules that are partially shared with the vertebrate tumor necrosis factor receptor (TNFR) signaling pathway. These include dFADD and DREDD/Caspase-8, which are both present in bivalves (Gerdol and Venier 2015), but also the IKK complex and MAPK pathway, which can be activated by the cross talk between TNFR and TLR signaling. Crucially, however, the key IMD adaptor molecule is lacking and no functionally homologous component has been identified yet in bivalves (Gerdol and Venier 2015). Taking into account the relevant sequence divergence between the intracellular domain of arthropod and molluscan membrane-bound PGRPs (see section "Other Membrane-Bound Immune

Receptors"), the identity of the hypothetical key mediator of the IMD-like pathway in these animals remains presently unknown.

Signaling Pathways Activated in Response to Microbial Sensing in the Cytosol

The interconnected signaling pathways presented so far act at the crossroads with the cytosolic PRRs described in section "Cytosolic Pattern Recognition Receptors," which share several signal transducers with the TLR/NF-κB/MAPK/IRF circuitry, thereby resulting in the activation of the same transcription factors and in the production of similar effector molecules. Among these, the signaling by NLRs would hypothetically involve the mediation of *receptor-interacting serine/threonine protein kinase 2* (RIPK2) for the recruitment of TAK1 and the consequent activation of the IKK complex (Nembrini et al. 2009). However, the lack of a bivalve RIP2K homolog points out that a bivalve NLR-based cytosolic MAMP–sensing system, if it exists, should be based on molecules that are divergent from their vertebrate functional homologs.

In vertebrates, STING stimulates the phosphorylation of IRF3 through the action of the TANK-binding kinase 1 (TBK1) (Tanaka and Chen 2012), the gene of which has been recently characterized in *C. gigas*. The oyster homolog was strongly upregulated in response to *V. alginolyticus* and OsHV-1 infections and, most importantly, its direct interaction with STING was demonstrated by co-IP studies, thereby confirming a mode of signal transduction similar to those described in vertebrates (Tang et al. 2016).

RLRs, key sensors of viral nucleic acids (see section "Cytosolic Pattern Recognition Receptors"), require the *IFN-beta promoter stimulator* (IPS-1, also known as *CARD adaptor inducing IFN-beta*, or CARDIF, and *Virus induced signaling adaptor*, or VISA) to induce the expression of interferon and inflammatory cytokines via IRFs or NF-κB (Fredericksen et al. 2008). This adapter has remained elusive for a long time in invertebrates, until the very recent discovery of the *C. gigas* homolog CgMAVS. The functional characterization of the oyster protein confirmed its primary role in antiviral response, as (1) CgMAVS could be strongly upregulated in response to viral infections; (2) the interaction between the CARD domain of CgRIG-I-1 and CgMAVS was demonstrated by yeast two-hybrid and co-IP; (3) an interaction was similarly demonstrated with the downstream signaling adapter TRAF6; and (4) the inactivation of CgMAVS by RNAi in infected oyster spat determined a remarkable increase in mortality (Huang et al. 2017b). The demonstrated interaction with TRAF6 would imply the activation of NF-κB. However, the most important MAVS interactor in vertebrates is another member of the TRAF family, TRAF3, which can recruit TBK1, activating IRF3. The first molluscan TRAF3 homolog was recently identified in the freshwater mussel *Anodonta woodiana*. Although the physical interaction with MAVS and RLRs has not been demonstrated yet, bacterial and viral challenges triggered the overexpression of this molecule, supporting its involvement in RLR-mediated signaling (Qu et al. 2017c).

Altogether, these functional studies, supported by the identification of nearly all of the required signaling molecules in sequence databases (Philipp et al. 2012;

Green et al. 2015; Ren et al. 2017b), as well as by the observation of their significant upregulation in response to experimental OsHV-1 infection in oysters (He et al. 2015), highlight that bivalve molluscs are equipped with a well-developed molecular system for viral sensing in the cytosol.

Production of Cytokines

Elusive Regulators of the Molluscan Immune System

The complex signaling machinery described in detail in the previous sections ultimately leads to the production of effector molecules that are used to kill or to reduce the pathogenicity of invading microbes (see section "Humoral Immune Effectors") or to regulate immune response at a cellular level (see section "Cellular Immune Responses") and at a systemic level. Cytokines are small proteins with regulatory immune functions, which are the most important regulators of metazoan immunity, as they activate signaling elements leading to the expression of other cytokines, antiviral effectors, and other immune-related genes. Their action is very fast and powerful in the amplification of the immune response despite an extremely low concentration in body fluids. Furthermore, many cytokines have a pleiotropic effect and a somewhat redundant function (Nicola 1994). Despite the essential and long-known role of cytokines in vertebrates, their existence in invertebrate animals was long debated until the first molecules with a cytokine-like activity were first identified (Beschin et al. 2001; Herpin et al. 2004). Moreover, as explained in section "Other Membrane-Bound Immune Receptors," one of the most studied cytokines in the *D. melanogaster* model, Spätzle (Parker et al. 2001), is not present in bivalves and therefore TLRs are likely to act in a vertebrate-like fashion, by directly binding MAMPs with their extracellular LRR domains. Despite the availability of genomic sequence data, interferon-like factors remain elusive in all invertebrates, seemingly supporting the idea that vertebrate and invertebrate cytokines have a different evolutionary origin, despite sharing a similar mode of action and a quite conserved intracellular signaling machinery. For the most part, molecular studies on molluscan cytokines are limited to evolutionarily conserved factors, readily identifiable by sequence similarity.

Structurally Conserved Cytokines: Interleukin-17, Macrophage Migration Inhibitory Factor, and Allograft Inflammatory Factor-1

The first bivalve cytokine to be identified was interleukin-17, produced at significant levels in oyster hemocytes in response to bacterial exposure (Roberts et al. 2008). IL-17 sequences have been subsequently isolated in many bivalve species or detected as highly responsive transcripts to bacterial challenges and abiotic stimuli (Wu et al. 2013; Moreira et al. 2014; Xin et al. 2015, 2016b). Genomic studies have further revealed that oyster IL-17 proteins are the product of a multigenic family, which comprises at least five members (Li et al. 2014). Although IL-17 signaling requires further study in bivalves, homology-based inference suggests that because of its conserved structure (Fig. 12), the binding of IL-17 to its receptor stimulates

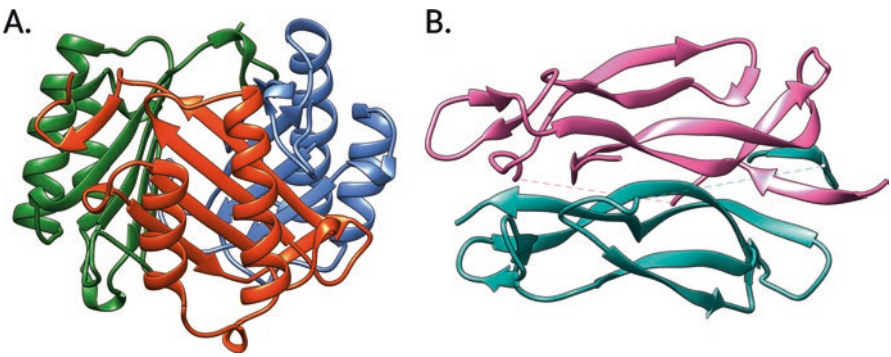


Fig. 12 Structure of two of the evolutionarily conserved cytokines found in bivalve molluscs. (**a**) Human macrophage migration inhibitory factor (MIF) trimer (PDB accession ID: 1MIF). (**b**) Human interleukin-17 dimer (PDB accession ID: 4HR9)

downstream CIKS/CIKSL proteins via SEFIR–SEFIR domain interactions and, subsequently, TRAF proteins related to both MAPK and NF-κB signaling (Rosani et al. 2015).

The macrophage migration inhibitory factor (MIF) and the allograft inflammatory factor-1 (AIF-1) are two other proinflammatory cytokines that have been identified in bivalves by sequence similarity. The former is a CD74 ligand, which stimulates the acute phase response. Despite the clear difference between bivalve and vertebrate circulating immune cells, the *M. galloprovincialis* MIF displays a well conserved three-dimensional fold (Parisi et al. 2012) (Fig. 12). In contrast with expression data collected in mussels, the *A. farreri* MIF sequence was upregulated upon bacterial challenges in a study that also provided an important confirmation about the functional conservation this molecule, as the recombinant protein could induce fibroblast migration (Li et al. 2011b). In addition, single nucleotide polymorphisms of MIF have been connected with increased resistance to *Vibrio* spp. infections in *M. meretrix* (Zou and Liu 2016). AIF-1, on the other hand, is activated in macrophages upon tissue injury. In *O. edulis*, AIF-1 was upregulated in the hemocytes and mantle of oysters affected with heavy bonamiosis (Martín-Gómez et al. 2014), and its expression could be induced in *C. gigas* with multiple immune challenges (Zhang et al. 2013b). From a functional point of view, the similarity between vertebrate and bivalve AIF-1 proteins is remarkable. Indeed, the oyster homologs could stimulate phagocytosis in the granulocyte hemocyte subpopulation and a clear involvement in tissue damage could be also established (Li et al. 2013a).

Tumor Necrosis Factor-α: A Cytokine Acting at the Crossroads Between Immunity and Apoptosis

Following the identification of a *tumor necrosis factor* α (TNF-α) in disk abalone (De Zoysa et al. 2009), this multifunctional immune modulator was also described in *C. gigas*, *C. hongkongensis*, and *O. edulis* (Martín-Gómez et al. 2014; Sun

et al. 2014; Qu et al. 2017b). Oyster TNF-α transcripts are upregulated in response to immune challenges and bonamiosis and modulate phagocytosis and apoptosis in hemocytes. Furthermore, TNF-α recombinant proteins could induce the expression of NF-κB reporter genes in human cell lines. In bivalve molluscs, the conserved function of this cytokine, which acts at the crossroads between the immune system and the apoptotic machinery, is supported by the identification of conserved accessory factors, i.e., TTRAP (Yang et al. 2011a) and *lipopolysaccharide-induced TNF factor* (LITAF), a positive regulator of TNF-α transcription (Zhu and Wu 2012; Yang et al. 2013a). As mentioned in section "Other Immune Signaling Pathways," TNF-α would exert its function through a signaling pathway partially shared with the arthropod IMD pathway, which includes the key evolutionarily conserved components dFADD and DREDD (Gerdol and Venier 2015). The transduction of immune signal inside the cell is enabled by the binding of TNF-like molecules to their receptors, collectively known as TNFRs. Functional tests carried out in many bivalve species support the involvement of bivalve TNFRs in the establishment of immune response, despite their limited homology with vertebrate receptors (Li et al. 2009; Su et al. 2011; Xing et al. 2016; Xiang et al. 2016). Another cytokine involved in the regulation of cell death, the *TNF-related apoptosis-inducing ligand* (TRAIL), is ubiquitously expressed in various tissues in *H. cumingii* and *Crassostrea ariakensis*. The few experimental pieces of evidence collected so far point toward the involvement of the MAPK pathway in the activation of this cytokine and also suggest the involvement of caspase 3 as a downstream effector (Yang and Wu 2010; Yang et al. 2013b).

New Opportunities for Cytokine Studies in Bivalves

Many divergent molecules with a cytokine-like function in bivalve molluscs have only been recently identified or still remain to be uncovered. An important example is provided by myticin C, a long-known mussel antimicrobial peptide, which has also been shown to bear chemotactic properties, stimulating hemocyte migration and morphological changes (Balseiro et al. 2011). The discovery of a class II helical cytokine in *C. gigas* with remote homology with vertebrate IFN-like molecules further stimulates research efforts directed at the discovery of novel cytokines in bivalves. CgIFNLP was upregulated in response to poly(I:C) stimulation and the recombinant protein could sensibly enhance both apoptosis and phagocytosis in oyster hemocytes (Zhang et al. 2015).

In the vertebrate IFN signaling, the activation of IFN receptors stimulates the activity of downstream Janus kinases (JAK) and, consequently, the migration of the Signal transducer and activator of transcription (STAT) to the nucleus, with the consequent expression of *IFN-stimulated genes* (ISGs). This signaling pathway, whose presence in bivalves had been already assessed by a number of transcriptomic studies (Philipp et al. 2012; Green et al. 2014, 2015), has been conclusively implicated in the regulation of immune response by CgIFNLP through its newly isolated receptor (Zhang et al. 2016b).

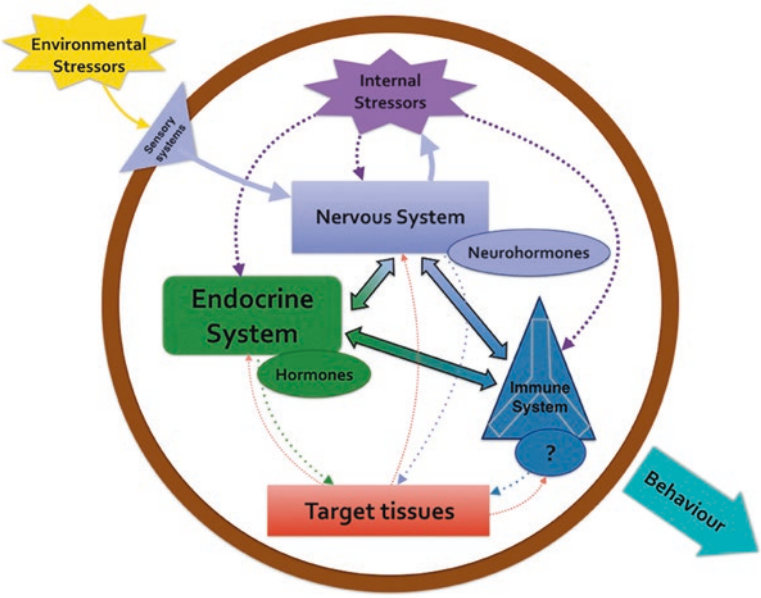


Fig. 13 Cross talk between the nervous, endocrine, and immune systems in response to an external stimulus. (Original Source: Di Cosmo and Polese 2016)

Connections with the Neuroendocrine System

The neuroendocrine immunomodulation (NEI) regulatory network encompasses the complex cross talk between the nervous system, the endocrine system, and the immune system to maintain homeostasis and to modulate innate immune response in all animals (Fig. 13). Although NEI appears to be simpler in invertebrates than in vertebrates, it is highly conserved and represents an efficient regulatory mechanism (Hartenstein 2006). From this point of view, molluscs are of particular interest, as they are the most primitive animals with a complete NEI system and there is evidence that points to hemocytes as a connecting link between the immune and the nervous system (Liu et al. 2017b). While cephalopods have long been considered as privileged molluscan models for the study of NEI because of their well-developed nervous system and amenability for laboratory research (Di Cosmo and Polese 2016), in recent years bivalve molluscs have been the subject of an increasing number of studies (Song et al. 2015; Wang et al. 2017a). The main components of the NEI are the cholinergic, catecholaminergic, and nitric oxidase systems, together with the action of the neuropeptides.

The Cholinergic and Catecholaminergic Neuroendocrine Systems

The cholinergic neuroendocrine system can be activated by pathogens and tends to negatively regulate the immune response on a long time scale. The main component of the cholinergic nervous system is acetylcholine (ACh), whose concentration has

been shown to significantly increase in the hemolymph of scallops upon stimulation with LPS or TNF-α (Shi et al. 2014). Acetylcholinesterase-like enzymes and muscarinic receptors of Ach have been detected in the hemocytes and other tissues of bivalve molluscs. Strikingly, the *A. farreri* acetylcholinesterase is thought to contribute to the rebalancing of the immune system following immune response in *A. farreri* (Shi et al. 2012). As a further confirmation in support of the existence of the cholinergic anti-inflammatory pathway in this animal group, the expression of a novel muscarinic acetylcholine receptor was regulated by LPS stimulation in *C. gigas*. The activation of this receptor seems to be crucial for the production of TNF and for the regulation of apoptosis in hemocytes (Liu et al. 2016b). Moreover, the subunits of the nicotinic acetylcholine receptor of *A. farreri* were subjected to a similar induction in response to LPS and TNF-α (Shi et al. 2015).

The catecholaminergic neuroendocrine system is mainly composed of catecholamines (dopamine, norepinephrine and epinephrine), their metabolic enzymes, and receptors. Catecholamines are among the first neurotransmitters to appear during the ontogenesis of molluscs to regulate cell proliferation, differentiation, and neurogenesis. In adults, the synthesis and release of catecholamines has been reported in the hemocytes, mantle, and gills. The first important evidence supporting the involvement of this system in the modulation of both the cellular and humoral immune response was provided by the observation of the induction of the alpha-1 norepinephrine receptor in response to LPS in *C. gigas*. This receptor could in turn modulate the expression of TNF and induce phagocytosis and apoptosis of hemocytes (Liu et al. 2016c). Furthermore, the catecholaminergic system is markedly activated after acute heat and bacterial stress in oyster larvae (Liu et al. 2017a).

Nitric Oxide, Neuropeptides, and Open Challenges in Neuroendocrine Immunomodulation Studies

NO synthase (NOS) is a fundamental enzyme for the production of nitric oxide (NO), a key signaling molecule involved in multiple processes, including immune defense. Unlike vertebrates, molluscs display only a single NOS isoform, pointing toward the existence of a unique prototypical enzyme that combines the functions of the three vertebrate isoforms. Recently, the mutual modulation between norepinephrine and nitric oxide during immune response has been demonstrated in scallops (Jiang et al. 2014), showing the intimate linkage among all of these regulatory systems.

Neuropeptides include a diverse class of cell signaling molecules. These molecules are produced and released by neurons, and their mechanism of action occurs through a regulated secretory pathway. As in vertebrates, various neuropeptides identified in molluscs could potentially play important roles in immune regulation. Although 74 possible neuropeptide genes have indeed been identified in the oyster genome (Zhang et al. 2012a), neuropeptide studies in the context of immunity are still lacking in bivalves.

As a final consideration about the regulation of NEI function in molluscs, the action of microRNAs also needs to be taken into account. In fact, several miRNAs (named NeurimmiRs) are highly responsive to acetylcholine and norepinephrine

stimulation in oyster hemocytes. The in silico–predicted targets for NeurimmiRs comprise over 300 genes with functions in cell death, immunity, and response to stimulus, which might therefore explain the observed decrease in phagocytosis and late apoptosis/necrosis in stimulated hemocytes (Chen et al. 2015). One of the identified miRNAs was subjected to further studies, which evidenced its role in repressing acetylcholine production and choline uptake in hemocytes (Chen et al. 2016).

Humoral Immune Effectors

Antimicrobial Peptides

Because of their fundamental role as a first line of defense in the molluscan innate immune system and potential biotechnological applications, antimicrobial peptides (AMPs) have been the subject of a considerable number of molecular studies. The first pioneer studies, targeting the hemolymph of mussels, provided the impetus for the characterization of novel antimicrobial compounds, using classical biochemical methods. This field of research is growing thanks to the application of in silico data-mining approaches, and bivalves have been one of the most extensively exploited sources of AMPs in the animal kingdom over the past 20 years.

Defensins, Mytilins, and Myticins: Main Players in Hemocyte-Mediated Immune Response

The story of antimicrobial research in bivalve molluscs dates back to 1996, when several novel cysteine-rich peptides similar to arthropod defensins were extracted from the active fraction of hemolymph in the marine mussels *M. edulis* and *M. galloprovincialis* (Hubert et al. 1996; Charlet et al. 1996). Two novel peptides, containing eight cysteine residues arranged in a slightly different pattern, were named mytilins and displayed significant activity mostly directed against Gram-positive bacteria (Charlet et al. 1996). Mytilins and defensins exert their antimicrobial action following the recruitment of a specialized subpopulation of circulating hemocytes to the site of infection, where they are intracellularly released from granules (Mitta et al. 2000b, c). Although these AMPs are clearly involved in the intracellular killing of bacterial cells phagocytosed by hemocytes, they also appear to secondarily participate in the systemic immune response when released in the hemolymph (Mitta et al. 2000d). A few years later, a new class of AMPs named myticins was identified in *M. galloprovincialis* plasma and hemocytes. These peptides displayed only limited antimicrobial properties in comparison with defensins and mytilins but shared eight conserved cysteine residues and high hemocyte specificity (Mitta et al. 1999). Although the antimicrobial activity of myticins is rather weak and it can be only attained at acidic pH (Martinez-Lopez et al. 2013; Domeneghetti et al. 2015), they might have alternative potential roles both as antiviral agents and as chemokine/cytokine–like molecules (Balseiro et al. 2011; Novoa et al. 2016).

Molecular and genetic studies revealed that these mussel AMPs are produced as secreted pre-propeptides. The highly cationic charge of the central mature peptide

region is balanced by an acidic C-terminal extension of the precursor protein, which is likely removed after its release from hemocyte granules. It was also revealed that these AMPs pertain to multigenic families that share a similar architecture, as they all comprise four exons and three introns, with fixed exon/intron boundaries (Mitta et al. 2000a). An aspect of mussel hemocyte-specific AMPs that has revealed somewhat counterintuitive patterns concerns unpredictable fluctuations in gene expression in response to bacterial challenges (Mitta et al. 2000a) and significant intraspecific variation, suggesting that genome–environment interactions play a major role in regulating AMP production (Li et al. 2010).

A few years after the original discovery of AMPs in mussel hemocytes, defensin-like AMPs with eight cysteines were also identified in circulating immune cells in the Pacific oyster, together with a second isoform mainly expressed in the mantle edge (Gueguen et al. 2006; Gonzalez et al. 2007a). Over the years, many other sequences labeled as "defensin" or "defensin-like" AMPs have been isolated in different bivalve species. Besides their structural differences, summarized by the presence of either three or four disulfide bonds, these AMPs are also often characterized by different spectra of activity, preferential tissues of expression, and accessory functions. For example, a foot-specific defensin-like peptide has been linked to byssogenesis in zebra mussels (Xu and Faisal 2010), whereas a gill-specific peptide with marked activity against Gram-positive bacteria has been isolated from gills extracts of *C. virginica* (Seo et al. 2005). Clam and freshwater mussel defensins display a spectrum of activity and tissue specificity similar to those of *Mytilus* AMPs, but they are reportedly upregulated following bacterial challenges (Peng et al. 2012; Wang et al. 2015c). These reports suggest that different cysteine-rich peptides currently classified with the same label could have slightly different biological properties depending on the species of origin.

From a structural point of view, all of the aforementioned defensin-like AMPs (including mytilins and myticins) share a common structural motif, the cysteine-stabilized α-helix β-sheet (CS-αβ) fold (Fig. 14). This conserved and successful compact domain consists of an α-helix and two antiparallel β-sheets, whose orientation and reciprocal position in the 3D space are fixed by intramolecular disulfide bridges (Yang et al. 2000; Gueguen et al. 2006). Crystallographic studies reveled that, in spite of a negligible primary sequence homology and a slightly different position of cysteine residues, defensins and mytilins share not only the same structural fold but also similar hydrophobic and hydrophilic areas (Roch et al. 2008). Although the 3D structure of myticins has not been experimentally determined yet, modeling approaches have unequivocally evidenced that they are also likely to adopt a CS-αβ fold (Domeneghetti et al. 2015).

Other Cysteine-Rich Antimicrobial Peptide Families

In recent years, data-mining approaches have led to the identification of macins, an additional group of bivalve AMPs in the CS-αβ peptide superfamily. Originally identified in other metazoan phyla, macins were first described as a multigenic family in *M. galloprovincialis* (Gerdol et al. 2012) and later reported in other bivalve species. Although the functional significance of the complex cysteine array of

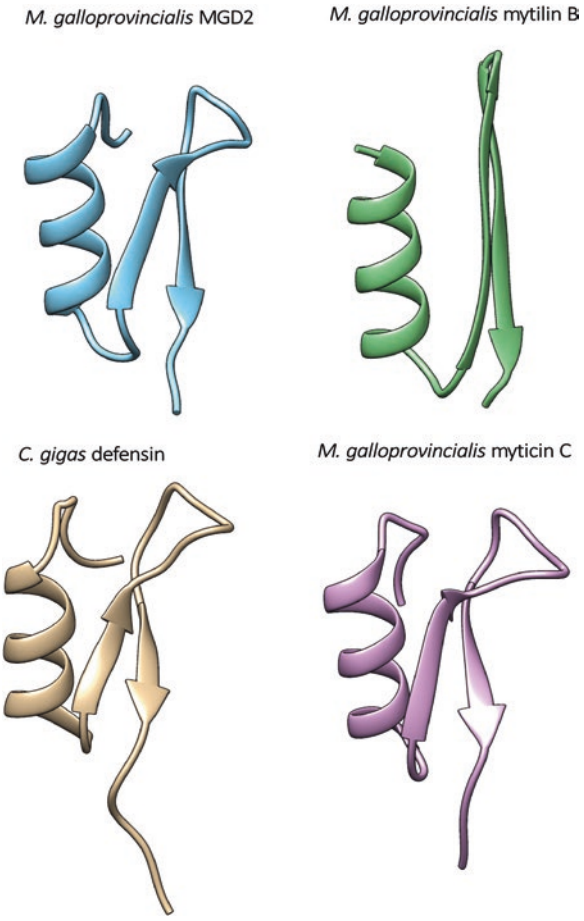


Fig. 14 Experimentally determined three-dimensional structures of *M. galloprovincialis* MGD2 defensin, mytilin B, and *C. gigas* defensin. The in silico–predicted structure of *M. galloprovincialis* myticin C is also reported. The conserved cysteine-stabilized α-helix β-sheet fold, comprising an α-helix followed by two antiparallel β-sheets, is easily detectable

macins is still poorly understood, these peptides are of great interest because of their role in wound healing, in addition to bacterial killing, and their widespread expression across all main tissues.

In comparison with canonical defensins, big defensins pertain to a structurally different but evolutionarily widespread class, also comprising vertebrate β-defensins. The characterizing six-cysteine array of big defensins is located in the C-terminal domain of these AMPs, and it is coupled with an N-terminal α-helical domain whose presence is also required for antimicrobial action. Big defensins have been isolated in many different bivalve species and, while all studies have evidenced the inducible expression of these AMPs, contrasting reports have been produced concerning the main tissues of expression (Zhao et al. 2010; Rosa et al. 2011; Gerdol et al. 2012; Li et al. 2012; Wang et al. 2014a; Yang et al. 2016). A more precise indication concerning the localization of big defensins has been provided by immunofluorescence studies carried out in *A. irradians*, which have evidenced a prominent abundance in the gill and mantle epithelia, strongly implicating a role in mucosal immunity (González et al. 2017).

The remarkable diversity of bivalve cysteine-rich AMPs is not limited to peptides with a known structure but also involves novel cysteine arrays and unknown disulfide connectivities. The first example is that of mytimycin, an antifungal peptide identified in mussel hemolymph extracts (Charlet et al. 1996). Like the other AMPs stored in granules, this peptide is produced as an inactive precursor, whose C-terminal extension contains an EF-hand domain. The mature peptide region can vary in terms of both the number and the arrangement of cysteine residues (Sonthi et al. 2011). More recently, three additional plausible AMP families—myticusins (Liao et al. 2013), mytichitins (Qin et al. 2014), and CRP-I (Gerdol et al. 2015a)—have been identified in *Mytilus* spp. but promising preliminary results still await a detailed functional characterization.

Improved Strategies Are Required to Discover Novel Antimicrobial Peptide Families

Although different molecules with heterogeneous evolutionary origins, amino acid compositions, and three-dimensional structures can act as antimicrobial agents, nearly all known bivalve AMPs pertain to a single large category, i.e., AMPs rich in cysteine residues engaged in disulfide bonds. This reflects the overwhelming prevalence of the scientific literature on the subject, as very scant information is available about AMPs devoid of disulfide bonds in Bivalvia. As a striking example, no AMP with an amphipathic α-helical secondary structure has ever been isolated, despite their widespread distribution and the important role these peptides cover in the innate immune system of other protostomes (Giangaspero et al. 2001). While it is possible that this lack of information mirrors a major shift toward the use of cysteine-rich AMPs in molluscs compared with other metazoans, other explanations are possible. For example, in silico similarity-based discovery methods are biased toward conserved disulfide arrays, whereas α-helical or linear AMPs do not necessarily present a primary sequence similarity significant enough to allow BLAST- or profile-based detection.

Some evidence supporting the involvement of peptides enriched in particular amino acids in bivalve immune response first surfaced with the report of short, secreted proline-rich peptides (CgPrp), which were found to be coexpressed with defensins in circulating hemocytes in *C. gigas*, synergistically enhancing their activity (Gueguen et al. 2009). A second, unrelated AMP was constitutively expressed in multiple tissues of the same species, and it was named molluscidin. This cationic peptide, similar to an AMP isolated in abalones, contained a series of dibasic repeats and exhibited broad-spectrum antimicrobial activity (Seo et al. 2013). The third and most recent case of linear cationic AMPs comprises myticalins and modiocalins from marine mussels pertaining to the *Mytilus* spp. and *Modiolus* spp. genera, respectively. These AMPs, identified thanks to an in silico approach, display a broad spectrum of activity against Gram-positive and Gram-negative bacteria. Myticalins are produced as pre-propeptides and display a gill-specific pattern of expression, suggesting a possible function as modulators of the microbial communities associated with this important filtrating tissue (Leoni et al. 2017).

The last major category of AMPs comprises peptides generated by fragmentation of larger precursors with various nonantimicrobial functions. Two important examples are provided by an antibacterial peptide isolated from *Anadara kagoshimensis*, which is a fragment of hemoglobin I (Chen et al. 2017b) and by the N-terminal highly cationic fragment of the histone H2B (named molluscin), which appears to modulate the bacterial community in the gills of oysters and possibly other bivalves (Seo et al. 2011). Histone H4 may also have a role in bivalve immunity (Nikapitiya et al. 2013).

Sequence Hyperdiversity as an Effective Weapon to Fight Microbial Infection

In addition to interspecies variability, several bivalve AMPs are characterized by an unusually high degree of intraspecific diversity. For example, the diversity of myticin C was first observed by denaturing gradient gel electrophoresis (DGGE), because of the presence of unique characteristic band patterns in individual mussels (Costa et al. 2009a). It was later found out that this variability also matched nucleotide variation at the mRNA level and that about 8% of the codons within the myticin C sequence evolved under strong positive selection (Pallavicini et al. 2008; Padhi and Verghese 2008). This high level of polymorphisms has been also observed in other (but curiously not in all) mussel AMPs with targeted massive parallel sequencing (Rosani et al. 2011). Similar considerations are also valid for oyster and clams defensins, whose sequence variability can be linked to relevant directional selection pressures (Schmitt et al. 2010; Wang et al. 2015c). It is still not entirely clear whether this remarkable sequence diversity is due to a high number of paralogous genes, high allelic variability, RNA editing, or all of these factors combined. Furthermore, evidence collected from both oysters (Rosa et al. 2015) and mussels (Leoni et al. 2017) strongly hints that complex phenomena of gene presence/absence variability might partially explain the extreme diversification of antimicrobial effectors. Certainly, the presence of such a diversified arsenal of AMPs, apparently driven by selective forces, suggests that amino acid variations might have been evolutionarily exploited to broaden the spectrum of action of these molecules, endowing bivalve populations with effective weapons to face the challenge of microbial infection.

Lysozymes, Bactericidal/Permeability-Increasing Proteins, and Other Pore-Forming Molecules

Lysozymes

The term “lysozymes” is used to collectively describe a group of heterogeneous and widespread proteins involved in the animal innate immune system, which display strong lytic action against bacteria. Although all lysozymes share a similar structural fold, they largely diverge in their primary sequence, which can therefore be used for classification purposes within three main classes: chicken-type (C-type), goose-type (G-type), and invertebrate-type (I-type) lysozymes (Callewaert and Michiels 2010).

From a genomic perspective, it is now clear that genes encoding all three major lysozyme types can be simultaneously present in the same species, sometimes with several different variants, which might cover slightly different biological functions (Gerdol and Venier 2015). In spite of their remarkable primary sequence divergence, all lysozymes share the same glycoside hydrolase enzymatic activity, which catalyzes the hydrolysis of peptidoglycan and, to a lesser extent, chitin. As PGN is a main component of the bacterial cell wall in Gram-positive bacteria but not in Gram-negative bacteria, lysozymes display stronger activity against the former.

The first studies on bivalve lysozymes were conducted on I-type sequences, with the purification of chlamysin in the Arctic scallop, *Chlamys islandica* (Nilsen et al. 1999). Highly similar sequences, implicated either in immune response or in digestive processes, were later reported in several other bivalve species (Matsumoto et al. 2006; La Peyre et al. 2010; Yue et al. 2011; Ren et al. 2012). The isolation of the complete gene sequence of bivalve I-type lysozymes allowed in-depth phylogenetic analyses, which revealed a remote homology between this class of enzymes and vertebrate C-type lysozymes, hinting at an evolutionary origin from a common ancestor (Bachali et al. 2002). The discovery that different I-type paralogous genes in hydrothermal vent mussels play a crucial role not just in antimicrobial response but also in the management of symbiotic communities (Detree et al. 2016a) is one of the most significant recent developments in bivalve lysozyme research.

In comparison, bivalve C-type lysozymes have been the subject of little scientific attention, with only a handful of studies reported so far. Following its initial identification in *M. galloprovincialis* (Venier et al. 2009), this enzyme was characterized as an inducible gene product, capable of targeting a broad range of bacteria (Wang et al. 2013c).

The presence of G-type lysozymes, previously thought to be taxonomically restricted to vertebrates, was demonstrated in 2007 in the scallop *A. farreri* (Zhao et al. 2007). In the following years, G-type lysozymes have been genetically and partly also functionally characterized in scallops and mussels (He et al. 2012a; Wang et al. 2013c; Li et al. 2013b), evidencing that paralogous gene copies might have acquired a specialized function in either digestive or immune functions. As a unique known case in nature, a chimeric protein combining a C-terminal G-type lysozyme domain with an N-terminal PGRP domain has been identified in *C. gigas*. This protein, which might combine bacteria binding and lytic properties, was inducible in hemocytes in response to *Marinococcus halophilus* and *V. tubiashii* exposure (Itoh and Takahashi 2009).

More recently, a fourth type of lysozyme was identified in veneroid clams. This novel antibacterial protein surprisingly shared significant similarity with lysozymes produced by bacteriophages to break the PGN chains of the infected bacterial cell walls and release mature phages (Ding et al. 2014). An interesting comparative study shed some light on the origin of this gene, revealing its co-option from viruses by horizontal gene transfer in two major bivalve groups, Heterodonta and Palaeoheterodonta. Following this event, the newly acquired sequences underwent complex genomic rearrangements, which overall contributed to increased antibacterial potential (Ren et al. 2017a).

Bactericidal/Permeability-Increasing Proteins

While lysozymes mainly target Gram-positive bacteria, a similar antibiotic action is exerted toward Gram-negative bacteria by Bactericidal/permeability-increasing proteins (BPIs), strong pore-forming agents found in nearly all metazoans. The specificity of action of BPIs is given by the recognition of LPS. The biological properties of *C. gigas* BPI (reminiscent of its vertebrate homologs) and its pattern of expression (broad distribution in different epithelia) suggested a role as a first line of defense in oyster mucosal immunity (Gonzalez et al. 2007b). Further genetic investigations revealed the presence of a second oyster gene copy, which displayed a slightly different expression pattern and functional specialization (Zhang et al. 2011d). Although the expression of BPIs can be positively regulated by LPS and bacterial challenges in oysters and ark shells (Zhang et al. 2011d; Mao et al. 2013), the molecular networks underlying this mechanism are still unknown. However, they are likely to be dissimilar to those involved in the production of lysozymes, which appear to be mostly downregulated under the same experimental conditions (Li et al. 2008; Ren et al. 2012), with some notable exceptions (He et al. 2012a; Wang et al. 2013c).

Might Pore-Forming Molecules Provide a Connection with the Complement System?

The possible connections with MAMP sensing by secreted and membrane-bound PRRs and maybe even with the primitive bivalve complement system remain to be fully elucidated. Because of the absence of convincing homologs of the molecular components of the terminal lytic pathway of the complement system, other pore-forming molecules are likely to cover a similar function in bivalve molluscs. While both lysozymes and BPIs could be involved, other options remain to be investigated.

A fascinating possibility is provided by several recently described cases. The first one, described so far only in the Mediterranean mussel, involves a protein containing a Membrane Attack Complex/Perforin (MACPF) domain structurally similar to that of C6/C7/C8/C9 proteins (Estévez-Calvar et al. 2011). Despite the negligible primary sequence similarity with these complement components, its upregulation strongly suggested an involvement in innate immune response. This observation gained even more importance with the report of over a dozen different similar gene products in the mussel transcriptome, which in some cases encode proteins where the perforin-like domain is associated with a PGN-binding ApeC domain (Gerdol and Venier 2015). The second class of molecules that might act as functional homologs to the complement terminal pathway are mytilectins (see section "The Role of Lectins in Immune Recognition"). Indeed, some mytilectins display a C-terminal aerolysin-like pore-forming domain, which could be employed in the lysis of microbial cells (Gerdol and Venier 2015). While both the Ricin B/aerolysin and ApeC/MACPF domain combinations could potentially result in highly efficient and concerted recognition and killing of invading pathogens, further functional assays will clearly be needed to investigate the possibility that these molecules are involved in pathogen recognition and clearance in mussels and other bivalve species.

Proteases and Protease Inhibitors

An Overview on the Role of Proteases and Their Inhibitors in the Bivalve Immune System

Several important immune processes are regulated by the concerted action of proteases and their inhibitors, which might act either on endogenous proteins, by cleaving regulatory subunits and enabling their biological activity, or on exogenous proteins produced by invading microbes and parasites, leading to their inactivation and degradation. Some of the fundamental immune processes described in other sections, such as the complement system (see section "Evidence of an Ancient Complement System in Bivalves?"), the prophenoloxidase cascade leading to melanization (see section "The Phenoloxidase Cascade"), and apoptosis (see section "Apoptosis and Autophagy"), are essentially governed by a cascade of proteolytic activations, initially triggered by the recognition of MAMPs by PRRs. Although the molecular players involved in such cascades have been comprehensively characterized in some invertebrates, such as in the case of melanization in insects (Tang 2009) or hemocyte clotting in horseshoe crabs (Iwanaga et al. 1998), the nature of such proteases has not been entirely clarified in bivalve molluscs.

This can be partly explained by the lack of specific studies on the subject, but also finds a justification in the fact that these molecules pertain to large and multifunctional families of proteases involved in a multitude of other cellular processes, often not linked with immune response. As an example, while the core components of the bivalve complement system, as well as a remarkable number of lectin-like molecules, have been characterized in bivalves, no MASP-like proteases has been identified with certainty (see section "Evidence of an Ancient Complement System in Bivalves?"), leaving a huge gap of knowledge about the link between MAMP recognition in the extracellular environment and the activation of C3, even though several similar uncharacterized serine proteases are present in bivalve genomes (Wang et al. 2017b). Similarly, the nature and specificity of action of the bivalve prophenoloxidase-activating enzymes (see section "The Phenoloxidase Cascade") and the identity of the proteases involved in the process of activation of AMPs (see section "Antimicrobial Peptides") still remain uncertain. Big defensins, CRP-I, mytimycins, and myticalins, for example, possess a dibasic cleavage site, which could be potentially cleaved off by proprotein convertases (Gerdol et al. 2012, 2015a; Leoni et al. 2017). However, other mussel AMPs such as defensins, mytilins, and myticins lack a clear consensus motif for propeptide cleavage and are therefore expected to be the substrates of other, still unknown, proteases.

Cathepsins

While all of the aforementioned proteases mainly exert their biological action in the extracellular environment, others are typically present in lysosomal compartments, where they aid the phagocytic processing of heterophagic and autophagic material. Among these, cathepsins have been the subject of multiple studies and linked to immune functions in bivalves, consistently with the well-known role these proteases have in the regulation of vertebrate immune and cell death processes

(Zavasnik-Bergant and Turk 2006; Repnik et al. 2012). In particular, multiple cathepsins have been characterized in the Chinese razor clam, *S. constricta*, where B-, C-, and L-type cathepsin were upregulated following *V. anguillarum* challenges in the mantle and, in particular, in the digestive gland (Niu et al. 2013a, b, 2014). Similar observations concerning tissue specificity and responsiveness to bacterial challenges have been also collected for a cathepsin L in *Cristaria plicata* (Hu et al. 2014), in contrast with a report from the Sidney rock oyster *S. glomerata*, where cathepsin B and L transcripts were mostly detected in hemocytes (Ertl et al. 2016).

Serine Protease Inhibitors: The Case of Oyster Perkinsosis

The infection process of many animal pathogens is also aided by a number of proteases, which target and inactivate host defense proteins and sometimes have more profound effects on the modulation of the host immune system (Armstrong 2006; Donnelly et al. 2011). In bivalve molluscs, this system has been best characterized in response to the parasite *P. marinus*, which produces proteases that specifically target defense plasma proteins, thereby impairing the immune response and creating favorable conditions for the establishment of infections by bacterial pathogens (Oliver et al. 1999; Tall et al. 1999). As a consequence, many bivalve species have developed large gene families of protease inhibitors to counteract the action of exogenous proteases produced by protozoans and other parasites (Romestand et al. 2002).

The serine protease inhibitors of the eastern oyster, *C. virginica* (CvSI) (Xue et al. 2009), pertain to the I84 family of serine protease inhibitors. These molecules have been implicated in resistance to *P. marinus* infections because of their high activity in oysters selected for increased survival in comparison with susceptible specimens (La Peyre et al. 2010) and their ability to inhibit the perkinsin pathogenic protease (Xue et al. 2006). Furthermore, a polymorphism located in the promoter region of the CvSI-1 gene was conclusively linked to its increased transcription and, consequently, to improved resistance to *P. marinus* (He et al. 2012b), and the expression levels of CvSI could also explain the interspecies differences in susceptibility to infection between *C. virginica* and the more resistant oyster species *Crassostrea corteziensis* (Gutiérrez-Rivera et al. 2015). Altogether, I84 serine protease inhibitors are part of a highly expanded and still rapidly evolving molluscan gene family (Xue et al. 2017a).

Kazal-Type Serine Protease Inhibitors and Tissue Inhibitors of Metalloproteinases

Kazal-type serine protease inhibitors are another large and widespread class of molecules that have been connected to immune functions in marine bivalves. These molecules were reportedly upregulated in the hemocytes of the scallop *A. irradians* following tissue injury and bacterial challenges (Zhu et al. 2006). Another Kazal-type protease inhibitor from *A. farreri* contained 12 tandemly repeated Kazal domains and was upregulated upon *V. anguillarum* challenges (Wang et al. 2008), and two similar but shorter proteins could be similarly induced in the hepatopancreas of *R. philippinarum* and in multiple tissues of the clam *Mesodesma donacium*

under similar experimental conditions (Maldonado-Aguayo et al. 2013; Yu et al. 2017). Like I84 inhibitors, Kazal-type inhibitors are produced by a multigenic family, whose members display different substrate specificity and sensitivity to stimulation (Zhang et al. 2014a).

The third large class of immunity-related protease inhibitors that has been studied in bivalves comprises the tissue inhibitors of metalloproteinases (TIMPs). Cg-TIMP, first identified in *C. gigas* because of its accumulation in hemocytes following shell injury and bacterial challenges (Montagnani et al. 2001), is activated through a DAMP-dependent pathway and is possibly regulated by NF-κB binding elements located in its promoter (Montagnani et al. 2007). The immune properties of TIMPs have not been investigated in other bivalve species, with the exception of the blood cockle *Tegillarca granosa*, where TgTIMP-4 is responsive to LPS, PGN, and *V. parahaemolyticus* challenges (Wang et al. 2012c).

These and other protease inhibitors might be involved in the management of microbial infections, as suggested by multiple reports of their upregulation from transcriptomic studies (Feng et al. 2010; Moreira et al. 2012a; Allam et al. 2014; Nikapitiya et al. 2014). However, the mode of action of just a few of these molecules has been properly functionally characterized. Therefore, protease inhibitors remain attractive targets for the study of host–pathogen interactions, in particular in the context of viral infections.

The Phenoloxidase Cascade

The recognition of MAMPs by PRRs, as well as various types of environmental stress, can trigger an extracellular proteolytic cascade, which leads to the conversion of prophenoloxidases (ProPO) to their active form, phenoloxidases (PO), copper-binding metalloproteins that catalyze the oxidation or hydroxylation of phenols. Different enzyme classes (tyrosinases, catecholases, and laccases) with low substrate specificity and similar activity exist in invertebrates, leading to a certain confusion in their unambiguous identification by biochemical tests on tissue extracts (Luna-Acosta et al. 2017). However, the activity of PO leads to the synthesis of the melanin pigment. This process, unique to a few invertebrate phyla, including arthropods and molluscs, enables the deposition of melanin on invading microbes, limiting the spread of infection. While the molecular players involved in the regulation of the melanization proteolytic cascade have been extensively studied and characterized in arthropods (Christensen et al. 2005; Tang 2009), limited information is available in molluscs (Luna-Acosta et al. 2017).

Secreted PGRPs are the main PRRs responsible for the activation of the ProPO cascade in *Drosophila* and other arthropods (Schmidt et al. 2008). However, as explained in section "Other Membrane-Bound Immune Receptors," while extracellular proteins with an N-acetylmuramoyl-L-alanine amidase domain are encoded by molluscan genomes, they seem to share closer similarities to those of vertebrates, where they play a direct bactericidal role. This divergence is in line with the major differences between arthropods and molluscs, which involve the interconnected

TLR (with the lack of Spätzle; see section "Canonical TLR Signaling") and IMD pathways (see section "Other Immune Signaling Pathways").

In bivalves, the melanization process has been known for a very long time as a normal physiological process linked to shell deposition in pallial mantle epithelia (Waite and Wilbur 1976). However, increased melanization, usually followed by a massive rearrangement of extracellular matrix deposition and alterations in shell mineralization, is also among the most distinctive features of some common pathologies of the bivalve mantle tissue (see section "Major Infectious Diseases Affecting Bivalve Molluscs") (Ford and Borrero 2001; Paillard 2004). Further evidence supports the involvement of the ProPO cascade in response to parasitic, bacterial, and viral infection, as PO activity appears to be strongly altered in *M. sydneyi*–infected Sydney rock oysters (Raftos et al. 2014; Luna-Acosta et al. 2017). Melanization is probably not merely an extracellular event, as it might also be implicated in the intracellular killing of encapsulated microbes (Butt and Raftos 2008). Moreover, the different rates of inhibition of PO activity in the hemocytes of *C. gigas* and *Geukensia demissa* in response to *P. marinus* infections could be linked to the different degree of susceptibility of the two species to infection (Jordan and Deaton 2005). These observations support the important role of the ProPO cascade as a system of defense against microbial infections in bivalve molluscs.

The existence of an extracellular ProPO cascade linked to components of the hemolymph has been demonstrated in *C. gigas* and *Perna viridis*, where it could be induced by LPS, zymosan, and laminarin (Asokan et al. 1997; Hellio et al. 2007). However, a proper functional characterization of POs is still lacking in most bivalve species and the sequences of very few PO genes have been identified. This is ascribable in part to the broad distribution of PO activity in different tissues and life stages, including the digestive gland, the mantle and shell, and the foot, where POs are likely to cover specific functions that are yet to be fully unveiled (Luna-Acosta et al. 2011b). For example, tyrosinases pertain to a gene family which underwent significant expansion in bivalves and has been implicated in the shell mineralization process (Huang et al. 2017c; Chen et al. 2017a). However, a tyrosinase-like protein significantly contributes to PO activity in *S. glomerata* hemocytes (Aladaileh et al. 2007) and a tyrosinase-like transcript whose expression level was significantly overexpressed in response to bacterial challenges has been reported in *A. farreri* (Zhou et al. 2012). In the same species, a 576-kDa protein with PO activity, selectively inhibiting the growth of *Vibrio* spp. and *Aeromonas salmonicida*, has been purified from hemocytes (Xing et al. 2012). Interestingly, a protein with a similar molecular weight (555 kDa), displaying *p*-diphenoloxidase activity, has been obtained from the hemocytes of a different scallop species, *A. irradians* (Jiang et al. 2011). Other studies have identified the hemocyte-specific PO enzyme as a laccase in *C. gigas* (Luna-Acosta et al. 2010, 2011a) and *R. philippinarum*, where only minor tyrosinase-like activity could be detected (Le Bris et al. 2013).

While the function of the ProPO cascade in the bivalve immune response has been related to different diseases, this topic has been the subject of limited

molecular studies and therefore still awaits detailed investigations to clarify which PRRs enable the melanization of invading microbes, both in the extracellular matrix and within phagocytic cells.

Cellular Immune Responses

Phagocytosis

Hemocytes Are the Main Cell Type Involved in the Phagocytic Process

Phagocytosis, encapsulation, and cell-mediated cytotoxicity have been extensively described in bivalves at a functional level and, more recently, at a genomic level (Schmitt et al. 2012; Soudant et al. 2013; Allam and Raftos 2015; Zannella et al. 2017; Schultz and Adema 2017) (Fig. 15).

During the early 1900s, the pathologist Metchnikoff used marine organisms, among other models, to describe and hypothesize the role of phagocytosis in digestion, immune defenses, and clearing of damaged cells (Gordon 2016; Schultz and Adema 2017). A dual role for bivalve hemocytes in digestion and immunity may be especially important during larval stages in bivalves, as suggested by evidence of phagocytic activity in early stages of larval development (Song et al. 2016). Moreover, hemocytes concentrate particulate material in the connective tissues surrounding the digestive glands in bivalve larvae (Dyachuk 2016). A more specific role for phagocytosis and encapsulation in disease resistance in bivalves has been hypothesized for Brown Ring Disease in clams, summer mortality in Pacific oysters, and QX disease (*M. sydneyi*) in Sydney rock oysters, based on in vitro observations of increased phagocytic function and/or upregulation of transcripts for genes putatively involved in phagocytosis in resistant bivalves compared with susceptible individuals (Allam and Ford 2006; Samain et al. 2007; Kuchel et al. 2010; Raftos et al. 2014).

Hemocytes are, by far, the best-studied phagocytic cells in bivalves. Flow cytometry has allowed for the development of high-throughput assays for the evaluation of hemocyte immune parameters in bivalves, including characterization of the populations of cells involved in phagocytosis of inert and biological particles and the subsequent stimulation of the oxidative burst response. Of the two major types of hemocytes described in bivalves on the basis of morphology, granulocytes in general seemed to be responsible for the majority of the phagocytic response and production of radical oxygen/nitrogen species (ROS/RNS), but this is highly dependent on the bivalve species and the nature of the stimuli (Schmitt et al. 2012; Soudant et al. 2013; Allam and Raftos 2015; Zannella et al. 2017; Schultz and Adema 2017). Moreover, differences in the timing of phagolysosome fusion between eosinophilic and basophilic hemocytes in deepwater mussels indicate that these two types of granulocytes may play different roles in phagocytosis, suggesting further definition of phagocytic capabilities within hemocyte populations (Tame et al. 2015). An additional type of hemocyte, a hemoblast-like cell, may be involved in phagocytosis,

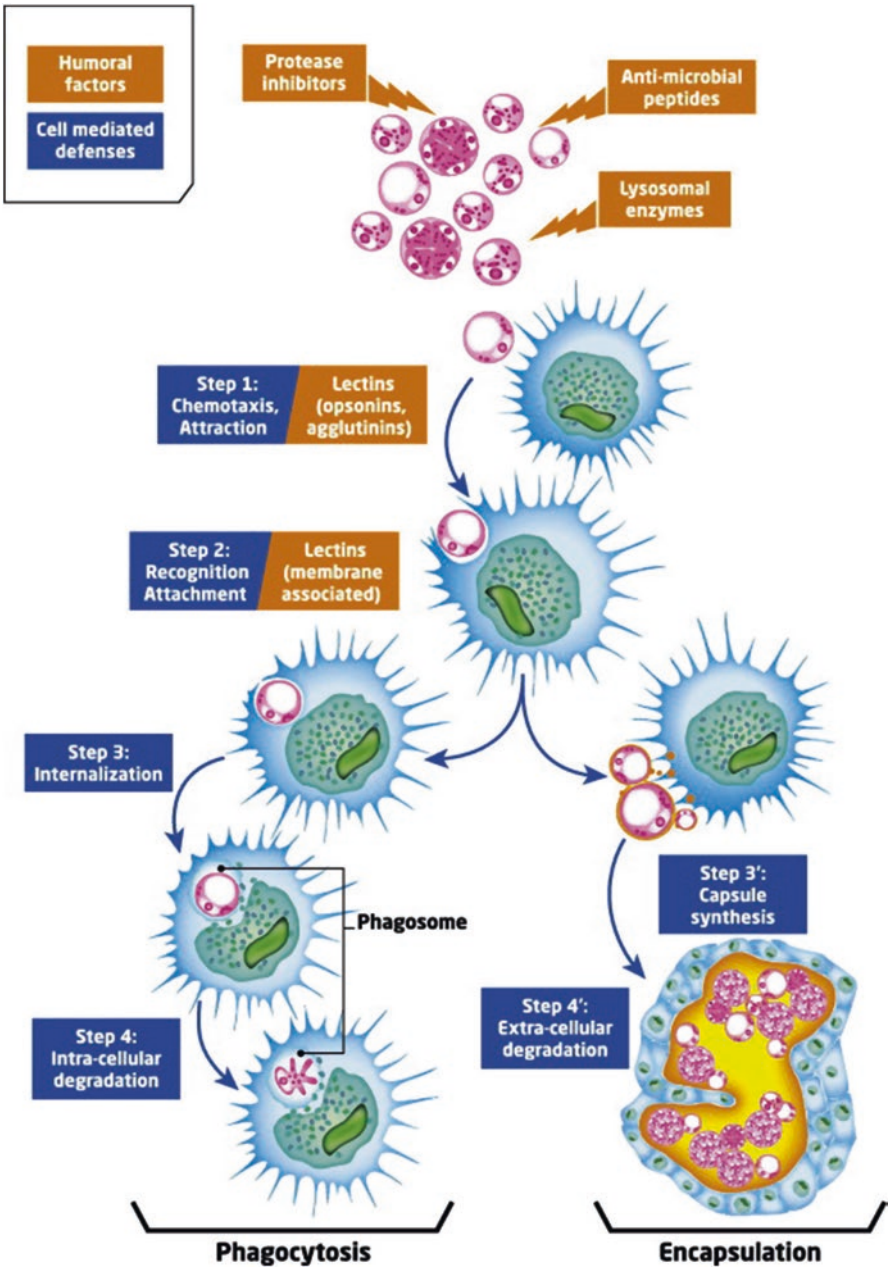


Fig. 15 The main humoral and cellular components of the bivalve immune response to microbial infection. The different steps of phagocytosis and encapsulation are shown in blue. Invading pathogens are indicated in purple, and humoral effectors (see section "Humoral Immune Effectors") are shown in green. (Source: Soudant et al. 2013)

composing a small percentage of all phagocytic cells in a hemocyte population and showing low levels of oxidative burst and lysosomal enzyme activity. Differences in the rates of phagocytosis by hemocytes also depend on the source of hemocytes within an individual (i.e., circulating hemocytes versus those present in the pallial or extrapallial spaces). Hemocytes have the ability to migrate through the epithelia into these cavities and then go back into the tissues, and those collected from the pallial cavity appear to have higher phagocytic activity than circulating hemocytes (Allam and Pales Espinosa 2016). These observations indicate that different populations of hemocytes may respond to selected stimuli and show different mechanisms of action (Evariste et al. 2016; Bettencourt et al. 2017; Vieira et al. 2017).

Other cells thought to have phagocytic capabilities are epithelial cells, with an ability that may be exploited by intracellular bacteria such as the Chlamydia- and Rickettsia-like organisms commonly seen in the gill and mantle epithelia of marine bivalves and gastropods (Allam and Pales Espinosa 2016). Development of specific cell markers will help us to understand if differences in phagocytic activity between cell populations within bivalves are due to the presence of specialized cell populations and/or the context in which these responses are occurring.

Phagocytosis in Detail: Chemotaxis, Opsonization, and Endocytosis

The process of phagocytosis involves the steps of chemotaxis, opsonization, endocytosis, formation of phagosomes, phagosome–lysosome fusion, respiratory burst, and exocytosis. Upon infection and injury, hemocytes migrate to the site of injury through the process of chemotaxis. Examples of bivalve pathogens causing massive focal infiltration of hemocytes at the site of infection include *V. tapetis* (Brown Ring Disease), *P. marinus*, and QPX. A chemotactic and/or chemokinetic response of hemocytes has been observed in response to several PAMPs, including bacterial endotoxins and extracts from trematodes and *P. marinus*. The nature of the chemotaxis/chemokinetic response depends on the type of PAMP (Schmitt et al. 2012; Soudant et al. 2013; Allam and Raftos 2015; Zannella et al. 2017; Schultz and Adema 2017).

Chemotaxis is followed by opsonization and phagocytosis. Transcriptomic analysis of Pacific oysters in response to LPS and other immune stimuli indicates that phagocytosis is promoted by a variety of opsonins (Zhang et al. 2012a). Several PRRs have been functionally demonstrated to mediate phagocytosis induction by immune stimuli through several signaling pathways (see sections "Recognition, Agglutination, and Opsonization" and "Signaling and Regulatory Pathways"). For example, an extracellular superoxide dismutase (Cg-EcSOD), highly abundant in oyster cell-free hemolymph, induces phagocytosis mediated by a β-integrin (Duperthuy et al. 2011). Lectins from Manila clams (MCL and MCL4) stimulate the opsonization of *P. olseni* parasite and *V. tubiashii* bacterial cells and subsequent phagocytosis by clam hemocytes in vitro (Soudant et al. 2013; Zannella et al. 2017). Competitive inhibition of a sialic acid–binding immunoglobulin-type lectin (CgSiglec-1) inhibits the stimulation of phagocytosis and apoptosis by LPS in oyster hemocytes, consistent with the role of siglecs as regulators of immune responses (Liu et al. 2016a). Expression of genes involved in signaling pathways associated

with integrin signaling and phagocytosis (PI3K, Rho J, MAPPK, PKC), phagosome maturation (Rab32), and respiratory bursts (NADPH oxidase) were upregulated upon secondary exposure to live *V. splendidus* after a primary challenge with killed *V. splendidus* (Zhang et al. 2014d).

Phagocytosis in Detail: Respiratory Burst and Exocytosis

The process of phagosome–lysosome fusion has been functionally observed in deepwater mussels (Tame et al. 2015). After phagosome–lysosome fusion, a respiratory burst ensues, followed by secretion of antimicrobial proteins (see section "Antimicrobial Peptides") (Soudant et al. 2013). On the basis of studies using enzyme activity measurements and the use of inhibitors, it appears that the mechanisms for production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are in general homologous to the ones observed in vertebrates (Soudant et al. 2013; Schultz and Adema 2017). The timing and extent of the respiratory burst in bivalve hemocytes, however, differs from those of the respiratory burst in vertebrate models. Moreover, bivalves and other marine invertebrates also show some differences from vertebrates in terms of the basal (not pathogen stimulated) generation of ROS as part of energy metabolism in organelles such as the mitochondria, endoplasmic reticulum, and peroxisomes (Donaghy et al. 2015). Sequencing studies indicate that, in addition to NADPH oxidase, bivalves contain genes similar to dual oxidase (DUOX, involved in immunity in *Drosophila*), which are upregulated in response to pathogenic vibrios. Bivalve hemocytes also show myeloperoxidase (MPO) activity (Schmitt et al. 2012; Donaghy et al. 2015). Radical nitrogen species, such as nitric oxide and peroxinitrite, also have an important role against pathogens in bivalves (Villamil et al. 2007). Nitric oxide also acts as an immune regulator (see section "Connections with the Neuroendocrine System"), enhancing phagocytosis, antibacterial activity, and apoptosis in bivalve hemocytes (Song et al. 2015). Expression of the single nitric oxide synthase (NOS) described in bivalve molluscs is modulated by immune stimuli (Song et al. 2015). In oyster hemocytes stimulated with zymosan, the NOS pathway is more active in hyalinocytes, while NADP oxidase activity is more prevalent in granulocyes (Lambert et al. 2007).

Antioxidant and detoxification enzymes are produced to protect cells from the toxicity of ROS and maintain redox homeostasis. Genome and transcriptome studies have led to the identification of the genes for five superoxide dismutases (SODs) in the Pacific oyster genome (He et al. 2015), two functional catalase genes in the oyster *C. hongkongensis*, and the genes coding for several glutathione peroxidases (GPxs) and gluthathione transferases (GSTs) (Sui et al. 2017; Wang et al. 2017a). Of the six known groups of superoxide dismutases, only manganese and copper/zinc have been characterized so far in bivalves. Little is known, however, about the specific roles of these enzymes in immunity and disease resistance. An extracellular SOD from Pacific oysters, CgEcSOD, a major component of oyster plasma, shows both antioxidant and PRR activities and is able to promote the phagocytosis of the bacterial pathogen *V. splendidus* (Wang et al. 2017a). The expression of Mn and Cu/Zn SODs is upregulated with both viral and bacterial challenge, and alleles in the

intracellular and extracellular Cu/Zn SOD have been associated with disease resistance to *Vibrio* infection in bay scallops (Wang et al. 2013b; Song et al. 2015; Wu et al. 2017).

Accessory Factors and Mechanisms of Regulation of Cell-Mediated Cytotoxicity

Other molecules shown to be involved in intracellular killing in the phagolysosome in bivalves include hydrolytic enzymes (β-glucuronidase, esterases, phosphatases, sulfatases, lipases), including unique versions of lysozymes showing tissue-specific patterns of gene expression (see section "Lysozymes, BPIs and Other Pore-Forming Molecules") and other antimicrobial molecules (phenoloxidases, antimicrobial peptides; see section "Antimicrobial Peptides") (Tanguy et al. 2013; Zannella et al. 2017). Phagocytosis and encapsulation are also aided by the prophenoloxidase system, a complex biochemical cascade occurring mainly in the hemolymph of bivalves, which is activated by microbial MAMPs, exogenous proteases, and environmental stress, leading to the formation of the antimicrobial molecule melanin (see section "The Phenoloxidase Cascade").

Little is known about the process of regulation of cell-mediated cytotoxicity in bivalves. A potential regulator of hemocyte function, thymosin beta-4, has been characterized in the oysters *C. hongkongensis* and *C. gigas*, and in the gastropod *Haliotis discus discus*. Treatment of oysters with recombinant protein led to increased numbers of circulating hemocytes, increased bacterial clearing, reduction of ROS production, and increased production of antioxidant enzymes, suggesting a potential role in wound healing (Li et al. 2016a). Dysregulation of the oxidative burst, on the other hand, may be involved in the pathogenesis of several diseases affecting marine bivalves. For example, oxidative stress resulting from a strong oxidative burst response, characterized by a strong upregulation of oxidase genes and downregulation of antioxidant genes, may contribute to the pathology seen in larval and juvenile oysters experimentally challenged with OsHV-1 μVar (He et al. 2015; Young et al. 2017) or infected with the bacterial pathogen *A. crassostreae* (McDowell et al. 2014).

Mechanisms of Evasion Adopted by Invading Pathogens

Several pathogenic and nonpathogenic vibrios, *Chlamydia* and Rickettsia-like organisms, and the protozoan parasites *B. ostreae*, *P. marinus*, and *P. olseni* appear to have evolved mechanisms to evade cell-mediated cytotoxicity in bivalves, exploiting that ability to survive within host tissues. Potential mechanisms used to evade phagocytosis and encapsulation include dysregulation of immune signaling through phosphorylation of p38-MAPK and induction of apoptosis of hemocytes (Ciacci et al. 2017; Burgos-Aceves and Faggio 2017). Other microbes can avoid intracellular killing by respiratory burst pathways in bivalve molluscs (Schmitt et al. 2012; Soudant et al. 2013; Allam and Raftos 2015). The enzymes arginase, alkaline phosphatase, ascorbate-dependent peroxidase, and superoxide dismutase are several of the factors potentially involved in the ability of *P. marinus* to inhibit ROS production in oyster hemocytes and survive in vitro exposure to ROS (Schott and Vasta 2003; Schott et al. 2003; Fernández-Robledo et al. 2008) (Fig. 16). The parasite is

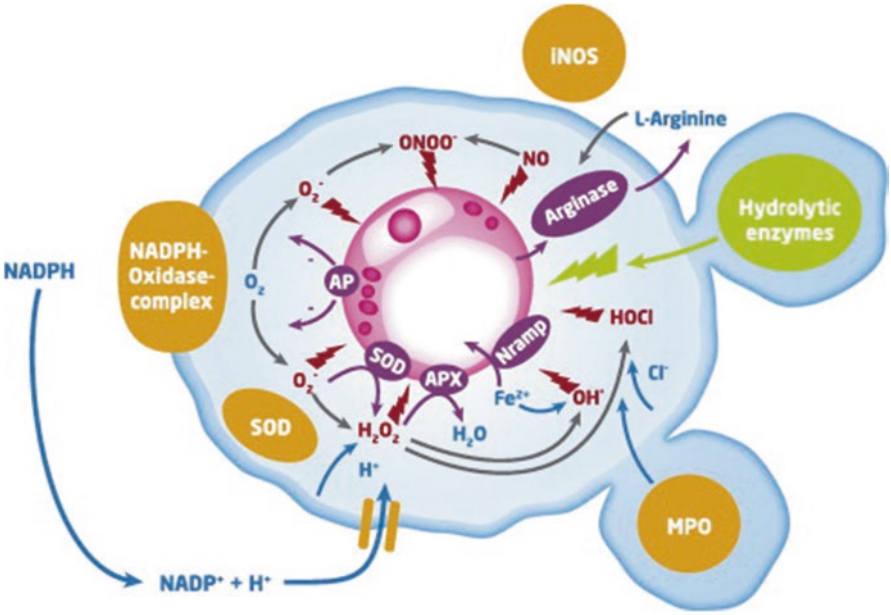


Fig. 16 Interaction between prooxidant (orange) and antioxidant (purple) activities in the phagosome of an hemocyte from the eastern oyster, *Crassostrea virginica* (blue), upon phagocytosis of the protozoan parasite *Perkinsus marinus* cell (purple). Prooxidant activities are exerted by hemocytes to kill the invading microbe by exposure to ROS (red), whereas antioxidant activities are used by *P. marinus* to escape these defensive measures. AP acid phosphatase, APX ascorbate-dependent peroxidase, HOCl hypochloride, iNOS inductible nitric oxide synthase, MPO myeloperoxidase, NO nitric oxide, Nramp Natural Resistance–Associated Macrophage Protein, O_2^- superoxide anion, $ONOO^-$ peroxynitrite, SOD superoxide dismutase. (Source: Soudant et al. 2013)

also resistant to high concentrations of nitric oxide (Villamil et al. 2007). The natural resistance–associated macrophage protein (NRAMP) in *P. marinus*, involved in iron uptake in *P. marinus* trophozoites, is hypothesized to deplete iron in hemocytes, limiting the ability of hemocytes to mount an effective respiratory burst (Lin et al. 2011). Moreover, the wall of parasites such as *P. olseni* appears to be resistant to proteolysis (Montes et al. 2002). Extracellular products from a pathogenic strain of *V. splendidus* inhibit phagocytic activity in *M. edulis* hemocytes, while those of a nonpathogenic strain do not (Ben Cheikh et al. 2016). Some metazoan parasites such as the digenean trematodes *Bucephalus* sp. and *Proctoeces maculatus* may also modulate hemocyte function in bivalve hosts, leading to decreased hemocytic infiltration in infected tissues (Carella et al. 2015).

Encapsulation and Granuloma Formation

The processes of encapsulation and granuloma formation occur when particles or pathogens are too large to be engulfed by hemocytes (e.g., in infection by trematodes) or the phagocytosis response is unsuccessful (e.g., in infection by *Perkinsus* spp. or *Nocardia* spp.). In the process of encapsulation, hemocytes recruited to

the site of infection surround and encapsulate the invading pathogen, secreting extracellular matrix products to prevent dissemination of the pathogen to other tissues and a variety of lysosomal enzymes and antimicrobial molecules to attempt to kill it (Soudant et al. 2013; Allam and Raftos 2015; Carella et al. 2015). This process can occur within the tissues, leading to granuloma-like formation, or within the extrapallial space between the mantle and the inner side of the bivalve shell, leading to conchiolin or pearl formation (Carella et al. 2015). Examples of diseases leading to granuloma formation include trematode infestations, Perkinsosis in *Ruditapes* clams, QPX in the quahog *M. mercenaria*, and fungal infections in Sydney rock oysters (Soudant et al. 2013; Allam and Raftos 2015). Diseases characterized by conchiolin formation include Roseovarius or Juvenile Oyster Disease and Brown Ring Disease in *Ruditapes* clams (Allam and Pales Espinosa 2016). On the basis of morphological differences it has been hypothesized that specialized populations of hemocytes may be responsible for encapsulation (Allam and Raftos 2015). In *Ruditapes* clams infected by *P. olseni*, granulocytes secrete (from membrane-bound granules) a polypeptide named p225, which surrounds encapsulated parasites and restricts their proliferation (Montes et al. 2002). Consistent with the importance of hemocytic infiltration in diseases characterized by granuloma-like formations, transcriptomic studies have shown differential expression of genes involved in hemocyte migration, pathogen recognition and binding, and inflammation (McDowell et al. 2014; Allam et al. 2014; Wang et al. 2016a, b).

The process of shell formation aids in encapsulation in the extrapallial cavity, playing an important role in immune defenses by preventing the penetration of pathogens through the mantle of bivalves. The process of shell formation in bivalves involves the secretion of organic molecules by secretory cells in the epithelium of the mantle outer fold, which provide a matrix for the deposition of calcium carbonate in a variety of structures, depending on the bivalve species. Hemocytes also play an important role in shell formation. A population of granulocytes containing calcium carbonate stored in granules migrate into the extrapallial space upon shell injury, forming aggregates at the biomineralization edge, which are incorporated into the shell as it forms (Mount et al. 2004; Zhang et al. 2012a; Li et al. 2016a). The fact that about 45% of the domains identified in the shell proteome of bivalves are related to immune function indicate the importance of the shell in bivalve immune defenses (Arivalagan et al. 2017). Among the organic compounds (1–5% of the total shell) that are embedded in the calcium carbonate structure that makes the shell, many immune-related molecules are worthy of mention, including PRRs such as galectins, scavenger receptors and C1q-related proteins, and effectors such as phenoloxidases, proteases, and protease inhibitors (Zhang et al. 2012a; Arivalagan et al. 2017; Calvo-Iglesias et al. 2017). Moreover, genes coding for the shell proteins are differentially expressed in oysters challenged with *A. crassostreae* and in Manila clams infected with *V. tapetis*. These two bacterial pathogens preferentially attach to the inner side of the shell in bivalves, and the diseases they cause are characterized by the formation of conchiolin (McDowell et al. 2014; Allam et al. 2014).

Apoptosis and Autophagy

The Profound Implications of Apoptosis in Bivalve Physiology and Pathology

Apoptosis, a form of programmed cell death, is a highly evolutionarily conserved process involving two major distinct but converging pathways, the death-receptor-mediated pathway (an extrinsic pathway) and the mitochondrial pathway (an intrinsic pathway). Apoptosis plays an important role in immune responses by preventing the proliferation of intracellular pathogens, limiting inflammation, and being involved in the activation of certain immune cells, such as neutrophils in vertebrates (Poon et al. 2014; Creagh 2014). On the basis of changes in apoptosis levels in response to a variety of environmental stimuli, apoptosis is thought to play key physiological roles in molluscs, such as maintenance of tissue homeostasis; processing and clearing of environmental pollutants; combating of bacterial, viral, and protistan pathogens; and adjustment to exposure to insecticides, herbicides, and pharmaceuticals (Kiss 2010; Moreau et al. 2015; Romero et al. 2015; Carella et al. 2015; Zhang et al. 2016a). The functional relevance of apoptosis modulation by pathogens and environmental stressors in bivalves, however, is still unclear, since the effect of challenge/exposure on apoptosis levels is not always consistent (Soudant et al. 2013). For example, exposure to *Perkinsus* spp. modulates apoptosis in oyster and clam hemocytes and tissues, but the nature of the modulation depends on the bivalve species and the stage of infection. Advanced stages of *P. marinus* infection in *C. virginica* are generally characterized by suppression of apoptosis, which is, on the other hand, enhanced at early stages of infection (Sunila and LaBanca 2003; Goedken et al. 2005; Hughes et al. 2010; Wang et al. 2017a). Interestingly, the protozoan parasite of eastern oysters *P. marinus* expresses many antiapoptotic genes in response to exposure to oyster pallial fluid, suggesting that this parasite may be able to regulate apoptosis in the host (Pales Espinosa et al. 2014). Basal rates of apoptosis in oysters also differ between the source of hemocytes, ranging from 5–25% in hemocytes in hemolymph to up to 50% in hemocytes within tissues (Sunila and LaBanca 2003; Goedken et al. 2005; Cherkasov et al. 2007; Sokolova 2009)

Main Molecular Players in the Apoptotic Process

Although the major molecules and pathways of apoptosis appear to be conserved between bivalves and other species on the basis of genomic studies (Fig. 17), only a few of them have been characterized functionally. These include the executioner caspase-3 and caspase-1 (caspase-7-like) from *C. gigas*, which appear to act as intracellular LPS receptors (Xu et al. 2016b; Wang et al. 2017a). Interestingly, bivalves may possess a caspase-independent apoptotic pathway, hypothesized to be involved in apoptosis induced by the protozoan parasite *P. marinus* (Wang et al. 2017a).

Several gene families involved in the apoptotic process have experienced lineage-specific expansions, including tumor necrosis factors (TNF), tumor necrosis factor receptors (TNFRs), caspase 8, inhibitor of apoptosis proteins (IAPs),

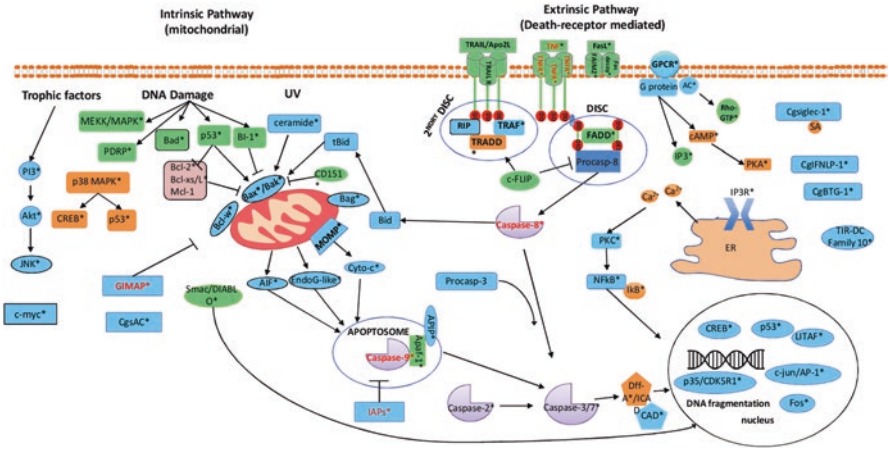


Fig. 17 Apoptosis pathway molecules, with those identified in molluscs indicated with asterisks. Genes identified only in *C. gigas* are prefixed by "Cg" and expanded gene families are shown in red *text*. Molecules that have been only preliminarily identified in molluscs via the eastern oyster genome annotation are denoted with "-like" and genes that been implicated in caspase-independent mechanisms (Kögel and Prehn 2013) are outlined in black

cysteine-aspartic proteases (caspases), and GTPase of the immune-associated proteins (GIMAPs) (Zhang et al. 2012a; Qu et al. 2015b; McDowell et al. 2016; Li et al. 2016b; Wang et al. 2017a). Enhanced genetic diversity of these apoptosis pathway gene families may allow for more diverse but also pathogen-specific functional responses to disease and therefore increase the ability of apoptosis pathways to aid in stress mitigation and increase survival. For example, while oyster *C. hongkongensis* Chcaspase8 is upregulated with bacterial challenge, *C. gigas* Cgcaspase8–2 responds to viral challenge but not bacterial challenge (Wang et al. 2017a).

Two of these gene families, coding for IAPs and GIMAPs (also known in plants as immune-associated nucleotide-binding genes, or IANs), are of particular interest because of their known critical apoptosis regulatory roles in other organisms, their high level of transcript diversity in bivalves, and their demonstrated differential expression in bivalves after immune challenge. The GIMAP/IAN family has 26 annotated members in *C. gigas*, similar to the predicted 26–28 GIMAPs in the eastern oyster, several of which are downregulated in eastern oyster juveniles after challenge with Roseovarius Oyster Disease (ROD), suggesting an upregulation of apoptosis (McDowell et al. 2016). The functional significance of this expansion in bivalves is unknown, but GIMAPs are known to play key roles in regulation of lymphocyte survival, T-cell selection and homeostasis, phagolysosomal processing and membrane trafficking in vertebrates, and pathogen resistance in the model plant system *Arabidopsis* (Weiss et al. 2013; Webb et al. 2016).

The CgIAP family represents another expanded apoptosis-related family in oysters, with 48 gene members, likely the result of tandem gene duplications (Qu et al. 2015b; Zhang et al. 2016a; Wang et al. 2017a). IAP proteins have known roles in apoptosis inhibition by interacting with caspases, and direct evidence of this

interaction has been shown for CgIAP2, where its characteristic BIR2 domain directly interacts with Cgcaspase-2 (Zhang et al. 2011b; Qu et al. 2015b). Bacterial challenges of the Pacific oyster with the bacterial pathogen *V. anguillarum* have shown increased gene expression over time (Zhang et al. 2011b; Qu et al. 2015b). When two families of Pacific oyster with different susceptibility to ostreid herpesvirus-1 (OsHV-1) were exposed to this virus, CgIAP expression was significantly upregulated in both families though with higher levels of expression in the family most sensitive to OsHV-1 (Zhang et al. 2016a). Another gene family with potential roles in apoptosis worth mentioning here is the TIR-DC family 10, characterized by the presence of two baculovirus inhibitor of apoptosis protein repeat (BIR) domains. This gene family has been found only in bivalves (Gerdol et al. 2017).

Potential Involvement of Autophagy in Immune Response

Not much is known about the role of other forms of programmed cell death in innate immune responses in bivalves. Autophagy, which is involved in innate immunity against intracellular pathogens in vertebrates, is induced in oysters in response to bacterial and viral challenge, as well as environmental stimuli such as changes in salinity, hypoxia, toxins, or lack of nutrition (Carella et al. 2015; Wang et al. 2017a). Genes in the autophagy (ATG) pathway have been described in Pacific oysters, and autophagy is involved in survival after challenge with OsHV-1 and *V. aestuarianus*, two pathogens commonly associated with summer mortality in the Pacific oyster, *C. gigas*. Interestingly, while challenge with OsHV-1 led to induction of autophagy, challenge with *V. aestuarianus* resulted in inhibition of autophagy (Moreau et al. 2015).

Overview of the Immune System of Other Molluscan Classes

We have so far outlined the main molecular and cellular components of the immune system of Bivalvia, the second largest molluscan class. Bivalves have been the subject of extensive immunological research over the past few decades, motivated by the high socioeconomic importance of edible species, their widespread distribution, and their amenability for laboratory research. The largest molluscan class in terms of the number of species, gastropods, has attracted considerable attention for similar reasons. These animals—adapted to the freshwater, marine, and terrestrial environments—present astounding morphological diversification, including snails, slugs, limpets, nudibranchs, and others. This diversity can be correlated with the adaptation of lineage-specific strategies for immune defense, which in some cases has led to the acquisition of unique traits and advanced mechanisms, such as the somatic diversification of FREPs. The main features of the gastropod immune system are presented in detail in Chap. 12.

Unfortunately, very little information is available concerning several aspects of the basic biology of the other molluscan classes, such as aplacophorans, monoplacophorans, polyplacophorans, and scaphopods. Consequently, the immune systems of these animals and the possible peculiar survival strategies that might have been

developed in these taxa during their evolution are presently unknown. The few data collected so far concern cellular immunity of chitons, where phagocytic cells located in circulating hemolymph, as well as in connective tissue, seem to bear remarkable immune recognition properties (Crichton et al. 1973; Crichton and Lafferty 1975).

The exception is represented by cephalopods, which have historically attracted major scientific attention, in particular due to their complex nervous system, intelligence, and learning skills. However, immune studies are also emerging, as evidenced by the conspicuous amount of literature produced on this subject over the past few years. The following sections will review the most distinctive peculiarities of the cephalopod immune system of these fascinating animals.

A Short Journey in the Immune System of Cephalopods

Cephalopods (i.e., nautiluses, cuttlefish, squids, and octopuses) comprise over 800 living species (Sweeney and Roper 1998), about 300 belonging to Octopodidae (Jereb et al. 2016) and including several species complexes (Allcock et al. 2011; Amor et al. 2014; Cheng et al. 2014; Sales et al. 2017). They are considered to rival vertebrates (Packard 1972) for physiological adaptations, complex neural organization, and behavior (Jereb and Roper 2005, 2010; Huffard 2013; Jereb et al. 2016; Marini et al. 2017). The immune system of cephalopods consists of innate mechanisms and includes cellular and humoral defenses (Ford 1992; Castillo et al. 2015; Pila et al. 2016).

The Highly Complex Circulatory System of Cephalopods

This molluscan taxon is the sole group of animals, other than vertebrates, to enjoy a fully enclosed high-pressure blood system, an example of convergent evolution. Three hearts (one systemic and two branchial) move blood through an extraordinarily complex network of arteries, veins, and capillaries (Fig. 18), thus representing "a triumph of engineering over design" (Wells and Smith 1987). An overview on the physiology of the circulatory system and its development is available in a number of works (Naef 1928; Boletzky 1968; Wells 1983; Budelmann et al. 1997).

Morphology and Function of Cephalopod Hemocytes

In contrast to bivalves, the circulating blood (hemolymph) in cephalopods turns blue when oxygenated (Wells 1983) because of the presence of hemocyanin. The hemocytes—also named leukocytes (Bolognari 1949, 1951), amoebocytes, or granulocytes (Budelmann et al. 1997)—are the "key" cellular components of the immune system of cephalopods. In an analogy to other molluscs, the identification of cellular types in cephalopods and their characterization is often contradictory, since their classification may be biased by the technique that is utilized (Vieira et al. 2017). Furthermore, the variability in observed cells may reflect the physiological status of the animals (Bolognesi and Fenech 2012; Locatello et al. 2013; Castellanos-Martínez et al. 2014b). Attempts to develop a consensus on the nomenclature of

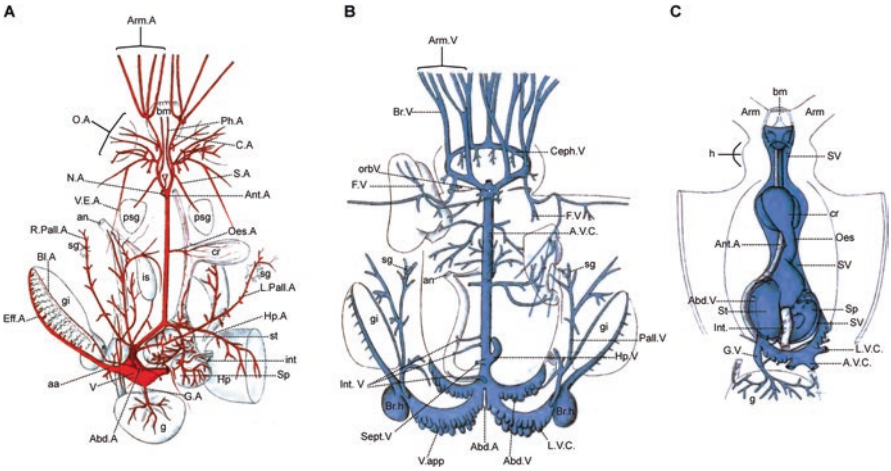


Fig. 18 General outline of the cephalopod circulatory system as exemplified for *Eledone cirrhosa*, modified after Isgrove (1909). In coleoids (cuttlefish, squid, and octopuses), three hearts exist: the systemic heart pumps oxygenated blood (red); the two branchial hearts (Br.h) move blood through the capillaries of the gills (Wells 1983). An extraordinary network of arteries (red, in A), veins, and capillaries exist in cephalopods. The venous system (blue, in B and C) is shown with the principal cephalic vein (Ceph.V), pallial veins (Pall.V), three venae cavae (A.V.C, L.V.C), and a large perivisceral blood sinus (in C). In *Nautilus* the circulatory system (not shown) is characterized by large venous spaces, i.e., the pericardium (Owen 1832), differently from what occurs in coleoids. Abbreviations: general - bm buccal mass, Arm arms, h head, psg posterior salivary gland, an anus, Oes oesophagus, cr crop, is ink sac, sg stellate ganglion, gi gills, st stomach, int intestine, Hp hepatopancreas, Sp spiral caecum, g gonad. Abbreviations: arterial system (A) - Arm.A brachial artery, O.A optic artery, Ph.A pharyngeal artery, C.A cephalic artery, S.A salivary artery, N.A nuchal artery, Ant.A anterior aorta, V.E.A artery to visceral envelope, R.Pall.A right pallial artery, Oes.A oesophagal artery, Bl.A branchial artery, L.Pall.A left pallial artery, Eff.A efferent artery, Hp.A hepatic artery, aa auricle, V ventricle, G.A genital aorta, Abd.A abdominal aorta. Abbreviations: venous system (B, C) - Arm.V brachial veins, Br.V interbrachial vein, Ceph.V cephalic vein, orb.V vein to orbital sinus, F.V infundibular veins, A.V.C anterior vena cava, Pall.V pallial vein, Hp.V hepatic vein, Int.V intestinal veins, Br.h branchial heart, Sept.V septal vein, V. app venous appendage, Abd.A abdominal aorta, Abd.V abdominal vein, L.V.C lateral vena cava, SV venous sinus, GV genital vein

hemocytes have been made for some molluscan species (Cheng 1984) but are still lacking for cephalopods. However, we outline their general description on the basis of the few reports available (Fig. 19).

Budelmann et al. (1997) described two types of cells in cephalopod hemolymph. The first type of hemocytes are round or oval cells, with an elongated V-shaped nucleus, known to extend large pseudopods producing amoeboid locomotion and capable of a phagocytic response and the secretion of pore-forming lysins and cytotoxic oxygen radicals by exocytosis of small granules (Budelmann et al. 1997). The second type include vacuolized round cells, which are relatively sessile (they do not display pseudopods), accumulate into large agglomerates, and are similar in size and shape to hemocytes. Each cell has either numerous small lysosomes or a single

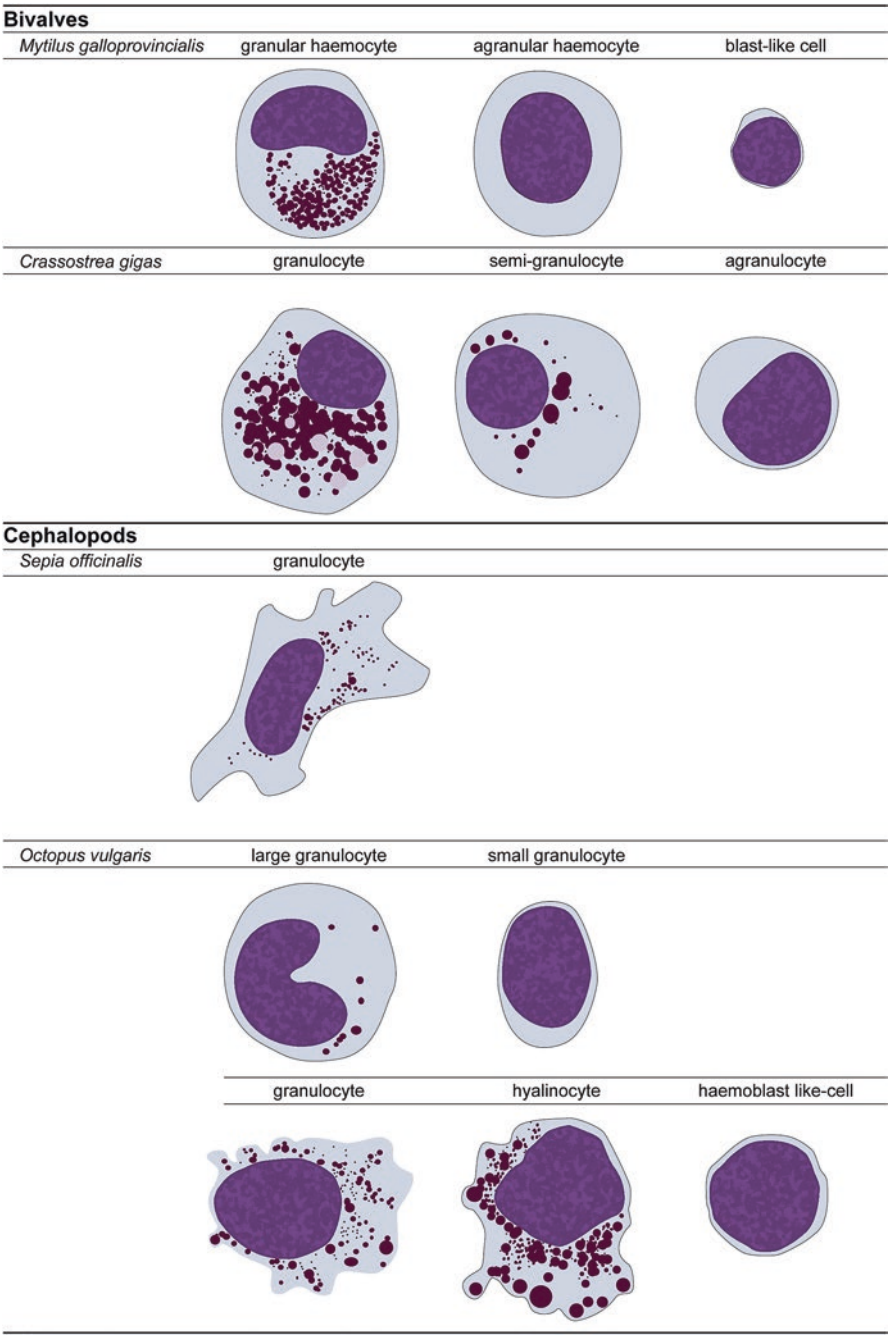


Fig. 19 A schematic overview of the different types of hemocytes identified in cephalopod molluscs, compared with those from some bivalves. The drawings are based on the original descriptions provided for mussels by Bolognesi and Fenech (2012) and by Yang et al. (2015), for oysters by Wang et al. (2017a), and for the cephalopods *Sepia officinalis* and *Octopus vulgaris* by Le Pabic et al. (2014a) and by Castellanos-Martínez et al. (2014b) and Troncone et al. (2015), respectively

large lysosome. They are able to incorporate particles through micropinocytosis. Vacuolized round cells are thought to correspond to the pore cells of other molluscs and to the monocyte–macrophage system of vertebrates (Budelmann et al. 1997).

Troncone and colleagues (2015) recognized three types of hemocytes in *Octopus vulgaris*: hemoblast-like cells, hyalinocytes, and granulocytes. According to those authors, the hemoblast-like cells are the smallest ones, not motile and without pseudopodia. Hyalinocytes are described as variable in size, with a rounded or oval nucleus, and no or few granules and vacuoles of different diameters in the cytoplasm. The cells are capable of amoeboid movement and can form pseudopodia. Granulocytes are variable in size, highly amoeboid, and able to form many long filopodia. Granulocytes are described as being characterized by an eccentric oval nucleus and numerous cytoplasmic granules of different sizes (endoplasm), while no granules are found in the ectoplasm (Troncone et al. 2015).

In coleoids (cuttlefish, squid, and octopuses) the hemocytes originate from the white body (Bolognari 1949, 1951; Cowden 1972; Cowden and Curtis 1973), a multilobed organ covered by a thin layer of connective tissue surrounding, as cushions, the optic lobes and located in the "orbits" in the head of the animal. White bodies extend between the medial external surfaces of the eyes and the skull, and encapsulate the "central brain." The morphology, structure, and function of this organ were originally described by Bolognari (1949, 1951). A pioneering attempt to isolate the cellular components and to estimate their mitotic activity and culturing in vivo was carried out by Necco and Martin (1963). Further characterization of this organ in the octopus was provided by Cowden (1972), including ultrastructural analysis (Cowden and Curtis 1974). A functional description of the white bodies is also available for *S. officinalis* (Claes 1996) and for sepiolids (see below), while no analogous structures are known in *Nautilus*, to the best of our knowledge.

After histological examination, the white bodies appear as a network of connective fibers, blood vessels, and vascular varicosities in which a mass of cellular strings is observed. These are believed to be precursors of the hemocytes (Bolognari 1949, 1951; Cowden 1972). Leukocytes at different stages of "maturity" are identified in the white bodies of *O. vulgaris* (Cowden 1972). According to the classical ultrastructural description, the hemocytoblasts (or reticulum cells of the white bodies) are characterized by an abundant "rough" endoplasmic reticulum, mitochondria, and Golgi, and an irregular large vesicle reported to "contain some internal fibrillar material condensed" in some areas (Cowden and Curtis 1974). These authors also provided a thorough description of other cellular characteristics, and of the transformation of hemocytoblasts to form primary and secondary leukoblasts, and finally mature leukocytes, which in turn possess a folded nucleus containing an abundance of condensed chromatin and dense extrachromosomal aggregates. The cytoplasm contains a number of electron-dense, rounded inclusions, possibly derived from the reduction of vesicles characterizing the hemocytoblasts (Cowden and Curtis 1974).

Two main groups of hemocytes are recognized in cephalopods: cells containing many granules (granular hemocytes or granulocytes), and cells with few or no granules (agranular hemocytes, agranulocytes, or hyalinocytes). These correspond to the two types of cells described by Budelmann et al. (1997).

The octopus hemocytes (sensu lato) act as immunocompetent cells in the hemolymph (Ford 1992). They are involved in the recognition and elimination of potential pathogens through phagocytosis, encapsulation, infiltration, and production of reactive agents with oxidizing capacity (i.e., reactive oxygen species (ROS) and reactive nitrogen species (RNS)). Hemocytes are also involved in scar formation, wound healing, and tissue repair by migrating to the site of injury, increasing in number and activity and forming plugs at the wound site to prevent hemolymph loss (Polglase et al. 1983; Féral 1988; Shaw et al. 2016; Imperadore et al. 2017).

The composition and number of hemocytes are highly variable both among species (Le Pabic et al. 2014a) and between individuals (Malham et al. 1998, 2002; Locatello et al. 2013; Roumbedakis et al. 2017) in an analogy to other molluscs (Anisimova et al. 2017). The number of circulating hemocytes appears variable among different individuals following "stressors" such as handling (Malham et al. 1998, 2002), immune challenge (Locatello et al. 2013), or life stages (Roumbedakis et al. 2017). Phagocytosis is known as the primary immune response of hemocytes and has been reported in various species, e.g., *S. officinalis* (Le Pabic et al. 2014a), *O. vulgaris* (Novoa et al. 2002; Rodríguez-Domínguez et al. 2006), and *Eledone cirrhosa* (Malham et al. 2002).

Molecular Immunology Studies Are Still at Their Embryonal Stage in Cephalopods

The humoral defense is achieved through soluble molecules (Castillo et al. 2015) such as opsonins, agglutinins, proteolytic enzymes, protease inhibitors, antimicrobials or cytotoxic compounds, phenoloxidase, and its intermediate synthesis products, which are in part similar to those described in detail for bivalve molluscs in the previous sections (Rögener et al. 1985; Lacoue-Labarthe et al. 2009; Alpuche et al. 2010; Le Pabic et al. 2014b; Roumbedakis et al. 2017). However, as evidenced by recent transcriptomic approaches, a relevant fraction of lineage-specific genes with unknown function exists in cephalopods. This observation is particularly relevant considering the large number of unknown mRNAs identified in the transcriptomes obtained from *O. vulgaris* hemocytes (Castellanos-Martínez et al. 2014a) and the white bodies of the sepiolid *Euprymna tasmanica* (Salazar et al. 2015).

Different studies have provided a description of putative *Euprymna* immune-related genes, identifying—for example—NF-κB and components of the Toll signaling pathway, pattern recognition proteins, TNF-receptor-associated factors, and proteins denoting membrane attack complex/perforin domains, which in large part mirror those described in bivalves (see sections "Recognition, Agglutination, and Opsonization", "Signaling and Regulatory Pathways", and "Humoral Immune Effectors") (Salazar et al. 2015; Goodson et al. 2005; Troll et al. 2009, 2010).

Although the cellular and "humoral" components of cephalopods have been studied extensively (Castillo et al. 2015), our knowledge of cephalopod immunity is still in its infancy. In brief, evidence exists for (1) a possible role of the white bodies as a hematopoietic and immune organ, and (2) the presence of different types and numbers of circulating cells after challenges. Molecular fingerprints for the immune response have been so far explored only in a limited way (Collins et al. 2012b;

Castellanos-Martínez et al. 2014a; Salazar et al. 2015). Preliminary evidence collected over the past few years suggests that cephalopod immunity, like that of other molluscs (see Chap. 12, section "Molluscs Exhibit Immune Priming with Intermediate Degrees of Specificity, and Involving a Plethora of Mechanisms" for a detailed discussion), may show some form of memory. The analysis of the plasticity of innate immune responses in these fascinating organisms is one of the most important future avenues for cephalopod science and, in particular, for immunological studies.

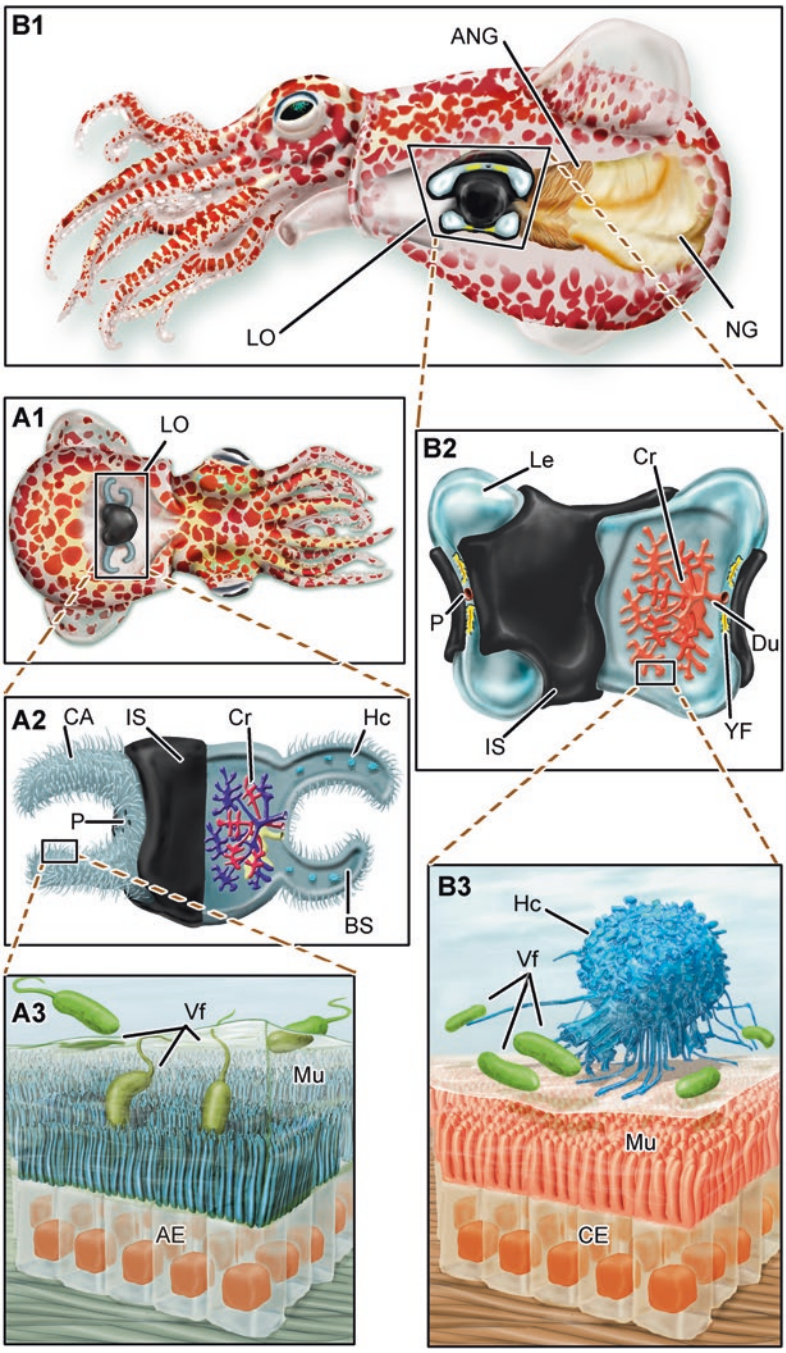
Bobtail Squid as a Model for the Study of Bacterial Symbiosis

The capacity of an animal's immune system to recognize and remove nonself is crucial for its survival and, by tradition, this has been the context in which we have defined immune components, and even how we have designed experiments to understand their roles. This is easy to envision when one considers the detrimental presence of microorganisms to the host, either because of nutrient competition or tissue damage. This kind of association is, by definition, usually considered pathogenic, but this is just one of the three types of symbiotic relationships an animal can establish with another species. The other two types are commensalism (where one species benefits and the other neither benefits nor gets harmed) and mutualism (a type of beneficial relationship between two species, in which both obtain some type of benefit). An animal can establish any one of these associations with the immense variety of microorganisms that share its ecological niche, i.e., bacteria, protozoans, helminths, fungi, or viruses. This section focuses on the major findings resulting from 30 years of study of one of these beneficial interactions, the *Euprymna scolopes–Vibrio fischeri* symbiosis. This model has somewhat challenged our vision on the role of the immune system in metazoans.

The squid–*Vibrio* symbiosis is one of the most studied and better understood binomial associations between an animal and its bacterial symbionts (McFall-Ngai 2008; Castillo et al. 2015; McAnulty and Nyholm 2017; Stabb and Visick 2013; Norsworthy and Visick 2015; Mandel and Dunn 2016). In addition, modern sequencing and proteomic technologies have recently allowed the identification of several molecular players participating in the squid's immune system (Chun et al. 2006; Wier et al. 2010; Collins et al. 2012a, b; Kremer et al. 2013; Salazar et al. 2015). The next paragraphs contain a brief description of this symbiosis, followed by specific information on the molecular players involved, with emphasis on the squid host immune components.

Main Features of the Squid–Vibrio Symbiosis

This mutualistic symbiosis involves the squid *E. scolopes*, also known as the bobtail squid, a relatively small (adult mantle length ~3–4 cm), nocturnal sepiolid species, native to the Hawaiian archipelago (Berry 1912) (Fig. 20, panel b1). The symbionts are Gram-negative marine Proteobacteria members of the Vibrionaceae family, capable of producing bioluminescence by means of luciferase activity under quorum-sensing conditions. The bacteria reside in the squid in a specialized bilobed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structure called the light organ (LO) (McFall-Ngai and Montgomery 1990). The LO is localized on the ventral side of the animal and inside the muscular mantle, just above the funnel or siphon (Fig. 20, panels a1-2, b1-2). In this location, the LO is flushed with ocean water during regular breathing or swimming movements of the mantle. Microorganisms present in the water, including *V. fischeri*, come in direct contact with the LO surface which, in response to bacterial compounds such as lipopolysaccharide (LPS) and peptidoglycan (PG), secretes mucus to which bacteria attach and start aggregating (Nyholm et al. 2000; Foster et al. 2000) (Fig. 20, panel a3). Several studies have found that the mucus contains chemoattractants (N-acetylgalactosamine and N-acetylneurominic acid) (Altura et al. 2011; Mandel et al. 2012), as well as soluble antimicrobials and nitric oxide (Davidson et al. 2004; Kremer et al. 2013). Together, these host-derived products are thought to favor *V. fischeri* attachment while discouraging nonsymbiont organisms from collecting at the site. In addition, the LO of juvenile *E. scolopes* is characterized by having on either side a pair of appendages made from densely ciliated epithelial cells where the mucus is held (Fig. 20, panel b2). The beating cilia help to move aggregated bacteria and particles toward the three open pores that serve as the entrance to the internal part of the LO (Nyholm et al. 2000). As *V. fischeri* cells enter the LO through a pore, they encounter a narrow, ciliated duct that eventually opens into a series of branched and closed-ended spaces known as crypts. Here, the bacteria reach their final place of residence. The lumen of the crypts is covered by epithelial cells with multiple microvilli that secrete mucus and other host molecules, and that, once the squid is colonized, will be in close contact with the bacterial symbionts. Not many *V. fischeri* cells are necessary to seed the LO, as it has been estimated that as few as 3–6 cells can start the colonization of each lobe of this organ (Wollenberg and Ruby 2009). If the bacteria colonizing the LO are capable of producing light, about 12 h after their arrival in the crypts, the combination of light and microbial products is recognized by the host and a developmental signal for a series of programmed morphological changes is initiated. This program includes the following events: (1) apoptosis of the ciliated appendages; (2) fusion of the three pores and ducts into a single one; and (3) an increase in microvilli and swelling of the crypt epithelia (McFall-Ngai and Ruby 1991; Nyholm and McFall-Ngai 2004). The overall result is irreversible loss of the lateral appendages from the LO surface and physiological changes in internal structures over the next 4 weeks that will ensure the maintenance of the newly acquired symbionts (Koch et al. 2014) (Fig. 20, panel b2).

Fig. 20 *Euprymna scolopes* squid and tissues associated with bacterial symbiosis. (**a1**) Juvenile *E. scolopes* squid ventral view. (**a2**) Juvenile light organ with crypts and ciliated appendages. (**a3**) Host–symbiont interaction zone in juvenile squid, consisting of the surface of epithelial cells on the ciliated appendages. (**b1**) Adult female *E. scolopes* squid side view; the transparent window allows us to see the light organ and accessory nidamental gland locations. (**b2**) Adult light organ with crypts. (**b3**) Host–symbiont interaction zone in adult squid consisting of crypt epithelial cells with microvilli and migrating hemocytes. AE appendage epithelia, ANG accessory nidamental gland, BS blood sinus, CA ciliated appendages, CE crypt epithelia, Cr crypts, Du duct, Hc hemocyte, IS ink sac, Le lens, LO light organ, Mu mucus, NA nidamental gland, P pore, Vf Vibrio fischeri bacteria, YF yellow filters

Once this association between *E. scolopes* juvenile squid and bacteria is established, the symbiosis will be maintained for the duration of the animal's life (Nyholm and McFall-Ngai 2004). An important characteristic of this symbiosis is the diel rhythm, which consists, among other things, of daily expulsion of the majority (90–95%) of the bacterial population from the LO at dawn (Lee and Ruby 1994; Boettcher et al. 1996; Nyholm and McFall-Ngai 1998). This thick exudate contains live and dead *V. fischeri* cells and also some host hemocytes and epithelial cells (Graf and Ruby 1998; Nyholm and McFall-Ngai 1998). In the 8 h following the emptying of the LO, the remaining population of symbionts quickly grows and divides inside the crypts, until they reach a density high enough to enable quorum sensing, thereby becoming luminescent again at night (Nyholm and McFall-Ngai 1998). It is suggested that the squid uses this light to camouflage itself from potential predators and preys. This is suggested by the presence of several tissues in the LO, including a lens and a reflector, that allow the animal to control the amount of light emitted, with the purpose of replicating down-welling light from the moon and stars. This behavior is known as counterillumination and prevents the production of a shadow during swimming in the water column. (Ruby and McFall-Ngai 1992; Jones and Nishiguchi 2004).

The *Euprymna scolopes–Vibrio fischeri* mutualism offers advantages over other animal model systems for understanding of the physiology and molecular mechanisms of animal–bacterial beneficial associations (Ruby 1999; McFall-Ngai 2008; Lee et al. 2009). This is mainly because this it is a binary association (Ruby and Lee 1998; Mandel 2010), where both organisms can be cultured separately, thereby allowing manipulation of the bacterial introduction, and because the bacterial symbiont is genetically tractable and introductions of mutations and markers are modifications relatively easy to achieve (Ruby 1999; McFall-Ngai 2008; Lee et al. 2009). Moreover, the direct contact and interaction between the two players (host and bacteria) in this symbiosis occur extracellularly, meaning that the bacteria never breach the epithelial integrity of the host tissues. Thus, their interaction occurs via secreted molecules and by means of cell surface molecules both at the level of juvenile squid ciliated appendages (Fig. 20, panel a3) and inside the juvenile and adult LO crypt epithelia (Fig. 20, panel b3).

The Fundamental Role of Hemocytes in the Establishment of Symbiosis

Hemocytes play a major role in the establishment and maintenance of this interaction. As detailed in the previous section, these are motile cells that circulate through the squid vasculature and can reach sites where the bacteria are located, and interact with them. For a review on the role of hemocytes on the squid–*Vibrio* symbiosis, the reader is directed to a recent publication by McAnulty and Nyholm (2017). The squid hemocytes play a pivotal role right from the initial stages of colonization. First, the presence of the symbiont causes the proliferation of hemocytes, the number of which peaks about 36 h postcolonization (Koropatnick et al. 2007). Furthermore, these cells play an active role during the apoptotic regression of the LO epithelia, a behavior that is accredited to the presence of *V. fischeri* products

released in the LO crypts. Specifically, and in response to *V. fischeri* outer membrane vesicles (OMV) (Aschtgen et al. 2016) and PGN-tracheal cytotoxin (TCT) (Koropatnick et al. 2004), squid hemocytes move from the circulation and migrate to the sinus space in the ciliated appendages. This migration is also accompanied by upregulation of transcripts involved in protein degradation, suggesting that these cells are involved in facilitating the apoptosis and restructuring of epithelial cells during the LO metamorphosis (Koropatnick et al. 2007). This process is aided by the activity of a matrix metalloproteinase (Koropatnick et al. 2014), as suggested by the upregulation of this enzyme in hemocytes and the LO tissues of symbiotic squids (Chun et al. 2006; Collins et al. 2012b; Schleicher et al. 2014).

In vitro studies have also shown that *E. scolopes* hemocytes can selectively recognize, bind, and engulf bacteria, while showing a degree of tolerance of *V. fischeri* in comparison with other marine bacteria (Nyholm and McFall-Ngai 1998; Nyholm et al. 2009). This recognition is modulated by unknown factors secreted by the symbionts (Nyholm et al. 2009). In addition, to discriminate between bacterial species, hemocytes of adult squids also appear to be "trained" to tolerate the symbiont, as hemocytes from antibiotic-treated squids lose their symbiont recognition capacity and bind *V. fischeri* cells more readily (Nyholm et al. 2009).

Several transcriptome and proteomic studies comparing hemocytes from colonized and noncolonized animals have been performed, which enabled the sequence identification of a number of soluble immune factors (Collins et al. 2012b). Among these, a matrix metalloprotein, a cephalotoxin, a galectin, and a soluble peptidoglycan recognition protein (EsPGRP5) were found to be downregulated in cured hemocytes, while EsC3 transcripts could not be detected in symbiotic animals. These results suggested that the presence of the symbiont modulates the host immune system to avoid its removal (Collins et al. 2012b). The complement component C3 and other complement-like molecules—including CD109 antigen (Yazzie et al. 2015), other thioester-containing proteins, and alpha-2-macroglobulin (Collins et al. 2012b; Castillo 2017, personal observations)—have also been identified in hemocytes, but their specific role in symbiosis have not been described yet. Like C3, some of these transcripts appear to be modulated in symbiotic squid compared with those not exposed to bacteria, as was the case for CD109 antigen (Yazzie et al. 2015). Furthermore, several transcripts with homology to known PRRs have been identified in hemocytes, including PGRPs and TLRs (Collins et al. 2012b). Hemocyte–proteomics studies have also revealed at least 37 differentially expressed proteins in the adult symbiotic animals compared with cured squid. Some of these are known to be involved in immune-related functions, most notably cathepsins, lysosomal proteins, and various proteases (see section "Proteases and Protease Inhibitors") (Schleicher et al. 2014). It is also worth noting that—as mentioned in section "A Short Journey in the 'Immune System' of Cephalopods",—like all other cephalopods, squid appear to possess a well conserved immune signaling machinery. It is, however, still unclear how these immune sensors and effector molecules modulate or are modulated by the presence of the bacterial symbiont.

Hemocytes are not only important during the squid colonization process; they are also central to the homeostatic maintenance of the symbiosis. Recent studies

have found that hemocytes have cytoplasmic vesicles that contain chitin (Heath-Heckman and McFall-Ngai 2011). Chitin is an abundant carbohydrate polymer in marine environments and a food source for many planktonic organisms, including bacteria. It has been suggested that hemocytes deliver this nutrient into the LO crypts during the evening and night hours, when the bacteria population is at its higher density, to provide nutrients to the symbionts. In return, the symbionts utilize this resource via fermentation and, as a consequence, acidify the crypt spaces to a pH of about 5.5 (Kremer et al. 2014). Furthermore, hemocyanin, the squid's blood pigment and oxygen carrier (Markl 2013), releases oxygen under acidic conditions. Since bacteria need oxygen to produce light, as in the luciferase reaction, the hemocytes are providing a source of food to the bacteria that will in turn promote the formation of the proper environment for light production, which the host uses for its nocturnal activities (Kremer et al. 2014).

The large number of putative immune molecules identified in the aforementioned sequencing studies confirm the involvement of hemocytes in the host response to *V. fischeri* colonization. It is also interesting to note that multiple genes associated with cytoskeletal and lysosomal activities are modulated, reflecting the developmental and morphological changes the host undergoes in response to its association with its bacterial partner. For more information, the reader is directed to the primary study sources (Goodson et al. 2005; Collins et al. 2012b; Schleicher et al. 2014; Salazar et al. 2015).

The Immune Role of the Light Organ

In addition to hemocytes, other squid tissues express immune-related molecules. Many of these were originally discovered during an extensive analysis of expressed sequence tags (ESTs) from the juvenile LO at different times after colonization (Chun et al. 2006), in the transcriptomes of adult LOs at different times during the diel rhythm (Wier et al. 2010), or in a data set of LO transcripts differentially expressed in animals exposed for 3 h to the symbiont (Kremer et al. 2013). The following paragraphs will describe these molecules and their suggested role in the symbiosis.

Receptors and Sensor Molecules

Several receptors were identified in the juvenile LO, including four PGRPs (PGRP1–4) (Chun et al. 2006), whose general role in invertebrate immunity is summarized in section "Other Membrane-Bound Immune Receptors." PGRP1 was found to be localized in the cytoplasm of surface epithelial cells and translocated to the nucleus, a change associated with the apoptosis of the LO appendages (Troll et al. 2009). PGRP2 was secreted in mucus and found to have PGN-catalytic activity, suggesting an antimicrobial purpose (Troll et al. 2010). Furthermore, PGRP2 was also secreted inside the LO crypts but only after colonization, possibly to aid in removal of PGN products released by the symbionts. Finally, PRGP3 had a glycophosphatidylinositol (GPI)-anchoring site, and PRGP4 was a true transmembrane receptor (McFall-Ngai et al. 2010). Additional PRRs identified in *E. scolopes* are members of the LBP/BPIs family of proteins (see section "Lysozymes, BPIs and Other Pore-Forming

Molecules"). Not much is known about the function of these sensor/effector molecules in squid, other than the fact that a BPI transcript was upregulated during LO apoptosis in symbiotic squid. Because of its localization in the LO crypts, this BPI might play a similar antimicrobial role to the PRGPs (Krasity et al. 2011).

Complement System

As mentioned earlier, bivalve molluscs possess a prototypical complement system (see section "Evidence of an Ancient Complement System in Bivalves?"). Furthermore, C3-like transcripts have been found in squid hemocytes (Collins et al. 2012b; Schleicher et al. 2014). Transcripts for this and other complement-like molecules were first identified in ESTs from juvenile LOs (Castillo et al. 2009; McFall-Ngai et al. 2010). Immunocytochemical analysis detected the expression of C3 in epithelial cells of several tissues of juvenile squid, including the LO, gills, and skin (Castillo et al. 2009). Other complement homologs have also been identified in *E. scolopes* and its sister species *E. tasmanica* (Castillo, 2017, unpublished data), including C1qDC proteins, C1qBP, and an MBL-like transcript (McFall-Ngai et al. 2010). Preliminary data also point toward the presence of several serine proteases with similarity to MASPs and Factor C (Salazar et al. 2016, unpublished data), although biological activity for these and the other complement-like proteins remains to be confirmed. Furthermore, TEPs similar to C3 have been identified in *E. scolopes*. Initially thought to be a representative of the insect TEPs (iTEPs) subgroup, Es-CD109 was found to be expressed in several squid tissues, and its transcript was downregulated in the LO of juveniles harboring *V. fischeri* (Collins et al. 2012b; Yazzie et al. 2015). This suggested that, similarly to C3, this microbial sensor is modulated in order to avoid the removal of symbiont cells (Collins et al. 2012b; Yazzie et al. 2015).

Soluble Effector Molecules

One of the first immune-related molecules identified in *E. scolopes* was a halide peroxidase (Tomarev et al. 1993). This enzyme, localized to vesicles in the epithelial cells, was secreted on the ciliated appendages of symbiotic juveniles, possibly as an antimicrobial factor (Weis et al. 1996). Transcripts of enzymes such as chitinase and lysozyme have also been described as upregulated in the first hours of exposure to *V. fischeri*, suggesting a possible involvement in the symbiont selection process (Kremer et al. 2013). The finding of NOS in the squid LO represented another possible antimicrobial source (Davidson et al. 2004). Immunocytochemical studies found NOS and NO in vesicles localized to the mucus on ciliated epithelial cells, where the bacteria aggregate and symbiont selection starts. In addition, NOS was expressed in the crypt ducts and antechambers (Davidson et al. 2004). Furthermore, it was shown that the presence of the symbiont or its products (LPS and TCT) downregulated the expression of NOS and the production of NO (Davidson et al. 2004; Altura et al. 2011). The authors proposed that in this case, the attenuation of NO production was a response by the host, enacted to modify the crypt environment to ease colonization upon symbiont recognition (Altura et al. 2011).

Although hemocyanin is mainly expressed in gills and the branchial heart, it was also detected in the symbiotic LO crypts, where it was suggested to release oxygen, thereby promoting bacterial growth and bioluminescence (Kremer et al. 2014). Moreover, the detection of a hemocyanin isomer in the mucus secretions of the juvenile LO suggests that this molecule may have a dual role and serve in the symbiont selection process as an antimicrobial agent against nonsymbiotic marine bacteria (Kremer et al. 2014). An additional antimicrobial and bacteriostatic molecule recently reported in *E. scolopes* is galaxin, one of the most highly upregulated transcripts in colonized LOs (Chun et al. 2008; Wier et al. 2010), whose encoded protein is localized to the epithelial cells and mucus secretions of the LO (Heath-Heckman et al. 2014). In vitro assays showed that a peptide fragment of galaxin had inhibitory effects mainly against Gram-positive bacteria, although the growth of *V. fischeri* was also affected (Heath-Heckman et al. 2014). As mentioned earlier, the sensor molecule PGRP2, which binds and degrades bacterial peptidoglycan, is localized to epithelial surfaces exposed to the environment and secreted into the LO mucus, suggesting a role during the initial stages of colonization and selection of the symbiont (Troll et al. 2010). This protein is also detected in the crypt lumen, suggesting that it also assists in modulating host–bacteria interactions once the symbiosis is established (Troll et al. 2010). Another soluble protein with antimicrobial properties found in this squid species is alkaline phosphatase (ALP) (Rader et al. 2012), whose enzymatic activity was upregulated in symbiotic hosts possibly in response to bacterial MAMPs. Indeed, the addition bacterial lipid A and TCT induced the enzymatic activity of ALP, while the addition of an inhibitor reduced bacterial colonization by more than 80%. Overall, it was suggested that ALP has a supporting role in the colonization and maintenance of symbiosis (Rader et al. 2012).

Signaling Molecules

Following the preliminary annotation of the LO-EST database, several molecules pertaining to the canonical TLR signaling (see section "Canonical TLR Signaling") were identified (Goodson et al. 2005). In a related study, three p-63-like (a member of the p-53 family of tumor suppressor proteins) transcripts were identified and localized to the nuclei of LO cells in symbiotic animals, suggesting a role in the apoptosis of appendages (Goodson et al. 2006).

This is a topic that warrants further study, as the capacity of the host to recognize the correct bacterial symbiont from the multitude of bacterial cells in the water may reside in the signaling cascades triggered by *V. fischeri*. One interesting aspect that has been learned since the early studies of this symbiosis is that at first glance, *V. fischeri* bacteria do not seem to contain any evident "symbiont marker" that could help the host to discern the symbionts from other bacteria. Surprisingly, the same molecules present in nonsymbiotic bacteria, including pathogens, are used to communicate with the animal host. These MAMPs, such as LPS and PGN, should be readily recognized by the innate immune system as foreign and as usual elicit a response resulting in microbial removal (see section "Phagocytosis"). Similarly, the host interacts with the symbionts using PRRs and signaling pathways known to be usually activated by pathogens. Nonetheless, there is still the potential of

discovering novel markers on the symbionts and receptors on the host, especially considering the scarce genomic resources currently available and the unknown function of most cephalopod genes (see section "A Short Journey in the 'Immune System' of Cephalopods"). The described studies suggest that attention needs to be paid to the context, timing, and very possibly the effector mechanisms elicited in response to the bacterial signals that can make the difference between removal and accommodation.

Accessory Nidamental Gland

E. scolopes is also used to study another very interesting case of symbiosis, in this case involving a consortium of symbionts that may be acquired in different ways. This particular interaction occurs in the accessory nidamental gland (ANG) (Fig. 20, Panel B1) (Collins et al. 2012a). The ANG is part of the reproductive organs in female squid. This structure is formed by a series of epithelial tubules containing a mixture of bacterial species dominated by Rhodobacteriaceae (Barbieri et al. 2001; Collins and Nyholm 2011; Collins et al. 2012a, 2015). It is thought that some of the components of the ANG bacterial community are added to the jelly coat of eggs during their formation, and that the function of these microorganisms is to protect the developing embryos from environmental infections (Barbieri et al. 1997; Collins et al. 2012a, 2015). In a recent publication, Gromek and colleagues (2016) isolated one of the ANG bacteria (*Leisingera* sp.) from the jelly coat of *E. scolopes* eggs, and in in vitro studies demonstrated that it had antimicrobial activity, producing a pigment that selectively inhibited the growth of several marine bacteria, including *Vibrio* species.

Altogether, the knowledge obtained from the study of these two types of symbiosis has the potential to provide an improved understanding of the complex bacterial associations between animals and microbes. In particular, this might bring new elements to interpret the mechanisms of regulation of bacterial symbiosis in various organs, such as the digestive, respiratory, and urogenital tracts of mammals, further serving as a productive research field for deciphering the multifaceted roles of the immune system in metazoans, which are still not well understood.

Conclusions

The application of -omic tools to the study of bivalve and cephalopod immunology has recently led to exciting discoveries about the extent of the diversity of immune genes in these groups of diverse species. Comparative functional studies using natural and selectively bred disease-resistant strains of bivalves, and in-depth analysis of the powerful model system of the bobtail squid–*Vibrio* symbiosis, as well as the application of gene-editing technologies, have the potential to provide exciting insights into the functional relevance of immune gene family expansion in molluscs and the potential role of this diversity in the specificity and plasticity of immune responses. Owing to the lack of tools and resources, other areas of molluscan immunity have been understudied until now. These include the elucidation of the process

of hematopoiesis, the molecular characterization of hemocyte subpopulations, and a thorough characterization of mechanisms underlying maternal immunity and immune priming.

Molluscan immunobiology is gaining renewed importance from the growing challenges posed by human activities, which have a significant impact in particular on anthropized coastal regions (for a detailed discussion, see Chap. 12, section "Challenges for Molluscs in the Anthropocene Epoch"). This, together with the current trends of global climate change, is currently leading significant shifts in the structure of benthic communities due to the introduction of non-native species, more resistant to the presence of pollutants and therefore outcompeting native species. Continuous research will be certainly needed to improve our knowledge of the immune system of molluscs, both to preserve endangered endemic populations and to face the challenges posed by emerging diseases targeting commercially and ecologically important species (see Chap. 12, section "Molluscan Conservation Immunology" for a detailed discussion on molluscan conservation immunology).

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Molluscan Immunobiology: Challenges in the Anthropocene Epoch

Eric S. Loker and Christopher J. Bayne

Introduction

The diversity encompassed by the phylum Mollusca has much to offer with respect to the study of innate immune systems. The phylum (Fig. 1) includes the largest of all invertebrates and some of the largest of all animals (*Architeuthis*, a giant squid up to 13 m long). The marine bivalve *Kuphus polythalamia*, only recently found living in 1- to 1.5-m calcareous tubes in marine mud, is among the largest of the known living bivalves (Distel et al. 2017). By contrast, the land snail *Acmella nana* packs everything it needs into a 0.7-mm shell. The ocean quahog (*Arctica islandica*) is estimated to live for more than 500 years, the longest known life-span of any noncolonial animal (Butler 2012). Yet the giant squid (*Architeuthis* sp.) and giant octopus (*Enteroctopus dofleini*) are believed to live for only 3–7 years (Cosgrove and McDaniel 2009), and several behaviorally and neurologically complex octopus and squid species routinely live for only a year. Some molluscs such as squids are highly mobile and pelagic, whereas many are benthic and slow moving or even cemented in place and thus completely sedentary. Some are carnivores, some graze on highly complex mixed diets of epiphytes and decaying vegetation, and some are suspension feeders. Gastropods that have adopted parasitic lifestyles (*Entoconcha*) have become so modified anatomically as to be unrecognizable as molluscs. Some molluscs have obligatory associations with specific populations of symbionts that have unique metabolic capabilities (such as cellulolytic bacteria in *Bankia* shipworm bivalves, and chemoautotrophic bacteria in gastropods and bivalves living near deep sea vents or in marine sediments). Saccoglossan sea slugs such as *Elysia*

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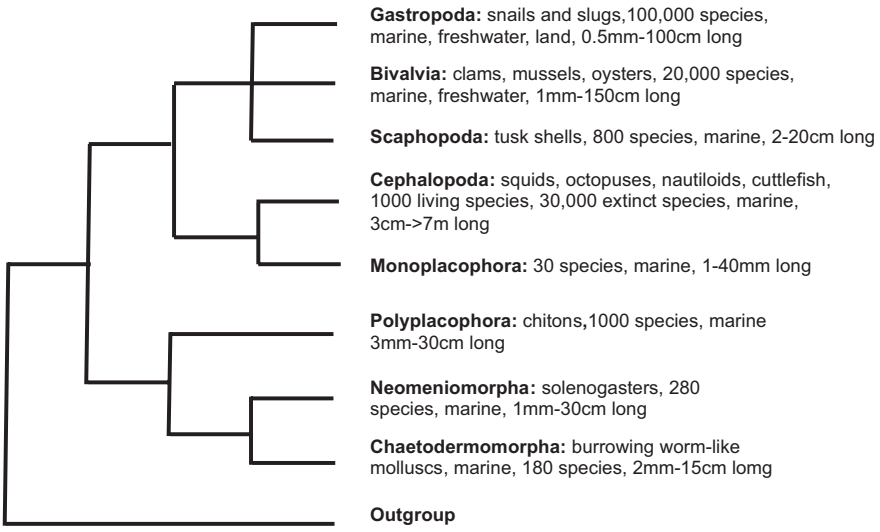

Fig. 1 Within the phylum Mollusca, relationships among the classes remain contentious, with no two among the eight classes consistently being joined by all trees constructed by Haszprunar and Wanninger (2012). The tree shown here is a consensus tree based on three separate studies (Kocot et al. 2011) but is just one of several less than fully resolved hypotheses. The remarks regarding the eight groups of molluscs are from Haszprunar and Wanninger (2012)

chlorotica and bivalves such as *Tridacna maxima* challenge the notion that animals are not photosynthetic. The extent to which particular immunological accommodations must be made to facilitate all of these diverse molluscan lifestyles will be a rich vein for evolutionary immunologists to mine for many years to come.

The development of a valid evolutionary perspective on molluscan immunobiology is served by consideration of the extensive molluscan fossil record (Ponder and Lindberg 2008), which begins in the early Cambrian 541 million years ago, if not even earlier. In addition to dramatically illustrating the abundance, inventive plasticity, diversity, and novelty in the molluscan body plan over time, fossils also provide evidence of endoparasitism in molluscs such as extinct ammonites (DeBaets et al. 2010); encounters with parasites have occurred for hundreds of millions of years. It is also a reminder that whatever extant molluscs are doing immunologically, it has been strongly influenced by prehistoric events and we are looking at numerous robust survivors, such as the gastropods, with as many as 100,000 living species. Although shells dominate the molluscan fossil record, it is worth trying to envision what is not accessible in this record—namely, the soft parts of these animals. Molluscs are distinctive in having soft, moist surfaces directly exposed to the external environment, and it is reasonable to conjecture that a persistent challenge for them has been to evolve means of protecting these surfaces from becoming portals of entry for pathogens—a topic we discuss below.

In this account of molluscan immunobiology, we first provide a brief overview of some of the pathogens and parasites with which molluscs must contend. This serves

to highlight the broad range of immune challenges faced by molluscs and makes the point that many of these parasites are from phylogenetically distinct groups of parasites that do not routinely infect other animals. Next, rather than reporting a class-by-class compendium of all we know regarding molluscan immune systems—with long lists of immune genes, signaling pathways, and the like—we endeavor to elucidate some of the general features emerging about molluscan immune systems and how they operate. It goes without saying that, as pointed out in several recent reviews (Bayne 2009; Loker 2010; Castellanos-Martinez and Gestal 2013; Raftos et al. 2014; Adema and Loker 2015; Buckley and Rast 2015; Castillo et al. 2015; Guo et al. 2015; Coustau et al. 2015; Allam and Raftos 2015; Zhang et al. 2015a, b; Guo and Ford 2016; Allam and Espinosa 2016; Mitta et al. 2017; Pila et al. 2016a, 2017; Schultz and Adema 2017; Portet et al. 2017; Gerdol 2017; Wang et al. 2017a, b), the surge of new information coming from genome projects, transcriptional profiling, and proteomics studies is providing an embarrassment of riches—indeed we are living in the golden age of discovery for comparative immunology!

Although there is much to cheer about with respect to the pace with which new knowledge is being acquired, the world's biodiversity—molluscs by no means excepted—currently faces an unprecedented set of threats in what has come to be known as the Anthropocene epoch. These threats come in many forms: climate change, ocean acidification, aquatic hypoxia, introduction of exotic species, rapid global movements of potential pathogens, pollution from diverse sources, and habitat loss and fragmentation. Such disturbances will influence many aspects of the biology of molluscs, including their production as sources of human food, their roles as vectors of disease, their species ranges, and losses of both abundance and species diversity in the sea, in freshwater, and on land. The immune systems of molluscs will be challenged in many ways by these changes, some no doubt mimicking changes that have occurred in past extinction episodes, but others are distinctive and unique to the Anthropocene. It seems likely that there is considerable potential for environmental changes to cause stress that, when coupled with exposure to even normal pathogen loads, could spell disaster for some molluscan species. We provide some examples of how molluscan immunobiology has a role to play in helping us to understand and hopefully alleviate some of these concerns.

The chapter ends by drawing attention to several points about which we need to know more. These include perspectives on distinctive kinds of studies that are needed and a note of caution to keep an open mind with respect to what comprises "the molluscan immune system." It seems likely that further study may reveal aspects of molluscan immunity that none of us has yet been able to envision.

An Overview of Infectious Agents with Which Molluscs Must Contend

To begin to grasp the scope of molluscan immunobiology, one needs a general appreciation for the range of infectious agents with which molluscs must contend. It is hardly surprising for a group as old as the Mollusca that this list is long and contains some agents that infect only or mostly molluscs, and that

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the agents vary from one molluscan class to the next. It is surprising, though, that for many major groups of infectious agents, our understanding of their diversity (Lohan et al. 2016) and impact on molluscs is still surprisingly meager.

Viruses: Viruses represent a particularly good example of a major group of pathogens whose overall occurrence in molluscs is very poorly known. Although molluscan virology is in its infancy, this is quickly changing with the continued application of next-generation sequencing studies to more molluscs. The best-known molluscan viruses are those infecting abalones and bivalves, in part because of their commercial importance. In bivalves, transmission may be favored by filter-feeding habits and by dense and sometimes suboptimal culturing conditions (Guo and Ford 2016). Also, when die-offs do occur, they attract attention because of the economic consequences (Meyers et al. 2009; Savin et al. 2010). Most prominent among bivalve viruses is the *Ostreid herpesvirus 1* (or oyster herpesvirus, OsHV-1), the first herpesvirus described from an invertebrate, representing a distinct lineage (Malacoherpesviridae) within the Herpesvirales (Farley et al. 1972; Davison et al. 2005). OsHV-1 and its variants (Burioli et al. 2017) can infect several bivalve species (Arzul et al. 2001; Guo and Ford 2016; Ren et al. 2013). Other known bivalve viruses include the gill necrosis virus (GNV), the hemocyte infection virus (HIV) (Comps 1988), and the oyster velar virus (OVV) (Elston and Wilkinson 1985), all of which infect oysters. As these viruses appear to be highly contagious, their geographic ranges are expected to expand.

A viral etiology also is suspected but not proven for two types of neoplastic disease affecting marine bivalves: disseminated neoplasia and gonadal neoplasia (Carballal et al. 2015). The first is characterized by the proliferation of cells probably of hemocyte origin, whereas the latter involves proliferation of undifferentiated germinal cells. Both result in metastasis and often kill the affected bivalve. The involvement of disseminated neoplastic cells in the phenomenon of transmissible tumors in marine bivalves (Metzger et al. 2016) is discussed further below.

Among gastropods, most of our information again concerns commercially valuable species, including the abalone herspesvirus (AbHV-1)—a distant relative of OsHV-1 (Savin et al. 2010; Corbeil et al. 2016)—and abalone shriveling syndrome–associated virus (AbSV) (Zhuang et al. 2010). Several transmission electron microscopy studies of gastropods have reported virus-like particles. Genome sequences have been reported for four picorna-like viruses and one member of the Totiviridae infecting the schistosomiasis vector snails *Biomphalaria glabrata* and *B. pfeifferi* (Adema et al. 2017; Galinier et al. 2017). There are few reports as yet of viruses from cephalopods (Rungger et al. 1971; Hanlon and Forsythe 1990) or the smaller molluscan groups.

As molluscan virology advances, major challenges include documenting that the virus sequences identified and assembled by next-generation

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sequencing are actually bona fide viral sequences and are actually viruses infecting the mollusc (i.e., are not derived from food sources or symbionts), and ascertaining if the identified viruses are in fact pathogenic in the molluscan species in which they occur.

Bacteria: Among the multitudes of bacteria in the environments in which molluscs live, a few species have been identified that seem to be consistently associated with pathology in molluscs. Various *Vibrio* species are often associated with disease in bivalves, gastropods, and cephalopods (Richards et al. 2015). Prominent among these is *Vibrio tapetis*, causing brown ring disease in Manila clams (*Venerupes philippinarum*). The alpha-proteobacterium *Roseovarius crassostreae* causes juvenile oyster disease (or roseovarius oyster disease) in the eastern oyster (*Crassostrea virginica*). An actinomycete bacterium, *Nocardia crassostrea*, causes foci of infection in the tissues of oysters and is responsible for significant mortality in some areas.

Lesions on the skin, mantle, gills, and even hearts of cephalopods have been associated with a variety of bacteria, including *Vibrio alginolyticus*, *Vibrio lentus*, other *Vibrio* species, *Aeromonas*, and *Pseudomonas* species. No repeated and definitive association of cephalopod disease with a single distinctive bacterium has yet been made (Castellanos-Martinez and Gestal 2013).

With respect to gastropods, withering abalone syndrome—a fatal disease of both wild and cultured abalones—is caused by the obligate intracellular Anaplasmataceae rickettsia *Candidatus* Xenohaliotis californiensis, which is found in vacuoles within the cytoplasm of gastrointestinal epithelial cells (Friedman et al. 2000). The presence of a newly discovered bacteriophage (Siphoviridae) infecting this bacterium (Cruz-Flores et al. 2016) indicates that the factors governing pathogenicity of some molluscan disease agents may prove complex. Another gastropod bacterium worthy of note, and quite different from the intracellular organism causing withering disease, is Candidatus Paenibacillus glabratella, reported from *B. glabrata*. This large bacterium grows extracellularly and is capable of forming massive colonies throughout the body of the snail, seemingly without provoking obvious cellular responses yet eventually causing mortality (Duval et al. 2015). It also occurs in the snail's eggs and kills embryos.

Eukaryotes: Parasitic eukaryotes from several different lineages infect molluscs but, as indicated below, representatives of these lineages by no means infect representatives of all molluscan classes. This creates a situation whereby particular groups of molluscs—for example, gastropods, bivalves, and cephalopods—experience quite different arrays of threats from eukaryotic parasites.

Phylum Haplosporidia: Members of this phylum are spore-forming parasites of marine and freshwater invertebrates. About 40 species infect molluscs, with three genera represented: *Haplosporidium*, *Bonamia*, and *Minchinia* (Arzul and Carnegie 2015). Their greatest known impacts are on

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marine bivalves. *Haplosporidium nelsoni* is responsible for MSX, a major disease of *Crassostrea virginica* oysters, in which multicellular plasmodia develop in host tissues. Members of this genus are typically extracellular parasites. Other species of the genus infect bivalves and gastropods, including some freshwater species, but have not been known to cause major die-offs (Vea and Siddall 2011).

The five or six known species of *Bonamia* are all intracellular parasites in the hemocytes of oysters. *B. ostreae* has been implicated in major epizootics in *Ostrea edulis* oysters. They are believed to have direct life cycles and to be fairly host specific because of their intimate intracellular habits (Arzul and Carnegie 2015).

The approximately seven known species of *Minchinia* infect marine clams (*Mercenaria* and *Cyrenoida*), chitons, scaphopods, shipworms, and oysters (Ford et al. 2009). They produce both plasmodia and sporogonic stages in their host tissues but are not associated with heavy mortality in cultured marine bivalves.

Phylum Cercozoa, order Paramyxida: Paramyxeans are parasites of marine invertebrates with development featuring a peculiar "cell-within-cell" arrangement as a result of endogenous budding. At least five species of *Marteilia* are parasites of bivalves (Carrasco et al. 2015). *M. refringens* is associated with mortality of oysters in Europe, and *M. sydneyi* is associated with oyster die-offs (QX disease) in Australia. The complete life cycles are not known and may involve alternative nonmolluscan hosts (Raftos et al. 2014). They mainly infect the host digestive gland or gonadal tissues, where they attract large numbers of hemocytes (Garcia et al. 2009).

Superphylum Heterokonta, family Labyrinthulomycetes, order Thraustochytrida: Within the poorly known osmotrophic Labyrinthulomycetes, thrausochytrids are sometimes implicated as parasites, particularly of molluscs (Raghukumar 2002). Thruastochytrids have been associated with skin and gill infections in cephalopods. *Schizochytrium* can infect nudibranch gastropods (*Tritonia*) and cause large subdermal yellow spots, and *Aplanochytrium haliotidis* can infect and kill abalones (*Haliotis*). An unnamed thraustochytrid is responsible for causing QPX, which can provoke large hemocyte aggregations (Allam and Raftos 2015) and cause mass mortality in quahogs (*Mercenaria*). It is likely a facultative parasite (Guo and Ford 2016).

Phylum Perkinsozoa: Most members of this alveolate phylum are in the genus *Perkinsus*; all seven or eight species infect marine molluscs, mostly bivalves. At least one species (*Perkinsus olseni*) also infects abalones. Two species, *Perkinsus marinus* and *P. olseni*, have severe impacts on bivalve populations, and the World Organization for Animal Health lists them as reportable diseases among aquatic animals (Ramilo et al. 2016). These parasites live intracellularly, including in hemocytes but also in several other tissues. Dermo, the disease caused by *P. marinus*, results from destruction of infected

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host cells. Direct transmission is achieved by flagellated zoospores, which are ingested by a new host.

Phylum Apicomplexa, suborder Eimeriorina: One distinctive lineage of eimeriorine apicomplexans is *Aggregata*, a genus in which at least 12 species are known, all either squids, octopuses, or cuttlefish. *Aggregata* is noteworthy because it infects the epithelial cells of the digestive tract of these cephalopods, undergoing sexual reproduction and spore production there. Spores are passed in the feces and ingested by crustaceans, which serve as intermediate hosts. *Aggregata* infections can lead to ulceration of the gut lining and malabsorption syndrome in the infected cephalopod (Castellanos-Martinez et al. 2014).

Unranked Filozoa clade, unranked Filasteria clade, family Capsasporidae: The one known species in this family, *Capsaspora owczarzaki*, is a symbiont of *B. glabrata* (Hertel et al. 2002). It is a single-celled organism which, as a member of the unranked Filasteria clade, is sister to a group containing the choanoflagellates and the animals, and so it occupies a pivotal phylogenetic position in our understanding of the unicellular origins of animals. It is also of interest because it can attack and kill sporocysts of *S. mansoni* when cocultured in vitro (Owczarzak et al. 1980). While snails injected with live suspensions of *C. owczarzaki* did not appear to suffer, its interactions with the gastropod immune system, if any, are unknown.

Kingdom Animalia, phylum Platyhelminthes, class Trematoda, subclass Digenea (known as digenetic trematodes, digeneans, or flukes): There are 18,000 nominal digenean species (Olson et al. 2003). With the exception of a handful of digeneans that undergo their proliferative larval development in polychaete annelids, the vast majority depend on molluscs to complete larval development. Digenean sporocysts and rediae grow to occupy large volumes of the extracellular tissue spaces of molluscs (Fig. 2) and can persist for a decade or more in long-lived molluscan hosts. The majority of digeneans use gastropod species for their larval development, though some species develop in bivalves and a few also utilize scaphopods. Host specificity is one of the hallmarks of such infections. The close relationship among these three molluscan lineages is evident in the consensus phylogeny shown in Fig. 1. As far as is known, members of the other molluscan classes do not support the proliferative asexual developmental stages of this group.

Digeneans are common and establish intimate infections in their preferred molluscan hosts, which suffer severe fitness consequences as a result of infection: they are typically castrated and have high rates of mortality. The adult stages of digeneans usually occur in vertebrate hosts but are found rarely in invertebrate hosts, including molluscs. Among the digeneans, several species cause significant global health problems for people (schistosomiasis, clonorchiasis), domestic animals (fascioliasis), and wild animals.

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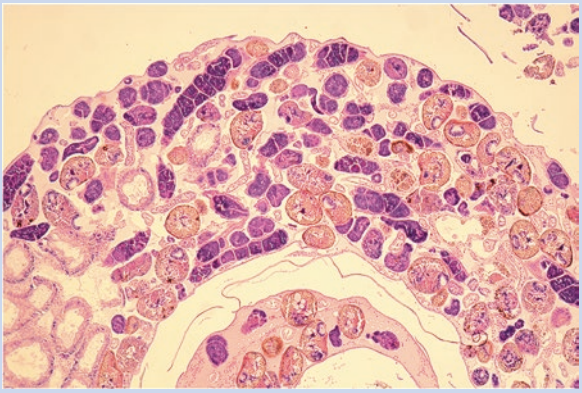

Fig. 2 Rediae (dark blue, oblong structures) and cercariae (clear oval structures) of a digenetic trematode (an amphistome) in the region formerly occupied by the digestive gland and ovotestis of the snail *Biomphalaria pfeifferi*. Note the absence of snail tissue and of any hemocytes either around or on the digenean larvae

Many other animal groups can infect molluscs but have less aggregate impact as pathogens. Included are the dicyemids (phylum Dicyemida), which colonize the renal sacs of benthic cephalopods and are generally not considered to be very pathogenic. Other animal groups such as mites (phylum Arthropoda, class Arachnida) often infect freshwater bivalves and snails, and several nematodes (phylum Nematoda) are known that either infect molluscs as their definitive hosts or encyst as juveniles in molluscs—often terrestrial gastropods—whose consumption by a vertebrate definitive host propagates the parasite.

Adopting a Realistic Perspective in Relation to Evolutionary and Molluscan Immunobiology

Prior to the ascendance of comparative immunology, many immunologists demonstrated an unwarranted degree of veneration of all things lymphoid. To this day, there remains a tendency to view nonlymphoid elements as existing to support the functions of the lymphoid. This "puts the cart before the horse" and skews attempts to reconstruct the evolution of immunity. Contemporaneously with or very soon after the origin of life, innate immune mechanisms arose, of necessity, and have subsequently experienced diversifying evolution. After hundreds of millions of years of animal evolution, lymphoid-mediated immune defenses arose to assist those highly successful ancestral innate immune mechanisms. So, study of the evolution of immune systems is very much the study of innate arms of immune systems. Molluscs have much to contribute here.

Some Salient Principles in Molluscan Immunobiology

In addition to the molluscan shell, the antiparasite and antipathogen defenses of molluscs encompass the mostly ciliated and muscular, mucoepithelial body wall; blood (hemolymph) cells (hemocytes); and plasma proteins. Intracellular mechanisms to ward off viral or genomic parasites are also relevant. Other than the evolutionarily advanced cephalopods, molluscs have circulatory systems that deliver hemolymph via arteries without capillary networks or veins; circulating hemolymph is delivered through arteries to irrigate the internal coelomic spaces, through which it constantly flows. The internal body spaces collectively comprise a hemocoel: a coelom filled with circulatory fluid. Any microbe or other foreign object that breaches the body wall of a mollusc finds no refuge, at least until (if it is small enough) it enters a host cell. Cephalopods are exceptional in that their blood, pumped by three hearts, is retained throughout its circulatory path in a closed vascular system.

Molluscan Immunity Begins at the Mucosal Surface, an Immunologically Active Site that Remains Understudied Allam and Espinosa (2016) note, "Of great concern is the fact that most studies on molluscan immunity focus on circulating hemocytes and the humoral defense factors in the plasma while most relevant host–microbe interactions occur at the mucosal surface." Molluscs, with their soft, moist body surfaces, would seem to be extraordinarily vulnerable to colonization by all kinds of pathogens, the infective stages of which reside in the water or soil that directly contacts the mucosal surface. Yet this surface is surprising resilient and clearly deserves much more attention from the standpoint of being the locus where many would-be infections could be initiated and terminated. Mucus is important for molluscs, in some cases accounting for 15% of their energy expenditure (Davies and Hawkins 1998). For instance, marine bivalves typically remain uninfected when immersed in water containing high concentrations of pathogens, yet injection of small numbers of pathogens will result in infection (Allam et al. 2002). Mucus forms an important barrier of cross-linked glycoproteins that resists easy microbial colonization. It contains antimicrobial peptides, lytic enzymes, and lectins, which can be upregulated following exposure to pathogens (Jing et al. 2011). When mucus-covered epithelia encounter heavy loads of particulates, secretion can increase and ciliated cells can transport the particle-laden sheet away for disposal. What are the stimuli that elicit these processes? Are responses modulated such that they are tailored to the specific provocateur?

In addition to secreting immunoactive molecules into the mucus, epithelial cells lining external body surfaces are phagocytic and can internalize biotic and abiotic particles (McLean 1980). Also associated with epithelial surfaces (including those of the gut) are specialized phagocytic hemocytes, whose surface markers differ from those on circulating hemocytes. These hemocytes can migrate either way across epithelia (Fig. 3); are associated with mucosa covering the mantle, gills, and palps (Yonge 1926; Allam and Espinosa 2016); and are thought to play a sentinel role in bivalve immunity (Allam and Espinosa 2016).

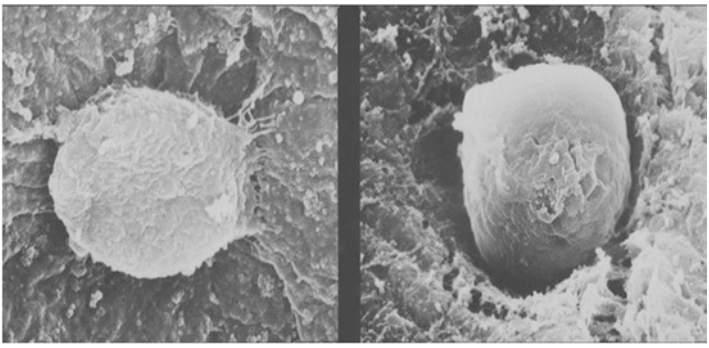

Fig. 3 Diapedesis of hemocytes across external epithelia. Scanning electron micrographs of hemocytes that have migrated to the pallial (external) surface of the mantle of a Manila clam (*Ruditapes philippinarum*). (Images courtesy of Bassem Allam)

The two-way movements of these hemocytes across epithelia suggest ways in which some infections such as *Perkinsus marinus* can be acquired—for example, by ingestion of the parasite followed by migration of the hemocyte into the host bivalve (Vasta et al. 2007; Allam and Espinosa 2016)—or possibly even ways that the disseminated neoplastic hemocyte-like cells in bivalves might exit from an infected host and gain access to the tissues of an uninfected bivalve (see discussion of transmissible neoplasia below). Clearly the soft surface epithelia of molluscs warrant a great deal more careful and extensive study. These surfaces may represent the single most important molluscan immune organ with respect to prevention of infections.

Hemocytes Play a Central Role in Molluscan Immune Responses—Some Basics Regarding Their Morphology and Origins It is notable that even though hemoglobin and hemocyanin occur commonly in molluscan body fluids, few species (some bivalves) confine their respiratory pigments within circulating cells. In by far the majority of molluscs, all hemocytes are leukocytic and these cells are to some degree both amoeboid and phagocytic. Those that respond as defenders of the internal milieu are typically granulocytes. They have roles in phagocytosis, encapsulation, diapedesis, wound healing, shell formation, and the production of soluble immune effector molecules. Facilitated by the animals' open circulatory systems, hemocytes enter and wander through extrahemocoelic spaces, where they can respond within seconds or minutes to exogenous entities such as pathogens.

Although the number of species in which the variety of hemocytes has been described remains tiny, we know enough to state with confidence that the numbers and types of molluscan hemocytes defy succinct description, and the classifications may change as better cell separation techniques are developed (Martins-Souza et al. 2009) and screening of cell populations for specific mRNAs, and/or single-cell transcriptomic studies, are reported. Unlike some of the varied and distinctive hemocyte types found in groups such as echinoderms, crustaceans, and insects, molluscan hemocytes are not filled with large vacuoles or inclusions that contain crystals or

rare metals, and molluscan hemocytes do not obviously degranulate upon contact with objects or surfaces. The numbers of hemocyte morphotypes described in the most discerning studies are three or fewer (reviewed in Pila et al. (2016a)). The two predominant types are hyalinocytes and granulocytes. The term "hyalinocyte" is usually used to describe a nonspreading, spherical cell with a high nucleus to cytoplasm ratio, presenting a refractive "hyaline" appearance under the phase contrast microscope and with a limited capacity to extend pseudopods. Granulocytes may mature from hyalinocytes or be independently derived, and they tend to spread and crawl on surfaces, produce filopodia, are highly phagocytic, and possess obvious cytoplasmic granules. Sometimes, spreading hyalinocytes are also recognized; these are generally less phagocytic than granulocytes and possess fewer granules. In common with phagocytic macrophages, neutrophils, and other innate immune cells in other animals, molluscan hemocytes contain enzyme-rich lysosomes that fuse with phagosomes, synthesize antimicrobial peptides (Mitta et al. 2000), and have the machinery to produce reactive oxygen and nitrogen derivatives (see Adema et al. 1993; Hahn et al. 2001a, b; Pila et al. 2016a; Adema et al. 2017).

It is commonly stated that round cells (hyalinocytes, agranulocytes) are young cells that differentiate into granulocytes as they mature. For example, hemocytes from the oyster *Crassostrea gigas* were recently identified as agranular cells, semigranulocytes, and granulocytes (Wang et al. 2017a, b). Trends toward higher levels of phagocytic activity, enhanced production of reactive oxygen and nitrogen species, expression of immune genes encoding toll-like receptors (TLRs), antimicrobial peptides and lysozyme in granulocytes were noted and offered as evidence that granulocytes were the main immunocompetent cells in *C. gigas* and that a developmental transition occurs from agranulocytes to granulocytes (Wang et al. 2017a, b).

Whereas molluscan hemocyte morphology is in general quite stereotypical, their genesis via hematopoiesis shows striking differences within and among molluscan classes (Pila et al. 2016a). In some cephalopods, a distinct region of specialized tissue (the "white body") surrounding each optic nerve is a source of hemocytes, though it remains to be discovered whether or not blood cells are produced in additional sites in cephalopods (Claes 1996). Mature hemocytes in cephalopods have typical molluscan diversity (Salazar et al. 2015) but may be predominantly macrophage-like (Koropatnick et al. 2007). In some gastropods (planorbid pulmonates), an amoebocyte-producing organ (APO) has been identified (Lie et al. 1975; Jeong et al. 1983) (Fig. 4), but this is by no means a universal attribute of the class. In *Biomphalaria tenagophila*, the APO has been verified by transplant studies as the site of hemocyte origin: recipients of genetically distinct APOs possess hemocytes bearing genetic markers distinctive of the donor (Barbosa et al. 2006). Sullivan and Spence (1994) transplanted the APO of *B. glabrata* snails from a strain resistant to *S. mansoni* into individuals of a strain susceptible to the parasite and showed that the recipients became more resistant, evidencing the importance of this organ in immune defense. The responsiveness of the APO to bacterial PAMPs or schistosome-mimicking fucoidan has also been monitored using a *B. glabrata* microarray; genes responding to these stimuli

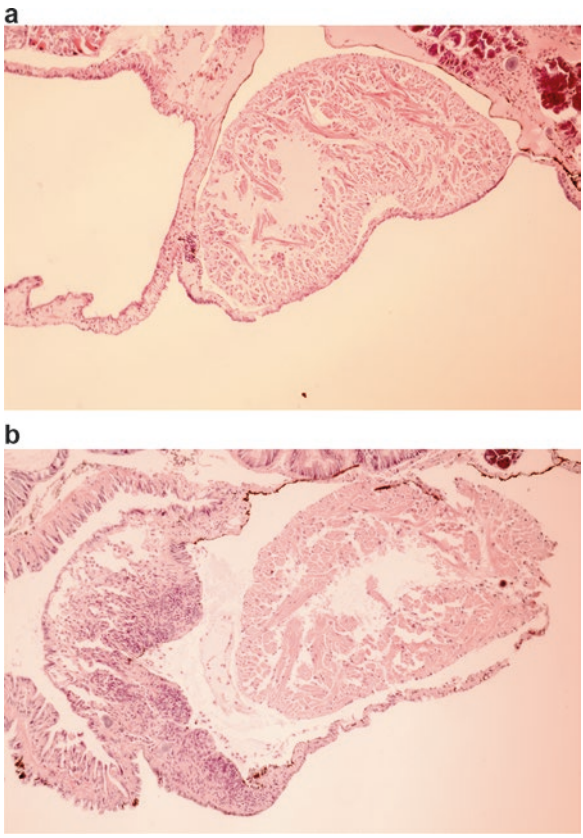

Fig. 4 (**a**) Amoebocyte-producing organ (APO) of an uninfected individual of the snail *Biomphalaria pfeifferi*. Note its thin and deflated appearance. It lies adjacent to the pericardium, which contains the ventricle. (**b**) APO derived from a *B. pfeifferi* infected with the rediae of an amphistome digenetic trematode. Note that the APO is swollen and laden with hemocytes. Hemocytes also can be seen in the nearby pericardium and ventricle

included several involved in regulation of mitosis (checkpoint 1 kinase), protein synthesis, and immune and stress responses, again indicative of an important role of this organ in immune system activation (Zhang et al. 2016).

In their review on hematopoiesis in molluscs, Pila et al. (2016a) reported that some gastropods appear to produce hemocytes in locations near the mantle, and in these species, hemocyte proliferation has been noted on the surface of vessels closely associated with the pericardium. Some gastropods such as *Physa* seem to lack distinctive hematopoietic structures altogether, and proliferation of hemocytes in the peripheral circulation is certainly possible. Hemocyte proliferation can be driven in pulmonates by the snail homolog of granulin (Pila et al. 2016b). Injection of this growth factor into snails induced an expansion of a population of hemocytes that participate in antischistosomal responses, and this was accompanied by an increase in resistance to the parasite.

With respect to bivalves, an important site of hemocyte production in the oyster *C. gigas* is a tissue called the IFS (irregularly folded structures) within the gills (Jemaa et al. 2014; Li et al. 2017). Generally, sites of hematopoiesis in bivalves remain to be discovered, and it may be that in some species, diffusely distributed precursors proliferate in distributed sites in the hemocoel (Tirape et al. 2007).

The numbers of hemocytes in circulation in molluscs are dynamic, increasing and in some cases decreasing in response to stimuli such as stress and infectious agents (Feng et al. 1971; Jeong et al. 1980; Amen et al. 1991; Malham et al. 1998; Renwrantz and Spielvogel 2011) (see also section below on molluscs in the Anthropocene). Surprisingly, hemocyte numbers were unchanged in squid after they encountered symbionts (Koropatnick et al. 2007). The numbers of circulating hemocytes might be influenced both by de novo production of hemocytes and by release of tissue-dwelling hemocytes into the circulation. The extent to which the latter actually occurs remains to be clarified.

It is beguiling to think that the number of hemocytes available to a mollusc might have a major influence on its immune competence, and in at least some instances this does seem to be the case. The number of hemocytes available correlates with the relative insusceptibility of adult versus juvenile *B. glabrata* to infection with *E. paraensei* (DeGaffe and Loker 1998) or with the ability of inbred variants of the 13-16-R1 strain of *B. glabrata* to resist *S. mansoni* (Larson et al. 2014). However, other differences of a qualitative nature clearly exist among hemocytes, whether they result from ontogenetic changes in the mollusc (Larson et al. 2014) or following exposure to infection (Hanington et al. 2010) or specific growth factors such as granulin (Pila et al. 2016b). It has long been suspected that functionally specialized subpopulations of molluscan hemocytes exist, and some evidence now supports that suspicion (Yoshino et al. 2013b; Hanington et al. 2010; Pila et al. 2016c), yet much more work remains to be done to verify and extend these observations.

Some Inherent Attributes of Molluscan Hemocytes One way to gain novel perspectives on hemocyte behavior and function is to examine them under in vitro conditions. It is notable that bivalve and gastropod hemocytes consistently and quickly change from a state in vivo in which they remain freely suspended as individual cells to a state in vitro of "aggregation competence" in which they stick to one another. At least in hemocytes from mussels (Chen and Bayne 1995) and from *B. glabrata* (Fryer and Adema 1993), the change is difficult to block and remains to be explained. A putative dermatopontin has emerged in several relevant transcriptome or proteome studies with *B. glabrata* (Bouchut et al. 2006; Wu et al. 2017); it may well be involved in hemocyte preparation for defensive roles such as encapsulation and phagocytosis. The factors promoting this change are important because they likely play a role in encapsulation responses in vivo. Also, by observing hemocyte behaviors in vitro in the absence of homologous plasma, one can identify functions whose execution does not require plasma components. Such knowledge can then be used to discover synergistic as well as independent roles for humoral factors.

In vitro approaches have shown that molluscan hemocytes are capable of chemotaxis: mussel (*Mytilus edulis*) (Schneeweis and Renwrantz 1993), clam (*Mercenaria mercenaria*) (Fawcett and Tripp 1994), and oyster (*C. virginica*) (Cheng and Howland 1979) hemocytes responded chemotactically to bacterial peptides or LPS, and parasite extracts reduced the motility of *B. glabrata* hemocytes (Lodes and Yoshino 1990). When snail hemocytes were exposed to molecules secreted by

parasites in vitro (Adema et al. 1994), the response was not directional migration but rounding up, lessening attachment to the substrate.

Phagocytosis by hemocytes has been demonstrated by in vivo studies in which opaque particles or bacteria were injected into various molluscs, and has been further studied in vitro (Tripp 1974; Anderson 1977). The topic has been extensively reviewed (Allam and Raftos 2015). One excellent recent example of the importance of phagocytosis in protection is provided by studies of granulocytes of the Sydney rock oyster (*Saccostrea glomerata*), which phagocytoses sporonts of the QX organism (*Marteilia sydneyi*), in the process sequestering them in phagolysosomes, where they are melanized and killed (Butt and Raftos 2008). Furthermore, resistance to QX has been associated with oyster stocks in which a distinct variant of phenoloxidase contributes to higher phenoloxidase activity within oyster hemocytes (Newton et al. 2004). In vivo, other objects and particles are either sequestered in the tissues or, if they have been phagocytosed, may be taken to the body surface (Fig. 2) for disposal (Allam and Espinosa 2016).

With respect to encapsulation responses, both in vivo and in vitro—except in special cases such as compatible parasites (Loker 2010) and certain grafted snail tissues (Sullivan et al. 1995a, b)—hemocytes accumulate on the surfaces of objects larger than themselves and encapsulate them (Fig. 5). Hemocytes are recruited to capsules by cytokines such as macrophage migration inhibitory factor (MIF) (Baeza Garcia et al. 2010) and allograft inflammatory factor (Larson et al. 2014). Multiple hypotheses have been proposed for how encapsulated organisms are killed, ranging from asphyxiation to molecular damage to toxic oxygen/nitrogen products or metabolites (such as superoxide anion or hydrogen peroxide) to physical dismemberment via hemocytes stripping off and then ingesting the thin exterior surfaces of encapsulated parasites (Hahn et al. 2001a, b; Bender et al. 2007; Loker et al. 1982). Biodegradable objects are broken down and the ingestible-sized debris is cleared away by the hemocytes.

One indication of the primacy of hemocytes in molluscan defense responses is provided by a transcriptional study of the response of oysters to *Perkinsus marinus* challenge. Of 107 genes found to be differentially expressed between control and

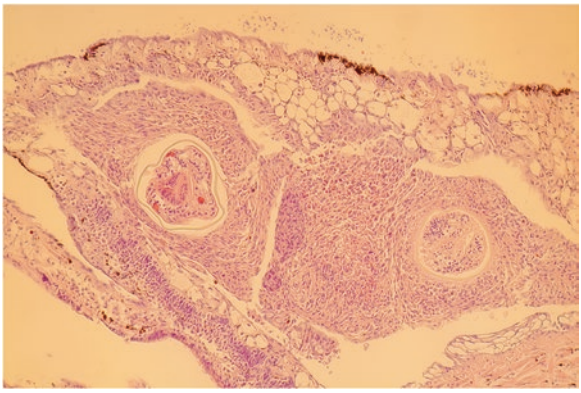

Fig. 5 Two metacercariae of echinostome digenetic trematodes that have been encapsulated by hemocytes in the pericardium of the snail *Biomphalaria pfeifferi*. In this case, the wall of the encysted metacercariae protects them from immediate destruction by hemocytes

parasite-challenged oysters, 70 of them were deemed to be hemocyte specific and another 24 were shared between gill tissues and hemocytes (Tanguy et al. 2004). Similarly, transcriptional profiles change in snail hemocytes following encounters with parasite-derived molecules (Lockyer et al. 2008). In an Illumina-based study of *Octopus vulgaris*, Castellanos-Martinez et al. (2014) found that several immune factors were produced by hemocytes, with TLR2, peptidoglycan recognition proteins (PGRPs), and peroxiredoxin being differentially expressed in animals suffering from *Aggregata* infections. Lists of those proteins whose transcripts increase after pathogen or parasite encounters have been both enticing and fruitful sources of candidates for functional studies (Galinier et al. 2013; Castellanos-Martinez et al. 2014; Schultz and Adema 2017; Buddenborg et al. 2017).

Molluscan Immune Receptors Knowledge of molluscan immune receptors, signal transduction, and regulation of immune-relevant gene transcription has increased rapidly with recent advances in molecular techniques (see reviews by Buckley and Rast 2015; Guo et al. 2015; Schultz and Adema 2017; Pila et al. 2017; Portet et al. 2017). With respect to receptors, candidates among molluscs generally include TLRs, retinoic acid–inducible gene (RIG-1)–like receptors (believed to recognize viral nucleic acids), PGRPs, lectins from at least three different families (C-type, P-type, and S-type), fibrinogen-related domain–containing proteins (FReDs), TNF receptors, C1q domain–containing proteins, and scavenger receptors (Guo et al. 2015). A component of what has been called the Guadeloupe Resistance Complex (Grctm6; a potential cell surface receptor (Allan et al. 2017)) and FREPs (fibrinogen-related proteins, which are FReDs comprised of a combination of one or two N-terminal IgSF domains and a C-terminal fibrinogen domain) appear to be distinctive for gastropods (Schultz and Adema 2017; Pila et al. 2017), as are CREPs (FReDs with an upstream C-type lectin domain and downstream fibrinogen domain) and GREPs (FReDs with an upstream galectin domain and downstream fibrinogen domain). FREPs, CREPs, and GREPs have been collectively termed variable immunoglobulin and lectin domain–containing molecules (VIgLs) (Dheilly et al. 2015). The extent of involvement of most of these molecules in recognition events that lead to effective defense against pathogens is a topic that is now beginning to gain increasing attention, with some progress forthcoming in pinpointing specific candidates (Hanington et al. 2010; Pila et al. 2016b, c, 2017; Portet et al. 2017).

With respect to identification of receptors on hemocyte surfaces, the work of Vasta et al. (1982, 1984) in recognizing the role of hemocyte membrane–bound lectins as receptors for pathogens in oysters represented a significant step forward. Some proteins that have been called receptors in molluscan hemocytes remain candidates, since naming has been based only on inferred structural similarities with other known receptors (Allan et al. 2017; Adema et al. 2017), while evidence of function remains to be documented. Other claimed receptors have been inferred because cells respond to the administration of their ligands (Renwrantz and Richards 1992; Fryer et al. 1989; Hahn et al. 2000; Humphries and Yoshino 2006; Costa et al. 2008) or their normal responses are reduced or blocked by putative ligands (Castillo

and Yoshino 2002). Future proof of receptor presence and function should require direct evidence (protein sequences) of their presence; their capture of the putative ligands; and the consequential transduction of signals via cytoplasmic pathways leading to effector functions, gene transcription, or both. Studies involving short interfering RNAs (siRNAs) to temporarily knock down gene expression have recently implicated hemocyte surface–associated TLRs in resistance of *B. glabrata* to *S. mansoni* (Pila et al. 2016c).

Molluscan Hemocytes Are Conformists in the Sphere of Signal-Transducing Pathways Providing an overview of the signal transduction pathways of molluscan cells in general, and of hemocytes in particular, is a daunting task worthy of its own separate review (see Green et al. 2015; Pila et al. 2016a; Epelboin et al. 2016; Ertl et al. 2016; Gerdol 2017; Huang et al. 2017 for some recent discussions). Approaches using inhibitory drugs showed that the hemocyte respiratory burst could be repressed (Adema et al. 1993) and that molluscan signaling pathways had similarities to those of mammals (Zahoor et al. 2009; Walker et al. 2010). Concerted characterization of the molluscan kinome—the protein kinases that regulate protein phosphorylation and signal transduction—has begun, with early studies identifying roles for protein kinase C in transduction of integrin-related signals or in production of immune effectors such as H_2O_2 (Bender et al. 2005) or superoxide anion (Gorbushin and Iakovleva 2007). MAPK/ERK pathways also seems to be important, as ERK inhibitors impair hemocyte encapsulation and H_2O_2 production by gastropod hemocytes (Zelck et al. 2007) and MAPK-encoding genes have been shown to be upregulated following bacterial infection of scallops (Sun et al. 2016). PIKK kinases (phosphatidylinositol 3-kinase–related kinases) influence the phagocytic activity of *Lymnaea stagnalis* hemocytes (Plows et al. 2006). Epelboin et al. (2016) reported the presence in the *C. gigas* genome of 371 protein kinases and that exposure to stressors such as heavy metals altered expression of 26% of these kinases.

Complete TLR-associated signaling pathways (with most components well conserved with the vertebrate pathway) leading to activation of nuclear factor-KappaB (NF-κB) and subsequent activation of immune genes have been documented in molluscs (Gerdol 2017). Schematic diagrams of still-emerging signaling pathways relevant to innate immunity for *C. gigas* (Guo et al. 2015), *S. glomerata* (Ertl et al. 2016), *Octopus vulgaris* (Castellenos-Martinez et al. 2014), and *B. glabrata* (Adema et al. 2017) have been presented. In light of earlier analyses revealing the origins of these pathways in less derived taxa (Asubel 2005), many of these findings were to be expected.

A particularly active area of investigation is the unraveling of the antivirus responses of molluscs, especially in bivalves, including the associated signaling pathways (Green et al. 2015; Huang et al. 2017). Although the molluscan equivalent of the interferon-like cytokine responsible for initiating an "interferon-like response" in molluscan cells is not known, molluscs do contain TLRs and RIG-like receptors capable of binding foreign nucleic acids, interferon regulatory factors, and

stimulator of interferon (STING), components of a Jak–STAT signaling pathway, as well as downstream ISGs (interferon-stimulated genes), some of which are similar to those produced during vertebrate antiviral responses (Green et al. 2015; Zhang et al. 2012). Oysters can sense virus-associated nucleic acids, mount transcriptional responses, and limit the harmful effects of infection (Green and Montagnani 2013), and oysters with heightened resistance to OsHV-1 infection have been identified (Segarra et al. 2014). Distinctive signaling pathways resulting in either apoptosis or autophagy may also be part of the repertoire of antiviral defenses shown by molluscs (Green et al. 2015), and pathways for both organelle and DNA apoptosis are present in oysters and snails (Adema et al. 2017). Yet another potential antiviral response, largely yet to be evaluated in molluscs, is RNA interference (RNAi). In molluscs it is thus far known to be effected only by microRNAs (miRNAs) which, for example, may influence translation of innate immune genes in scallops (Chen et al. 2014).

Defense-Associated Humoral Components Since the 1960s it has been known that snail plasmas (cell-free hemolymph) can agglutinate or lyse vertebrate erythrocytes (Boyd et al. 1966; Michelson and Dubois 1977), suggesting a role for humoral components in recognition of foreignness and defense in molluscs. Our knowledge of these humoral factors has grown exponentially since then, propelled by the early realization that carbohydrate-binding proteins, or lectins, were included among these humoral factors (Miller et al. 1982; Olafsen et al. 1992; Renwrantz 1983; Vasta et al. 2007, 2015). We now know that multiple families of lectins with diverse carbohydrate recognition domains are present in molluscs (Vasta et al. 2007; Gerdol and Venier 2015), individual lectin families exhibit gene expansion (Zhang et al. 2015a, b; Gerdol 2017; Adema et al. 2017), and individual members of these families respond in distinctive ways to different pathogens (Kang et al. 2006) and may even have site-specific expression, as in mucus or hemolymph (Xing et al. 2011). Heightened lectin activity has been associated with resistance to infection (LaPeyre et al. 1995), lectin activity increases following exposure to infection (Olafsen et al. 1992; Song et al. 2010), and lectin binding has been shown to enhance phagocytosis (opsonization) for some mollusc pathogens (Renwrantz and Stahmer 1983), with ever increasing resolution in our understanding (Tasumi and Vasta 2007; Feng et al. 2013).

Another group of humoral factors receiving considerable recent attention is the FREPs. Since their original discovery in *B. glabrata* (see Zhang et al. (2004) and references therein), the biology of FREPs has been extensively studied, as documented in several recent exhaustive reviews (Gordy et al. 2015; Adema 2015; Adema and Schultz 2017; Pila et al. 2017; Portet et al. 2017). Here we wish only to accentuate the fact that in *B. glabrata*, FREPs are abundant humoral factors with lectin properties that increase in abundance following exposure to digenean infection and can agglutinate soluble digenean antigens (Loker et al. 1994; Adema et al. 1997) and bind to sporocyst surfaces (Wu et al. 2017). They are believed to react with thioester-containing proteins (TEPs) to effectuate binding to digenean

sporocysts, a step believed to facilitate subsequent attack by hemocytes (Mone et al. 2010). Furthermore, sporocysts carry on their surfaces variable polymorphic mucins, which are the targets of FREP binding (Mone et al. 2010) and, as discussed by Portet et al. (2017), the dynamic interplay between FREPs (which are themselves diversified by somatic mutation) and parasite polymorphic mucins is believed to be an important underlying determinant in the compatibility of *S. mansoni* with *B. glabrata*. As noted in the reviews cited above, several other hypotheses exist to explain the underlying mechanism of compatibility (see Coustau et al. 2015; Pila et al. 2017), which deserve full consideration. Also, although the work with FREPs has stimulated a great deal of interest with respect to gastropod–digenean interactions, it leaves largely unresolved the role of the expanded FReD families in other molluscs (Guo et al. 2015; Allam and Raftos 2015), though FReD production is upregulated in scallop hemocytes following exposure to microbial products and recombinant FReD can bind both bacteria and fungi (Yang et al. 2014), suggestive of their role in defense.

Also included as prominent humoral components of molluscan defense are complement-like molecules. Although it seems clear that molluscs do not possess anywhere near the complete set of more than 30 components that participate in classical, lectin-mediated, or alternative pathways of complement activation in vertebrates (Castillo et al. 2015), molecules with homologs to complement components are present, including complement C3 (C3)–like and B factor–like homologs in clams (Prado-Alvarez et al. 2009), a C3-like molecule from *B. glabrata* (Adema et al. 2017), and a C3-like molecule in cephalopods (Castillo et al. 2009), as well as mannan-binding lectin–like and mannose-associated serine protease 1–like transcripts (Castillo et al. 2015) and C3R, C5R, C1S, ficolin, and C1q binding protein (Castellanos-Martinez et al. 2014). Especially noteworthy in bivalves is the large variety of C1q domain variants present (321 from the *C. gigas* genome), which have been shown to have complex patterns of responsiveness following exposure to both abiotic and biotic stresses (Zhang et al. 2012, 2015a, b).

Molluscs also have thioester-containing proteins (TEPs) and, as noted above, cleavage of a TEP following association with FREPs is believed to facilitate binding to schistosome sporocysts. The TEP α_2-macroglobulin antiprotease occurs in snail plasma (Bender, Fryer and Bayne 1992; Fryer, Bender and Bayne 1996), and genes encoding homologs occur in other molluscs. Some of the numerous TEPs encoded in the oyster genome may participate in an inferred complement-like system of opsonization (Wang et al. 2017a, b). With proteolytic enzymes implicated in both pathogenesis and defense, it is probable that humoral antiproteases play an underappreciated role in moderating their actions (see discussion of serine protease inhibitor from resistant oysters below).

As noted above, one of the first immune-related activities noted for molluscan plasma was an ability to lyse vertebrate erythrocytes. Among lytic humoral factors produced in *B. glabrata*, the best characterized is biomphalysin. This β-pore-forming toxin binds to and kills *Schistosoma mansoni* larvae (Galinier et al. 2013). Biomphalysin lacks a lectin-like domain. Mytilectins have both a pore-forming motif similar to biomphalysin and a ricin-like lectin domain, and are suspected of being pattern recognition molecules in mussels (Hasan et al. 2016).

Not to be overlooked are the major hemolymph proteins in molluscs, including hemoglobin, hemocyanin, and uncolored cavortin (in bivalves), which have been largely ignored by immunologists but will likely be found to participate in immunity (Coates and Nairn 2014; Green et al. 2014). Domains in these often enormous proteins include superoxide dismutase and phenoloxidase, neither of which transports oxygen, whereas both use oxygen to produce toxic molecules. In vitro, molluscan plasma can be altered such that it becomes toxic (Bender et al. 2002). This is thought to be a consequence of oxidation (possibly of hemoglobin or hemocyanin), since the toxicity is less if catalase (a scavenger of superoxide) is added or if the plasma is held under hypoxic conditions. Hemocyanins or their derived peptides are known to have antiviral properties in abalones (Zanjani et al. 2014).

One of the most interesting groups of humoral immune factors from a strictly comparative point of view is the antimicrobial peptides (AMPs). That is because—on the basis of what we presently know—they seem to be particularly well represented in bivalves, especially mussels (*Mytilus*), with relatively few representatives by comparison in gastropods (see discussion by Gerdol (2017)). This may in part reflect a lack of concerted looking for AMPs in molluscs other than bivalves, though available gastropod genomes do not nearly reveal the diversity of AMPs found in mussels (Rodriqugez de la Vega and Possani 2005; Adema et al. 2017). Mussels contain a superfamily of cysteine-stabilized alpha-helix beta-sheet (CS-αβ) AMPs, which includes defensins (also present in other bivalves), mytilins, and myticins. These are often found in hemocytes in inactive precursor forms and are believed to function primarily in killing microbes, especially gram-positive bacteria, but possibly with activity against viruses. Other apparently mussel-specific AMPs include mytimycins (possible antifungal activity), myticusins, and myticalins. The linear peptide molluscidin is thus far known only from oysters, whereas other AMP groups such as defensins, big defensins, and macins have been reported from multiple bivalve groups. Interestingly, abalones possess a broader spectrum of AMPs than other gastropods (De Zoysa et al. 2010), with the other gastropods thus far investigated seeming to possess only macins containing eight or ten cysteine residues (see Table 1 in Gerdol (2017)). The genome of *B. glabrata* reveals only a single macin-like family comprising six biomphamacins to be present (Adema et al. 2017). This snail does possess other antimicrobial proteins though, including biomphalysins and LBP/BPIs, which are discussed elsewhere. Macins appear to be broadly distributed in tissues and contribute to the antibacterial defenses of the mucus in *Achatina fulica* (Zhong et al. 2013).

It remains unclear where most of the various plasma proteins are synthesized. Hemocytes are implicated by mRNAs in the synthesis of several defense proteins that may be secreted (Tanguy et al. 2004; Lockyer et al. 2008). In pulmonate gastropods, the albumen gland has been implicated (Vergote et al. 2005; Baron et al. 2016), but as it remains a primordium in immature snails and slugs, such individuals may either lack those proteins or produce them elsewhere. Molluscs possess extensive midgut glands (also called digestive glands or hepatopancreas), and it would be surprising if no plasma proteins are produced there (Zhang et al. 2012; Ittiprasert et al. 2015).

Expansion and Diversification of Innate Immune Gene Families As more invertebrate genomes come to light—those of molluscs included—one of the most interesting features to emerge is the extent to which innate immune gene families that are associated with recognition of antigens or that function as immune effectors have expanded and diversified. In fact, it is not uncommon for innate immune genes to be an order of magnitude more diverse in invertebrates than in vertebrates (Zhang et al. 2015a, b). At the same time, the complexity of innate immune gene families poses many questions that must await definitive answers once better tools become available for clarifying the function of gene products and regulation of their expression. Demonstration of homology with known immune genes or documentation of transcriptional patterns such as upregulation following exposure to particular pathogens or stressors are not sufficient to provide the full functional picture.

On the basis of what has been revealed thus far for molluscs, especially on the basis of the bivalve *Crassostrea gigas* and the gastropods *Biomphalaria glabrata* and *Lottia gigantea* (Table 1), the following points emerge:

1. There has been extensive gene expansion within immune gene families, aided by the tandem arrangement of immune gene clusters. Studies of the oyster genome suggest that duplications in stress-related gene families are more likely to be retained, likely indicative of their survival value in variable environments (Zhang et al. 2012; Guo et al. 2015).
2. As exemplified by the TLR and FReD families (the latter also including FREPs, GREPs, and CREPs), functional expansion is associated not only with variations in sequence but also with modification of basic domain structures and creative juxtapositions of different domains to generate additional diversity (Zhang et al. 2004, 2012; Guo et al. 2015; Dheilly et al. 2015).
3. The extent of diversification within and among the different innate immune gene families varies remarkably among taxa.
4. There is evidence that within a particular gene family, some members might be responsive to both abiotic stresses (such as temperature or salinity changes) and extrinsic biotic stresses (such as exposure to pathogens), whereas other members of the same gene family might respond only to abiotic or biotic stresses but not both. As emphasized by Guo et al. (2015), and as discussed elsewhere in this chapter, the molluscan responses to abiotic and biotic stressors seem to be intimately intertwined.
5. As noted further below, exposure to different pathogens can provoke qualitatively and quantitatively different responses from the same gene family.

It should be particularly revealing to gain a better understanding of the evolutionary drivers that have favored immune gene family expansions, and to discover why different molluscs vary so much in their respective expansions. For example, between the two best-known molluscan models, the oyster *C. gigas* seems to have more expanded gene families with more representatives within each family than does the

Table 1 To highlight some of the differences in emphasis between one bivalve and two gastropods with respect to selected immune and stress-related gene families, we indicate known or estimated numbers of represented genes for the Pacific oyster (*Crassostrea gigas*), the freshwater snail *Biomphalaria glabrata*, and the owl limpet (*Lottia gigantea*)

Genes	*C. gigas*	*B. glabrata*	*L. gigantea*
Toll-like receptors	83	56	10–20
Myd88	10	1	Small number
C-type lectins	266	3	~100
FReDs	190		~50
FREPs	0	22	0
GREPs		2	
NF-κB	4	2	
TNFs	18	11	
GNBPs	5	1	
PGRPs	9	8	
C1qDC	321	1	~10
C3	1	1	
Factor B	1	0	
Mannose-binding protein	1	0	
HSP70	88	21	
Cytochrome P450s	136	99	
Caspase inhibitors	48	56	17
Antimicrobial peptides	60+	6	

The figures are compiled from associated genome papers and recent reviews (Schmitt et al. 2010; Zhang et al. 2012, 2015a, b; Adema et al. 2017; Gerdol 2017). Blanks indicate unknown to us and presence not yet specifically ruled out

C1qDC globular head C1q domain–containing protein, *C3* complement C3, *FReD* fibrinogen-related domain–containing protein, *FREP* Fibrinogen-related protein (meaning a molecule with one or two IgSF domains and a downstream fibrinogen domain), *GNBP* gram-negative binding protein, *GREP* galectin domain with a downstream fibrinogen domain, *HSP70* heat shock protein 70s, *MBP* mannose-binding protein, Complement Factor B, *MyD88* myeloid differentiation primary response gene 88, *NF-κB* nuclear factor-KappaB, *PGRP* peptidoglycan recognition protein

pulmonate gastropod *B. glabrata* (Zhang et al. 2012; Adema et al. 2017) (Table 1). These two molluscs, of course, differ in many respects, including long separate evolutionary histories, habitats, and feeding modes, and different sets of challenges from pathogens and parasites. Some of the differences in innate immune family representation, such as the more extensive set of heat shock proteins found in oysters than in *B. glabrata*, make sense given the temperature extremes that oysters must normally endure in intertidal and estuarine habitats. But what might explain why oysters have 321 putative genes encoding globular head C1q domain–containing proteins whereas *B. glabrata* has only one? Or, even though both molluscs have FReD families, why oysters have many more separate FReD genes (~190) whereas *B. glabrata* has a smaller number of FREPs (~22), which undergo distinctive diversification processes?

Our attempts to relate the extent of expansion of particular gene families in certain mollusc species with the pathogens those species encounter leave lots of questions unanswered. Consider the FREPs as an example. The case for the need for a diverse FREP response in defense of *B. glabrata* against digeneans (including *Schistosoma mansoni*) has been made (Hanington et al. 2010). Digeneans are especially common in *B. glabrata* and in many other gastropod species, and digeneans typically castrate their hosts and so have severe fitness consequences. This argument is convincing from the standpoint that FREP-enriched responses are mounted following exposure to digeneans (Loker and Hertel 1987), *S. mansoni* is capable of producing very diverse molecules that may need to be countered by the snail host (Mone et al. 2010), and knockdown of some FREPs influences susceptibility to *S. mansoni* (Hanington et al. 2010). However, some particular FREPs are also upregulated upon exposure in snails known to be fully compatible to schistosome infection (Buddenborg et al. 2017), and other gastropods such as *Littorina littorea* that regularly experience digeneane infection produce FREPs but they are not diversified (Gorbushin and Borisova 2015). Interestingly, although the limpet *Lottia gigantea* does have multiple FReD genes, it lacks bona fide FREPs (Gorbushin and Iakovleva 2011; Gorbushin and Borisova 2015). This basal gastropod species is—at least thus far—not known to harbor sporocysts or rediae of any digenean (e.g., Ching et al. (1991)).

In marine bivalves, although both mussels and oysters do become infected with larval digeneans, neither produce bona fide FREPs. In common with many other non-molluscs, they do produce FReDs. Interestingly, the FReDs produced by *Mytilus galloprovincialis* are highly variable within and among individuals (Romero et al. 2011), as are the antimicrobial peptide myticin C (Costa et al. 2009) and C1q-containing proteins (Gestal et al. 2010). Why this should be the case in *Mytilus* but not other bivalve genera is hard to explain. Of course, these bivalves also harbor viruses and very diverse arrays of both prokaryotic and eukaryotic pathogens, and they experience challenging environmental stresses, all of which may favor not only diverse FReDs but also diverse members of other families as well. The specific uses to which all of these different gene family members are put is a story mostly yet to be told.

With respect to innate immune gene family evolution, it is also a mystery why some molluscs have LBP/BPI (lipopolysaccharide binding protein)/bacterial permeability increasing proteins) whereas other invertebrate phyla tend to lack them. Five distinct LBP/BPI genes are present in *B. glabrata*, at least one of which is produced in the albumen gland and is found abundantly in egg masses, where it protects against colonization by oomycetes (Hathaway et al. 2010; Baron et al. 2013). The other four LBP/BPI genes have different structures, properties, expression patterns, and localizations (Baron et al. 2016).

The LBP/BPI genes highlight one of the most fundamental questions emerging from the presence of expanded gene families (including TLRs), and that is: how is their expression orchestrated using an apparently limited set of signaling molecules? With respect to the TLR-associated pathway, although limited isoforms of MyD88 (myeloid differentiation primary response gene 88) appear to be present in

molluscs, at least in bivalves there appear to be multiple cytosolic TIR domain–containing proteins. These possibly provide ways to transduce signals from specific engaged TLRs for distinctive downstream signaling events, thereby potentially allowing tailored responses to occur (Gerdol 2017). Any such explanations have to account for specific activation of particular genes following exposure to particular stressors, including in certain tissues, or at critical developmental stages. This will be a major challenge, going forward, as we seek to understand how complex arrays of invertebrate innate immune genes are deployed and regulated.

Molluscs Recognize, and Can Tailor Responses to, Particular Pathogens If exposed to a particular infectious agent such that an infection is initiated, a mollusc will mount a multifaceted response, which will probably include upregulation of genes associated with both generalized stress responses and genes in several categories associated with innate immunity (Zhang et al. 2015a, b; Buddenborg et al. 2017). Against such a potentially complex background response, is there evidence that molluscs can also generate responses tailored to particular infectious agents? We submit that this is possible, and we point out evidence consistent with this interpretation.

First, as noted in the previous section, genome studies are revealing that the diversity of receptor-encoding genes in invertebrates is surprisingly high. Furthermore, mechanisms to further diversify the spectrum of molluscan immune receptors have been identified. Both of these topics have been extensively discussed (Adema 2015; Zhang et al. 2015a, b). So, the innate immune machinery is not inherently precluded by a limited repertoire of receptor molecules from achieving some specificity in response. It also makes sense that "one size should not necessarily fit all" with respect to immune responses given the diverse array of pathogens and parasites encountered, from viruses to macroscopic metazoans.

Additionally, molluscs can be selected for resistance to particular pathogens; examples are *B. glabrata* resistance to *S. mansoni* and oyster resistance to MSX, juvenile oyster disease, and dermo. These selected animals are, of course, not rendered resistant to all pathogens, so clearly there is some capacity to select for tailored responses. Also, with respect to digeneans and their associations with gastropods, a particular digenean species typically has a limited spectrum of snail taxa it can infect (Adema and Loker 1997, 2015). This spectrum varies in its width, depending on the digenean species, often being a single snail genus, possibly multiple members of a single gastropod family, and occasionally extending to members of multiple gastropod families. *S. mansoni* successfully develops in snails of the planorbid genus *Biomphalaria* but not in the planorbid genus *Bulinus*, and vice versa for the closely related parasite *S. haematobium*. Not all species of *Biomphalaria* are compatible with *S. mansoni*, and the same is true for *S. haematobium* in *Bulinus*. Hence these parasites experience limitations in their colonizing ability among even fairly closely related snails, implying a fine-tuned discriminatory capacity among the snails species involved. We have previously argued that exposure of gastropods to digeneans may be a factor that has driven the specific nature of gastropod innate

immune responses in the first place, and this in turn may also be a factor in the diversification of digeneans (Adema and Loker 2015).

Exposure of molluscs to different infectious agents also provokes distinctive transcriptomic responses. For *B. glabrata*, wounding, gram-positive or gram-negative bacteria, or two different compatible digenean species all provoked different transcriptional responses as measured by a microarray (Adema et al. 2010). An llumina-based study of *B. glabrata* exposed to gram-positive or gram-negative bacteria or to yeast concluded that similar signaling pathways were activated by exposure to the various stimuli, but that different members of various expanded immune gene families were engaged by each type of stimulus (Deleury et al. 2012). Given the apparent conservatism of signaling pathways, how are stimulus-specific responses achieved? As noted in the previous section, particular cytosolic components of signaling pathways may also be diversified, thereby favoring—in ways not fully understood—the transduction of unique signals to activate particular members of diversified gene families.

For bivalves, among the 168 different C1qDC (globular head C1q domain–containing protein) transcripts produced in *Mytilus galloprovincilis*, different patterns of expression were apparent following exposure to gram-positive or gram-negative bacteria (Gestal et al. 2010; Gerdol et al. 2011). Similar results have been seen in other bivalve species exposed to different pathogens (Leite et al. 2013; Allam et al. 2014), suggesting that different groups of bacteria are bound and potentially opsonized by different C1qDC variants (Allam and Raftos 2015). Differential binding of variant forms of hemolymph lectins following exposure to bacterial or *Perkinsus* infections in Manila clams (*Ruditapes philippinarum*) have been reported (Kang et al. 2006). Zhang et al. (2015a, b) observed multiple expanded immune gene families respond in concert to both biotic and abiotic stimuli, but specific stimuli provoke responses of different individual members of such families. The issue of specificity in the immune response is directly germane to understanding of the significance and importance of immune priming as it has been reported in molluscs, discussed in the section to follow.

Although there is growing evidence that defense responses of adult molluscs to particular pathogens involve both generalized and specific components, we must also acknowledge that the diverse immune receptors encoded in the genome could also be selectively deployed in several other meaningful contexts. As noted by Allam and Raftos (2015), specialized phagocytic mucocytes patrolling mucosal surfaces may express different variants of immune receptors, such as a variant C-type lectin (CvML) in *C. virginica*, observed by Xing et al. (2011). Zhang et al. (2015a, b) also noted tissue-specific responses of oysters to various stimuli. Transgenerational provision of maternal immune factors to offspring, as noted for *B. glabrata* (Hathaway et al. 2010; Baron et al. 2013) and other molluscan species (Yue et al. 2013; Wang et al. 2015), is another context in which expression of different subsets of immune receptors might occur. Parenthetically, the extent to which maternal experience influences the expression of particular subsets of immune receptors, how effective they are, and how commonly this happens in molluscs are all attractive

questions. Additionally, pelagic larval stages are likely to experience very different challenges from pathogens than the benthic, sessile adults that produced them, so transcriptomic studies of their responses to pathogens and how they might compare with the responses of settled juveniles or adults are of interest. In trochophore larvae of *C. gigas*, Song et al. (2016) found upregulated levels of pattern recognition receptors such as C-type lectin 3 and TLR4, indicative of involvement of specific immune gene family members in defense during planktonic stages. Finally, like all animals, molluscs are not self-contained, sterile entities but harbor symbiont populations that vary radically in abundance and complexity among species. Accommodation to these symbiont populations represents another fruitful avenue to explore in pursuit of an understanding of the full nature of diversity of molluscan immune receptors and the specificity with which they are deployed. The accommodations of molluscs to their microbiomes is discussed further below.

Immune Priming

The phenomenon of immune priming (Milutinovic and Kurtz 2016) is both interesting and important on account of its potential to protect valuable populations from pathogen-caused epidemics (as in aquaculture) and because of the prominence of both memory and specificity as components of the phenomenon. Immune priming is evidenced when, on account of a prior life experience, an organism displays an improved capacity to survive a pathogenic challenge. Generalized (nonspecific) immune priming can be achieved by dietary intake or injection of *immune potentiators* (Hadden et al. 1979). In species with more highly evolved immune systems, priming can be quite specific or very specific. In such cases, immunity is enhanced by a restricted array of recognition and/or effector molecules or cells. But priming need not be limited to the two extremes—nonspecific or highly specific; it can exist anywhere along a continuum. Deciphering the story of highly specific immune memory was a major reason for interest in lymphoid immunity in earlier days. If less specific immune memory exists, it might be demonstrated by recall of less specific PRR-type recognition receptors (perhaps FREP-like) and cellular effectors. Molluscs, it appears, have the capacity for quite specific immune priming. Such studies are just beginning, and "Mechanistic knowledge (of immune priming and memory) is still not available for most observations . . . in invertebrates" (Milutinovic and Kurtz 2016).

Before envisioning the demise of the last bastion ascribed uniquely to "sophisticated" vertebrate immunity (i.e., specific memory), let us consider the meanings of *specificity* and *memory* in immunological contexts. "*Specificity*" is measured on a sliding scale: *highly specific immune recognition* occurs when

(continued)

rare templates or ligands are engaged by a receptor. In lymphoid immunology, "epitope" refers to such a structure. *Nonspecific immune recognition* occurs when a *common* template or ligand is engaged. Nonspecific recognition systems have the advantages of necessitating less diversification of germline-encoded receptors and of being able to recognize large numbers of organisms that all display the template externally. Milutinovic and Kurtz (2016) adopted the terms "sustained memory" and "recalled memory." *At its most nonspecific, immune memory is likely to be of the sustained type* and manifests itself as a heightened capacity to *immediately* confront a wide variety of potentially infectious agents on occasions of second or later encounters. Of course, memory is not implied unless the elicitors of the primary response have been cleared. *The most specific immune memory is the recall type*. It is understood to be that which occurs in avian and mammalian Ig-based systems and is well illustrated by the restricted immunity that is elicited by a single epitope vaccine. Recall memory could also potentially be nonspecific in its nature.

Specific memory responses are a justification for the commonly used descriptor "sophisticated" when lymphoid immune systems are mentioned. However, this type of memory response is constrained by delays required for expansion and maturation of limited clones of specific memory cells. Nonspecific memory can be characterized as analogous to "persistent anger" or, in the jargon of immunology, sustained cell activation, and persistently elevated humoral defenses (Milutinovic and Kurtz 2016). Specificity has its advantages, but its costs potentially include the inescapable delays that provide pathogens with windows of opportunity for the evolution of rapid growth and achievement of acute, high virulence. The phenotype of a mollusc with specific immune memory should be obvious: on a second or later encounter with a specific pathogen-associated epitope, it will have the capacity to inactivate more individual pathogens and/or to more quickly inactivate the same or similar pathogens. However, it is not obvious where to look for the cellular and molecular basis of even relatively nonspecific memory. The technology is available to find expanding clones of memory cells but, partly because hematopoietic tissues in most molluscs remain mostly shrouded (as discussed earlier), sites of clonal expansion have not been sought. Similarly, mRNAs for relevant genes can be, and have been, quantified before and after provocation (Pinaud et al. 2016), but it remains to be determined if changes seen in transcriptomic studies are persistent or faithfully predict eventual levels of their protein products. In any case, for how long must mRNAs and proteins remain elevated/depressed after stimuli are no longer around in order to be considered part of memory responses? Is the persistence of elevated mRNAs due to their greater longevity or to continuing transcription? Might epigenetic changes brought about as a consequence of first encounters prepare genes for rapid transcription following a repeated encounter? These kinds of questions must be asked also about proteomes.

Molluscs Exhibit Immune Priming with Intermediate Degrees of Specificity and Involving a Plethora of Mechanisms

By careful histological analyses, Lie and Heyneman (1976) discovered that the fates of digeneans in snails could be manipulated by the snails' pre-exposure to the same or related parasites (Lie et al. 1982, 1983). They described both "acquired resistance" like immunity and "interference" like immunosuppression. The former was what we now call immune priming. Both hemocytes and humoral factors were implicated. When digenean larvae entered snails that had been previously sensitized through infections by irradiation-attenuated larvae of the same parasite strain, they encountered a more rapid and more frequently lethal response from qualitatively altered hemocytes and often died. Lie and colleagues used irradiated larvae for sensitization (priming) and delayed the repeated challenges until after the apparent demise of the earlier parasites so as to circumvent the possibility that the killing of larvae in the second or later wave of infection was being effected by more mature parasites of the prior infection. However, lacking the means to do so, Lie and colleagues were unable to completely rule out the possibility that products of the earlier parasites remained in the snails at the time of rechallenge.

Scallops and oysters also demonstrate the capacity for immune priming, manifested in scallops (Cong et al. 2008) as improved survival of primed animals after repeated *Listonella anguillarum* challenge. In oysters, a primary encounter with *Vibrio splendidus* resulted in both quantitative and qualitative enhancements in hemocytes following a second challenge (Zhang et al. 2014). Improved phagocytosis was ascribed to increased expression of oyster integrin and to increased numbers of hemocytes.

The 1976 Lie and Heyneman discovery of "acquired resistance" was confirmed independently 13 years later in a different gastropod–digenean system (Hata and Kojima 1989) yet still remains to be fully explained. Using a DNA microarray, Hanington et al. (2010) monitored the transcriptional responses of *B. glabrata* with acquired resistance to *Echinostoma paraensei* (primed by exposure to irradiated parasites and challenged with normal ones). FREP3 was among the genes whose mRNA levels were strongly elevated in snails that had been sensitized. Observed somatic diversification of FREP3 in hemocytes may be instrumental in enabling "heightened levels of responsiveness" (priming) (Hanington et al. 2010) with some specificity (Lie and Heyneman 1976; Mone et al. 2010).

In another gastropod case (Dubief et al. 2017), abalone populations that either had or had not been subjected to natural selection for resistance to *Vibrio harveyi* were challenged then rechallenged with the same pathogen, with an interval of 4 weeks between the two challenges. Hemocytes taken from these two groups of animals differed in their capacities for immune priming. After the abalones were challenged the second time, those from the naturally more resistant population were less sensitive (as indicated by phagocytosis of latex beads) to a toxin in extracellular products from *V. harveyi* than were hemocytes from the less resistant abalones.

Recently, from the University of Perpigñan, there has emerged a series of studies on the relationships between digeneans and *Biomphalaria glabrata* snails,

including one (Portela et al. 2013) from the results of which its authors inferred that immune priming develops as a result of an initial infection and protects snails from infection by schistosome larvae in a second challenge. Starting at around 7 days after a primary infection, parasites in a second challenge met with less success. Refractoriness [our term] rose to 100% by 10 days (Pinaud et al. 2016) and remained at this level for at least 25 days. The refractoriness could not be elicited by surgical injury or by irradiation-attenuated larvae but was mildly elicited by an injection of parasite extract. As the refractoriness was evident for up to at least 25 days after the primary infections, memory merits evaluation. But addressing these issues requires knowledge of the cellular and molecular basis for the altered susceptibility (Pinaud et al. 2016; Milutinovic and Kurtz 2016).

An alternative explanation for the failure of larvae in a second challenge is that those in the primary infection, developing in the snail host, orchestrate the demise of the later arrivals (Sire et al. 1998; Portela et al. 2013). The explanation favored by Portela et al. (2013)—specific genotype-dependent priming of immunity—may well be valid, but the possibility remains that parasite-on-parasite antagonism affects the fates of larvae in the second wave. Yet several facts are consistent with the notion of an immune basis for the outcome: (1) the degree of resistance to repeated challenges follows a gradient of relatedness; (2) a recognition system (FREPs) (Zhang et al. 2004) is already known to be involved in this host–parasite system; (3) knocking down the mRNAs for FREPs 2, 3, and 4 lowered the levels of acquired resistance (Pinaud et al. 2016); and (4) some "protection" appeared to result from injection of parasite-derived molecules.

If the altered outcomes described are bona fide cases of immune priming, then they are likely due to both improved recognition and enhanced effector pathways. We say this because the degree of success of parasite development correlates faithfully with the degree of relatedness between the parasites of the priming and challenge infections (Portela et al. 2013). The more closely related the challenge parasites were to those of the primary infection, the higher the degree of "protection" observed in the snail. The several reports of schistosome-transmitting snails from the field infected with multiple *S. mansoni* genotypes (Adema and Loker 2015) seemingly provide evidence against meaningful immune priming in this system but may not violate the claim of Portela et al. (2013), because the field parasites are not closely related to one another.

Having observed that once snails are infected with *S. mansoni*, larvae in a subsequent challenge perish quickly, Pinaud et al. (2016) probed this more deeply. Larvae in primary infections met one of two fates: either they elicited no cellular response and went on to develop normally, or they were encapsulated by hemocytes and killed. In secondary infections, all larvae died without eliciting cellular attention. Pinaud et al. (2016) interpreted this as evidence of innate immune memory that was, to some degree, specific and humorally mediated (Portela et al. 2013). Injection into naïve snails of plasma from "primed" snails resulted in a lower prevalence of infection, so the immunity was at least partly, mediated by humoral factors. This could be an indirect effect: such plasma likely contains *provocateurs* of hematopoiesis and granulocyte maturation, such as snail granulin (Pila et al. 2016b). RNA-seq (mRNA)

and proteomic catalogs from infected snails have revealed a diverse array of molecules that could contribute to acquired resistance: mannose receptors, selectins, C-type lectins and FREPs, cytotoxic and antioxidant molecules, and others (Pinaud et al. 2016). These data focus attention on persistent questions for which the answers will be highly informative.

On the basis of the putative identities of defense molecules whose mRNA and protein levels change upon infection, and on the perception that the recognition system of FREPs is relatively but not highly specific, we suggest that any immune memory component of acquired resistance is manifested by increased concentrations of selective plasma recognition factors (e.g., FREPs) as well as by increased numbers of granulocytes, as implicated in two recent studies (Larson et al. 2014; Pila et al. 2016b) and supported by another (Baeza Garcia et al. 2010). Even if the targeting of their aggression is mediated by relatively specific recognition molecules, those immunocyte populations may include elevated numbers of activated "angry" cells (Lie and Heyneman 1976). This is implied by transcriptomic data showing elevated mRNA levels for both humoral and cell proteins such as macrophage mannose receptors, selectins, and C-type lectins (Pinaud et al. 2016). We speculate that parasite-secreted molecules and/or their effects may persist for days or weeks in snails even after the death and elimination of the parasite. It is premature to rule out a role for parasite antagonism as a factor that contributes to the fates of larvae in second and later waves of challenge.

To conclude, immune priming may be partially or even totally responsible for acquired resistance, but it remains possible that parasite-on-parasite antagonism strongly influences the outcomes of repeated infections. Both phenomena likely contribute to the phenotype of failed infections in already parasitized snails. The primed state is achieved undoubtedly by a plethora of cellular and molecular mechanisms.

The facts discussed here, and the questions raised, reflect the considerable complexity of molluscan immune systems. Evidently, immune memory can be achieved through a diversity of mechanisms. Moving forward, it is clear that to fully understand the nature of helpful enhanced secondary responses (or immune priming) in these animals, a number of criteria must be met. The kinds of things that must be quantified include:

- The *scale* of secondary responses relative to primary responses:
  - Neutralization of more entities
  - Greater numbers of recognition receptors, either cellular or humoral, or both
  - Recognition receptors with higher affinities for their ligands
  - Greater numbers of engaged molecular and/or cellular effectors
- The *speed* of response initiation: a shorter latent period

As a final thought here with respect to the effects of an initial exposure to digenean larvae on the fate of larvae from later exposures, digenean-mediated immune interference and the role it plays in facilitation of subsequent infections (Lie 1982; Loker et al. 1992) and direct antagonism (predation) directed by digeneans toward

later-arriving larvae (Soldánová et al. 2012) may assume much greater significance than immune priming in the interactive worlds in which digenean larvae find themselves within their molluscan hosts.

The Immune Systems and The Microbiomes of Molluscs Must Make Accommodations to One Another Like other metazoans (Weiss et al. 2012; Chu and Mazmanian 2013), molluscs engage microbes in complex symbiotic relationships, and these pose fundamental challenges to molluscan immune systems. How are distinctions made between mutualistic gut microbes and harmful microbial pathogens? How can molluscs allow some microbes—perhaps with specialized metabolic capabilities or with unique bioluminescent properties—to proliferate within their tissues when at the same time they clearly possess multilayered defenses to prevent microbial colonization? These are by no means new problems to solve. It seems likely that intimate contact with beneficial symbionts has always been an essential part of developing more complex multicellular body plans, and so it has been part and parcel of development of internal defense systems from the beginning (Chu and Mazmanian 2013).

There are several known instances in which molluscs—whether gastropods such as shipworms (Betcher et al. 2012) or sacoglossan sea slugs (Johnson 2011), bivalves such as *Solemya velum* harboring intracellular chemoautotrophic bacteria (Dmytrenko et al. 2014), or cephalopods with bioluminescent bacteria (McFall-Ngai 2014)—harbor specialized microbial populations on which they are partially or wholly dependent. One model system that has helped break new ground on this front is the squid *Euprymna scolopes* and its interactions with microbes. In one case, a single symbiont species, the gram-negative bacterium *Vibrio fischeri*, colonizes the squid light organ to the exclusion of other microbes and generates a protective, masking bioluminescent response. Additionally, the squid supports a more complex consortium of microbial species that colonize the accessory nidamental gland and are involved in populating the jelly coats of squid eggs, with the effect of protecting the eggs from attack by other microbes (Castillo et al. 2015; McAnulty and Nyholm 2017).

Juvenile squid engage in a remarkable process, involving specialized ciliated structures, in which they selectively harvest bacteria for colonization of their light organs. Just one bacterial species, *V. fischeri*, eventually populates the crypts of the light organ (Nyholm and McFall-Ngai 2004). LPS, a derivative of peptidoglycan, and outer membrane vesicles of *V. fischeri* all enable colonization (Koropatnick et al. 2014; Aschtgen et al. 2016). Once the light organ is colonized, then—in an effect that is apparently mediated systemically and may impact developing hemocytes in the squid's hematopoietic white body organ—hemocytes are then produced that have less ability to bind to *V. fischeri* than other bacterial species, which are bound and engulfed normally (Nyholm et al. 2009). If squid are treated with antibiotics and the founding population of *V. fischeri* is lost, the squid's hemocytes then seem to lose their "training" such that they will regain the ability to bind *V. fischeri* (Nyholm et al. 2009). The concept of training hemocytes to be "tolerant" is a

fascinating one and suggests we have much to learn about key regulatory steps in hemocyte development. It seems that some hemocyte molecules are downregulated to maintain the trained state, but further study is needed to pinpoint the nature of the tolerance-inducing molecules produced by *V. fischeri*, and that of the hemocyte molecules that bind to such factors (McAnulty and Nyholm 2017). The complicity of hemocytes in accommodating *V. fischeri* is all the more remarkable because they are apparently involved in removing the structures used to facilitate colonization of the light organ once that has been successfully achieved (Foster and McFall-Ngai 1998). Also, a proportion of hemocytes are regularly lysed within the light organ to provide chitin, which ultimately serves as a needed nutrient for the bioluminescent response produced by *V. fischeri* (Schwartzman et al. 2015).

The colonization of the accessory nidamental gland of squid is also of general interest for two reasons because it implies that accommodation can involve multiple bacterial species (different from *V. fischeri*) and that it can occur in a sex-specific fashion (only female squid are involved). So, within one host species, both a species-specific and a more generalized accommodation to bacterial symbionts can occur in different organs and for fundamentally different underlying reasons. It is also of interest, and likely a general phenomenon more common than usually realized, that symbionts are enlisted to protect the squid's progeny from microbial attack once in the external environment (Gromek et al. 2016).

Are There "Blind Spots" in Molluscan Immune Systems? That molluscan hemocytes can assume a state of tolerance with respect to the presence of specialized symbionts is reminiscent of another situation in at least some molluscs, and that is the extent to which they are able to tolerate the presence of allografts and even xenografts. In early studies of endocrine control of reproductive maturation in pulmonates, reproductive tract primordia from immature individuals were transplanted into the hemocoels of mature individuals, where they matured (Hunter and Runham 1970). An immunological angle on these results should elicit raised eyebrows, since precedent would lead to the expectation that allografts would be rejected, not matured.

Other gastropods such as *B. glabrata* have been shown to have remarkable tolerance for even xenografts. Heart allografts and xenografts from other *Biomphalaria* species—or even from other closely related genera of planorbid snails such as *Helisoma*, *Planorbula*, or *Planorbarius*—will continue to beat for as long as 6 months when inserted heterotopically into the hemocoel of *B. glabrata* (Sullivan et al. 1993, 1995a, b; Sullivan and Farengo 2002). Hematopoietic tissue from lines of *B. glabrata* that are resistant to *S. mansoni*, when transplanted into individuals that were susceptible to the same parasite, showed that the hemocytes endowed the recipient with resistance (Sullivan et al. 1995a, b). Furthermore, if the entire head–foot of a juvenile *B. glabrata* individual is transplanted into the hemocoel of a larger individual of the same species, the transplant will survive for at least 60 days, even showing signs of motility and feeding motions. Remarkably, no recipient host hemocyte responses to the complex, living transplant were noted. Similar results

were obtained when congeneric xenograft head–foot grafts of *B. obstructa* snails were placed in *B. glabrata* recipients (Sullivan et al. 1999). The authors concluded that some invertebrates (such as molluscs) that do not form colonies, and therefore do not suffer the risks of somatic or germ cell parasitism resulting from fusion with other individuals, have a less developed system of allorecognition. The ability to successfully transplant interneurons from one individual of *Lymnaea stagnalis* to another (Syed et al. 1992) is another indication of limited allorecognition capability. The extent to which this is generalizable across all molluscs remains to be seen.

An Hypothesis to Account for Failure to Reject Grafts: Negative Signaling in Immune Cells Responding to Encounter with "Self" Signals

Naturally, the surprising molluscan grafting experiments call for a mechanistic explanation (Loker and Bayne 2001). In brief, the reasoning goes like this: The interiors of cells, even those first evolved, comprise nurturing environments for any living entities that enter and thus require protection (defense). Within those cells, information-encoding molecules such as RNA and DNA constitute targets whose destruction can facilitate the survival of another living entity. Their protection is essential. The need for defenses, then, is contemporaneous with (or soon follows) the origin of cellular life. Hand in hand with the origins of destructive defenses, survival would have required the means to avoid self-inflicted injuries. In other words, self–nonself discrimination was essential if life was to avoid self-destruction. This necessity is universal; its failure has inescapable, catastrophic consequences. Our challenge is to explain how self-destruction is avoided.

In a word, tolerance—by avoiding aggression toward targets that display one's own self-markers. The identity markings of mating types in protists (Coleman 2000) and the recognition necessitated by self-reaggregation of separated, mixed sponge cells (Haseley et al. 2001) are based on carbohydrate ligands and lectins that recognize them specifically. Additional biological phenomena rely on the same system of recognition. Our notions are that for the carbohydrate motifs that identify molluscan cells, there are cognate receptors on immune cells and that these receptors signal negatively by means of phosphatase activity. This remains to be proven, but candidate receptor proteins with putative phosphatase motifs occur in the recently available *B. glabrata* genome (Kate Buckley, 2016, personal communication). If substantiated, this mechanism of tolerance will provide the basis for understanding the apparent blindness of at least some molluscan immunocytes when nonself is encountered.

The fact that sporocysts of *S. mansoni* display surface glycoconjugates that mimic glycoconjugates found on the surfaces of *B. glabrata* hemocytes (Yoshino et al. 2013b) supports the notion that mimicry of "self" markers provides a way for parasites too to escape detection and so establish and

(continued)

perpetuate an infection. The same parasite may be unable to match hosts with different arrays of self-glycoconjugates such that negative signaling to immune cells may no longer occur or may be less robust. Such parasites will no longer "fly under the radar." This echoes earlier reports from the 1980s (Yoshino and Bayne 1983), including one in which the degree of antigenic similarity between *Oncomelania* snails and strains of *S. japonicum* correlated with the efficiency with which those parasites would infect that host (Iwanaga and Tsuji 1985). In that case, "antigenic similarities" were scored by antibodies in rabbit antisera, implying that rabbits' and snails' perceptions of parasite antigens share some properties.

Another intriguing, recently discovered phenomenon in marine bivalves may represent a different manifestation of an immunological blind spot stemming from a lack of a strong allorecognition capability in molluscs. Although cancer does not typically spread beyond the body of the host in which it develops, recent studies of soft-shell clams (*Mya*) strongly suggest that large numbers of abnormal neoplastic cells not only are common in the circulation of these bivalves but also can be spread horizontally from an infected bivalve to uninfected individuals (Metzger et al. 2015). Further studies have since shown that neoplastic cells can also spread from one infected bivalve species to another (Metzger et al. 2016; Murchison 2016). In the latter study, four previously unidentified transmissible cancers were found: one in mussels (*Mytilus trossulus*), one in golden carpet clams (*Polititapes aureus*), and two in cockles (*Cerastoderma edule*). Genetic analysis of the neoplastic cells from *P. aureus* indicated they were more similar genetically to pullet clams (*Venerupis corrugata*), even though bivalves of the latter species have not been found to harbor neoplasia and may have evolved resistance to it. The high densities of bivalve populations and their filter-feeding habit may facilitate the spread of these cells, though how they enter and how they leave the bivalve tissues are not yet clear. Movement of hemocytes across epithelial membranes is well known among bivalves (Yonge 1926; Allam and Espinosa 2016). It is also not known if these transmissible cells are ancient or of more recent origin, possibly indicative of the involvement of anthropogenic stressors (Metzger et al. 2015).

High copy numbers and extensive expression of the retrotransposon *Steamer* have been associated with disseminated hemoplasia in the soft-shell clam (*Mya areneria*; family Myidae). A survey for the presence of *Steamer* among bivalve specimens from museum collections found numerous variants within and among specimens of the razor clam *Ensis directus* (family Pharidae). *Steamer* was also found in another unrelated bivalve, *Macoma balthica* (family Tellinidae) (Paynter et al. 2017). The authors concluded that the pattern of distribution of *Steamer* was consistent with a possible origin in *E. directus* with more recent horizontal transmission of nearly identical versions of *Steamer* to these other unrelated bivalves, rather than to an ancient origin of the retrotransposon that closely tracked bivalve phylogeny. For example, *Steamer* was found to be absent from close relatives of

M. areneria or *E. directus*. Similar habitat requirements of the three bivalve species involved may predispose them to acquiring infection horizontally.

The work with disseminated neoplasia in bivalves is of fundamental significance to comparative immunology for at least four reasons:

1. It provides additional important insights into cancer, including its potentially infectious nature, already noted in other models such as Tasmanian devil facial tumors but in this case afflicting invertebrates.
2. It forces us to rethink the whole topic of allorecognition and graft rejection in invertebrates. "Natural grafting experiments" mediated by waterborne, invasive cells that challenge the integrity of "self" can be an issue even for noncolonial invertebrates. However, the relative lack of potent allorecognition shown by many molluscs may suggest that this form of invasion has not been a common or widespread threat among molluscs.
3. Whereas histoincompatibility in vertebrates seems to be a secondary consequence of the role of the MHC in countering infectious diseases, for at least some invertebrates, transmissible malignancies could be a primary driver to favor recognition of nonself tissues (Metgzer et al. 2015).
4. The fact that some bivalves such as *V. corrugata* seem to have once been sources of neoplastic cells that currently infect other bivalves yet do not themselves currently suffer from neoplasia suggests that unique and fascinating resistance mechanisms have evolved in this species. What are they? The health of some mollusc species may depend on knowing this in the future.

Challenges for Molluscs in the Anthropocene Epoch

Although there are differences of opinion as to when the "golden spike" was forged that delineates the start of the Anthropocene epoch (Lewis and Maslin 2015), and stratigraphers debate whether we even should acknowledge or encourage the formal naming or recognition of the Anthropocene (Waters et al. 2016), there is no doubt that we live in a time of rapid global change that is impacting molluscan biology in many ways. Atmospheric levels of carbon dioxide have risen sharply, with at least two major consequences: rapid warming of the earth and increased ocean acidification, both of which can be expected to be stressful in molluscan environments. Globalization has resulted in rapid increases in the rate of introductions of exotic species of all kinds, including molluscs and molluscan parasites (Giannelli et al. 2016; Sohn 2017). Additional stressors permeate many molluscan environments, including micro- and nanoplastic particles, heavy metals, and organic pollutants, the latter including pharmaceuticals discarded into aquatic environments. Extreme overcrowding is often a feature of rearing of commercially valuable aquaculture species. Habitat fragmentation and loss further compromise molluscan environments. Lydeard et al. (2004) reported that 42% of 693 extinctions of animal species recorded since the year 1500 were of molluscs: 260 gastropod and 31 bivalve species. In 2016, the International Union for Conservation of Nature (IUCN) Red List indicated that 297 of 744 extinct

animal species (40%) were molluscs, but Cowie et al. (2017) have argued that the Red List estimates of species loss among molluscs were too low, with more realistic numbers being more like 638 extinct species, 380 likely extinct species, and 14 species extinct in the wild by one means of estimation, and >5000 molluscan species being extinct by another method of estimation.

Many molluscs—oysters are a good example—are highly resilient to environmental perturbation and routinely survive considerable changes in temperature, salinity, or air exposure (Bayne 2017), but the tolerances of even such hardy species might be severely tested. Given the changing environmental circumstances, many molluscs may find themselves in locations that have become marginal for their continued existence such that the low ends of their elevational ranges may be shifting upward and their latitudinal ranges may be moving toward the poles. The net result is that molluscs will experience difficulty in maintaining homeostasis, and energetically expensive activities such as spawning, maintaining growth or shell condition, or mounting stress responses may come at the expense of the maintenance of robust immune defenses (Sheldon and Verhulst 1996; Wendling and Wegner 2013; Asplund et al. 2014). Below, we outline some examples that call attention to the concerns and accentuate the importance of achieving a thorough understanding of molluscan immunobiology.

A Brief Digression—Stress, Immune, or Defense Responses? While preparing this review, we have learned from the literature that the term "defense responses" is usually considered to be the most inclusive term. "Stress responses" are usually considered to be more generalized and systemic, and to occur in response to both abiotic stressors (such as temperature or salinity changes) and biotic stressors, which might be intrinsic (as, for example, imposed by the rigors of spawning) or extrinsic (as imposed by an infectious agent). As we have indicated elsewhere, transcriptomic studies based on comprehensive next-generation approaches (e.g., Guo et al. (2015); Buddenborg et al. (2017)) indicate that exposure to infectious agents does indeed provoke responses from gene families normally associated with both stress and immune responses. Where the boundaries lie between stress and immune responses, and even whether it is meaningful to be concerned about this, we cannot resolve here. On the basis of extensive transcriptional studies in bivalves, as noted by Guo et al. (2015), the responses to abiotic and external biotic stressors—the latter being what we would recognize as immune responses—are inextricably interconnected in molluscs, with considerable cross talk in the course of their deployment. Expanded gene families involved in immunity include particular members that respond only to infection and others that respond only to abiotic stress, while others are responsive to both (Guo et al. 2015). It is also important, going forward, to appreciate more fully that molluscs are frequently exposed simultaneously to multiple stressors, whether abiotic or biotic, and that these impose competing demands on their resources such that unpredictable and far-reaching, unfavorable, synergistic effects may result.

Commercially Important Marine Molluscs Several oyster diseases—including those caused by *P. marinus*, *R. crassostrae*, and OsHV-1—tend to increase in severity

as water temperatures warm in the summer (see review by Ford and Guo (2016)). Furthermore, dermo seems to be extending its range northward along the North American Atlantic coast, following a warming trend that began in the mid-1980s and accelerated in the early 1990s (Ford 1996). Similarly, outbreaks of OsHV-1 in Chinese scallops (*Chlamys farreri*) are correlated with rising ocean temperatures in the Yellow Sea (Wang et al. 2013) and seem to be favored by high-density culturing methods. Crowding would potentially both stress the scallops and favor virus transmission (Ford and Guo 2016). Several studies indicate that higher water temperatures directly inhibit immune responses of oysters and other molluscs (Hegaret et al. 2004; Gagnaire et al. 2006; Hooper et al. 2007; Travers et al. 2009; Wendling and Wegner 2013).

The effects of exposure to elevated temperatures, spawning (an intrinsic biotic stressor), and *Vibrio* infection (an extrinsic biotic stressor) on summer mortality in Pacific oysters (*C. gigas*) were examined, and it was concluded that all three factors contributed additively to mortality (Fig. 6), with infection by a pathogenic *Vibrio* sp.

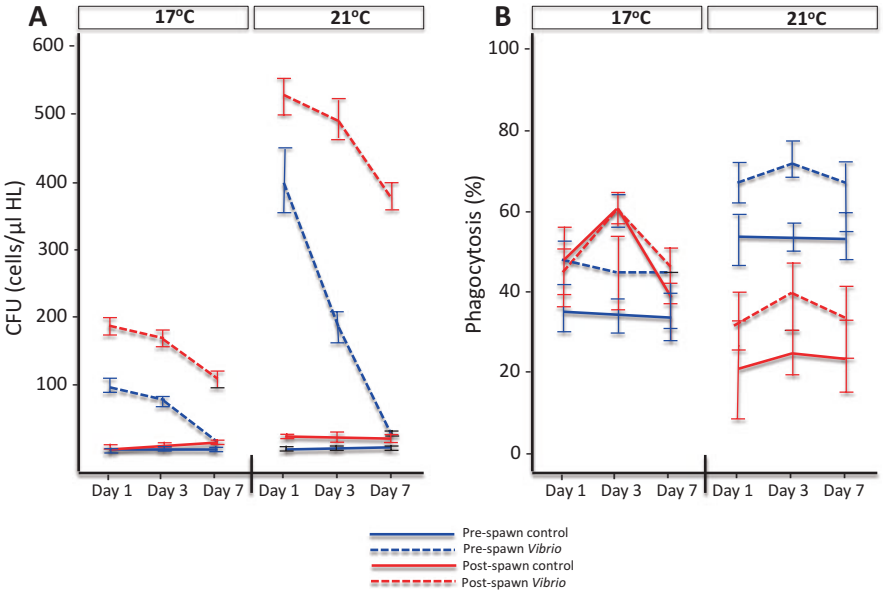

Fig. 6 Impacts of an abiotic stressor (temperature) and biotic stressors, whether intrinsic (spawning) or extrinsic (*Vibrio* infection), on (**a**) the number of colony-forming units (CFU) of *Vibrio*, and (**b**) phagocytic activity of hemocytes from Pacific oysters (*Crassostrea gigas*). The *panels on the left* (**a**) show CFUs for *Vibrio* sp. in oysters held at 17 or 21 °C, which were tested in prespawning (*blue*) or postspawning (*red*) condition, injected with *Vibrio* sp. (*dotted line*) or saline (*solid line*), and examined at 1, 3, or 7 days postinfection. The *panels on the right* (**b**) show the percentage of hemocytes able to phagocytose zymosan particles, for the same experimental groups. In general, postspawning oysters were less able to control *Vibrio* infection and had reduced phagocytic competence at the higher temperature. The authors note that spawning leaves oysters energetically depleted, thereby affecting hemocyte function, and that higher temperatures permit faster bacterial growth, all helping to explain high postspawning mortality following periods of high environmental temperature. (Modified from Wendling and Wegner 2013, *Aquaculture* 412–413: 88–96)

being the most important contributor. This was further attributed to a decreased ability of hemocytes to clear bacteria by phagocytosis, indicative of a shortage of energy reserves among postspawning and heat-stressed oysters (Wendling and Wegner 2013). Higher rates of pathogen proliferation at warmer temperatures also contribute to overmatching of the oyster immune system. This study illustrates the complexity of the situation; not only are there multiple abiotic and biotic stressors being brought to bear, but also some factors such as elevated temperature can have multiple adverse effects, including favoring increased pathogen growth rates and transmission, as well as impairing the host's ability to mount immune responses (Matozzo and Marin 2011).

The consequences of complex interplays between several simultaneous stressors on immune function also emerge from a series of studies of QX disease (*Marteilia sydneyi*) in Sydney rock oysters (*Saccostrea glomerata*) and of other bivalves such as pearl oysters (*Pinctada imbricata*) (Raftos et al. 2014). In these bivalves, environmental stress caused by reduced salinity (such as after heavy rainfall), physical agitation, or temperature extremes elicit a noradrenaline surge, which stimulates apoptotic responses (such as damaged mitochondrial membranes, DNA fragmentation, and plasma membrane blebbing) in hemocytes. This results in a reduction of hemocyte numbers, inhibition of hemocyte adhesion and phagocytosis, and decreasing numbers of phenoloxidase-positive hemocytes. The latter seem to be critically important in limiting intracellular QX growth, because the parasites are melanized within phagolysosomes (Raftos et al. 2014).

Ivanina et al. (2014) provided an example of how two different abiotic stressors—increases in partial pressure of CO_2 and elevated cadmium—can interact to impact immune responses of both oysters and clams. They found that moderately elevated CO_2 potentiated the negative effects of cadmium on hemocyte adhesion and phagocytic activity, and lowered expression levels of mRNAs for lectins, heat shock proteins, and lysozyme. They concluded that this could increase the vulnerability of clams and oysters to pathogens in acidified, polluted locations. Ocean pH is predicted to decline from 8.1 to 7.7 by 2100. Hernroth et al. (2016) studied AMP activities in the gills and hemocytes of the blue mussel (*Mytilus edulis*). AMPs of gill tissue were found to be less influenced by pH changes than AMPs in hemocyte extracts. Nonetheless, if mussels were exposed to a pH of 7.7 for 4 months, the bacteriostatic activities in gills were modulated. The authors concluded that weakened first-line defenses in the gills could favor *Vibrio* infections in future altered oceanic environments.

Several caveats apply with respect to foreseeing the possible future impacts of global change on bivalves and their immune systems. One is the potential emergence of poorly known or unknown threats, such as our growing awareness of the detrimental effects of micro- and nanoplastic particles in marine environments in particular. Canesi et al. (2015) noted dose-dependent decreases in phagocytic activity in *Mytilus galloprovincialis* hemocytes exposed to polystyrene nanoparticles, with evidence of cytotoxicity at higher concentrations. It was concluded that molluscan immune functions can be adversely affected by nanoplastics in marine environments.

Another caveat is that not everything necessarily worsens with climbing temperatures. The unnamed thraustochytrid QPX exemplifies a "cold water disease" (Perrigault et al. 2010, 2011). *Mercenaria mercenaria* mounts healing defense responses to QPX at higher temperatures (27 or 21 °C versus 13 °C), even though the pathogen grows faster at higher temperatures up to 23 °C. However, the ability of hemocytes to resist cytotoxic extracellular QPX products increases with temperature, as do the levels of plasma factors that neutralize these cytotoxic factors (Perrigault et al. 2011). Exposure to moderately high temperatures induces a heat shock response that is associated with remission of QPX in treated clams, and a deliberate brief exposure of clams with QPX to moderately high temperatures (27 °C for 4 h) promotes remission while minimizing stress to the clams (Wang et al. 2016). QPX is also instructive in that even though bivalve immune defenses to QPX seem to work best at higher temperatures, mortality outbreaks do occur in summer, likely a reflection of the fact that the infection is chronic and the mortality seen in summer is the end point of a long disease process (Perrigault et al. 2011).

Also, we should not underestimate the resilience of marine bivalves and their immune responses. When *Mytilus edulis* mussels were exposed to combinations of warm temperatures and cadmium, immunological perturbations were minimal and the animals showed no significant loss of general condition (Beaudry et al. 2016). Ivanina et al. (2016) found copper to be an immunostimulant for both oysters and clams, and suggested that copper treatments could be used for immune enhancements or disease protection of bivalve stocks. They also noted that even in combination with copper, hypercapnia at levels predicted by ocean acidification models was unlikely to significantly affect marine bivalve resistance to disease.

Marine bivalve genomes possess many genes involved in both stress and immune responses. Oysters have 5844 genes involved in response to abiotic stresses and 1405 immune genes (Guo et al. 2015). Oysters may employ additional genetic responses to stress, including alternative splicing. As many as 16% of oyster multiexon genes may be capable of alternative splicing with attendant creation of novel isoforms that may broaden even further the ability of oysters to respond to stress (Huang et al. 2016). Gavery and Roberts (2014) have advanced the interesting idea that DNA hypomethylation may be associated with "transcriptional noise" (alternative transcriptional start sites, exon skipping, or other unknown mechanisms) in bivalves such that they have greater phenotypic plasticity in the face of the unpredictable and variable environments they frequently occupy. They note that hypomethylation is a part of the plant immune response that facilitates transcription of immune genes associated with transposable elements (Yu et al. 2013). Marine bivalves have survived previous major extinction events, so it would clearly be premature to assume they will not survive the Anthropocene, but some of the anticipated challenges may not have been encountered previously, especially those imposed by pollution and by the intensity of our aquaculture practices.

Unionoid Bivalves About 860 species are recognized in the freshwater bivalve superfamily Unionoidea (Lydeard et al. 2004). More certain than the number of living species is the fact that unionoids are one of the most endangered groups of

animals. The 2017 IUCN Red List included 533 unionoid taxa, of which 194 were listed as vulnerable, endangered, critically endangered, regionally extinct, extinct in the wild, or extinct. The cause that is most frequently implicated in the decline of the unionoids is habitat destruction or alteration, with the construction of dams, pollution, and introduction of exotic bivalves such as zebra mussels (*Dreissena polymorpha*) and the Asiatic clam (*Corbicula fluminea*) all playing a role (Downing et al. 2010).

Like some marine bivalves, unionoids suffer from disseminated neoplasia, but the pathogens with which the two groups must contend appear to be quite different (Carella et al. 2016). This may in part reflect a paucity of studies of unionoids. Although there has not been a lot of work done to document the impact of human-caused environmental changes on unionoid immunity, it is reasonable to assume that unionoids respond to environmental stressors in ways that are broadly similar to those noted in marine bivalves, and that combinations of stressors can overwhelm their defenses (Falfushynska et al. 2016; Ferreira-Rodriguez and Pardo 2017). Exposure of several marine and freshwater bivalve species (including the unionoid *Elliptio complanata*) to four heavy metals revealed that each metal induced a dose-dependent inhibition of phagocytosis in all bivalves tested, though with most metals tested, unionoid hemocytes tended to be more susceptible (Sauve et al. 2002).

Exposure to municipal effluent waters induced stress responses in *E. complanata*, including increased mixed-function oxidase activity, metallothionein levels, DNA damage, and hemocyte bacteria counts (Gagne et al. 2002). Exposure of *Lasmigonia costata* to municipal effluents also provoked oxidative stress (Gillis et al. 2014). Hemocytes in *E. complanata* were more numerous in individuals exposed to waters downstream of municipal effluents, but they had diminished phagocytosis activity as compared with hemocytes from individuals upstream of toxic effluents (Blais et al. 2002). Maintenance of immune function is obviously important to unionoids. Even 32 days of starvation of *Lamellidens marginalia* did not change hemocyte function (numbers, phagocytic efficiency, nitric oxide production) or plasma protein levels, even as the enzymatic activity of vital organs such as the digestive gland significantly declined (Mahapatra et al. 2017). In the wake of widespread habitat loss, loss of fish host species needed by unionoid bivalves to complete their life cycles, and habitat degradation, lowering of immune competence may not garner much attention as a factor contributing to the demise of unionoid bivalves. However, combinations of multiple environmental stressors may open the door for opportunistic pathogens to overwhelm their defense responses, and this needs to be considered as a potential proximate cause in declining populations.

Disease-Transmitting Snails Snails are essential for the propagation of several species of digeneans and nematodes, which collectively infect ~300 million people worldwide. Snail-vectored helminths also compromise the health of domestic food animals, companion animals, and wild animals in many different contexts (Adema et al. 2012; Giannelli et al. 2016; Sohn 2017). Anthropogenic changes are predicted to have considerable impacts on the distribution and abundance of snail-transmitted

diseases because most of the snail species involved occur in freshwater and their body temperatures will increase as water temperatures increase (Mangal et al. 2008; Mas-Coma et al. 2009; McCreesh and Booth 2013; McCreesh et al. 2015). Higher water temperatures are expected to alter the geographic ranges of snails that vector the parasites responsible for schistosomiasis and fascioliasis, and to influence key parameters related to transmission, such as snail growth and mortality rates, rates of parasite development within snails, numbers of digenean cercariae produced by snails, and the life-spans of cercariae released from snails. Paull and Johnson (2011) argued that the pathological consequences of infection for snails with digeneans would be amplified at higher temperatures and that climate change could result in "phenological mismatches" between parasites and snails, with infections occurring in younger and more immunologically naïve hosts. As yet another factor to consider, exposure of snails to infection may alter the temperature at which they prefer to reside if given a choice (Lefcort and Bayne 1991; Żbikowska and Żbikowski 2015). Laboratory studies designed to mimic prolonged heat waves (longer than 1 week) provided evidence that the immune responses of *Lymnaea stagnalis* (hemocyte concentration, phenoloxidase activity, and antibacterial activity of snail hemolymph) were diminished at elevated temperatures (25 or 30 °C) relative to those of snails maintained at a nonstressful temperature of 15 °C (Seppälä and Jokela 2011; Leicht et al. 2013). Snails exposed to elevated temperatures acquired larger numbers of encysted metacercariae of the digenean *Echinoparyphium aconiatum* than snails maintained at normal temperatures (Leicht and Seppälä 2014).

As discussed in several recent reviews (Adema and Loker 2015; Pila et al. 2017), snails are known to mount both cellular and humoral immune responses to limit digenean infections, but when stressful anthropogenic changes put additional demands on vector snails, collateral effects on the abilities of the snails to fight parasite infections are anticipated (Nelson et al. 2016). Results consistent with this idea have emerged from work with juvenile individuals of strains of *Biomphalaria glabrata* that are either susceptible (NMRI strain) or resistant (BS-90 strain) to *Schistosoma mansoni* (Ittiprasert and Knight 2012). BS-90 snails normally mount active hemocyte encapsulation responses if penetrated by *S. mansoni* miracidia. Snails of both strains are normally maintained at 23–25 °C. It was discovered that if BS-90 snails were first exposed to a heat pulse of 32 °C for up to 4 h, followed by exposure to *S. mansoni* miracidia, their resistance was diminished such that 100% of the snails so treated would shed parasite cercariae by 7 weeks postexposure. This response was correlated with increased levels of transcription of heat shock proteins (HSPs) 70 and 90 and the reverse transcriptase (RT) domain of the retrotransposon *nimbus*. Furthermore, heat-pulsed BS-90 snails then treated with exposure to the HSP90 inhibitor geldanamycin did not lose their resistance to *S. mansoni*, whereas exposure of normally susceptible NMRI snails to geldanamycin prevented the snails from becoming infected with *S. mansoni*. The authors noted that NMRI snails mounted strong HSP70, HSP90, and RT responses following exposure to *S. mansoni*. The effect of geldanamycin in inhibiting these responses was not documented in the NMRI snails. From these experiments and others, Ittiprasert et al. (2009)

concluded that the induction of a stress response was associated with successful development of *S. mansoni* in juvenile *B. glabrata* (Ittiprasert and Knight 2012).

Nelson et al. (2016) confirmed that a 4- to 5-h heat treatment induced HSP70 and HSP90 production in BS-90 snails, but they were unable to document any increase in susceptibility to *S. mansoni* as a result of this heat exposure. They also found that exposure of *B. glabrata* to lower (20 °C) or higher (33 °C) than optimum temperatures did not induce an increase in circulating hemocyte numbers, and they concluded that abnormal temperatures can be inhibitory or stimulatory in regard to hemocyte numbers. Surprisingly, snails denied food for 2 weeks had higher hemocyte counts than fed snails at either sub- or supraoptimal temperatures—another indication of the importance of maintaining immune function even in the presence of starvation. Other studies using adult *B. glabrata* have found HSP70 to be preferentially upregulated in resistant compared with susceptible snails (Lockyer et al. 2004) following exposure to *S. mansoni*, and microarray and Illumina-based studies (Zhang et al. 2015a, b, 2016; Buddenborg et al. 2017) suggest that both *B. glabrata* and *B. pfeifferi* exhibit complex, multicomponent stress responses, so further studies are warranted to gain a better understanding of the relationship between induction of HSP and other stress response proteins and susceptibility/resistance to schistosome infections.

Indeed, the scope of studies documenting the impact of anthropogenic change on schistosome–snail relationships, and on other snail-borne parasites of concern, needs to be expanded, for it is clear that we have much to learn in this regard. As mentioned above, *B. glabrata* and other freshwater snails exposed to or infected with digenean larvae may respond by moving to cooler temperatures (Lefcort and Bayne 1991; Żbikowska and Żbikowski 2015). Lower temperatures prolong survival of infected snails but also, in the longer run, allow more cercariae to be produced. This and the fact that some digeneans provoke no movement of their snail hosts to lower temperatures have been taken to imply that any resultant behavioral changes are actually aiding the digeneans rather than permitting the snails to overcome their infections (Żbikowska and Żbikowski 2015). It is also instructive that even slow-moving snails can exhibit behavioral adjustments—such as moving to shallower or deeper water (McCreesh et al. 2015)—that may enable them to minimize some of the detrimental effects of altered temperatures.

Also, as emphasized above, multiple stressors may impact snails at the same time. Schistosome-resistant BS-90 snails have been found to be more susceptible to cadmium exposure than schistosome-compatible NMRI snails (Salice and Roesijadi 2002). Although it was clear that snails of both strains could develop enhanced tolerance of cadmium following multiple generations of exposure, they clearly carried a latent cost of cadmium tolerance because when exposed to elevated temperatures (36 °C) for 10 days, they had lower survival rates than control snails (Salice et al. 2010). This experiment indicates that trade-offs occur in how multiple stressors are dealt with, and it will be of particular interest to learn how susceptibility to *S. mansoni*, and the ability of snails to support the full program of *S. mansoni* development, might be altered as a consequence of multiple, co-occurring stressors.

Lastly, we need not only to better understand how changing climates will influence the future presence/absence of schistosome-transmitting vectors in major

endemic foci such as the Lake Victoria basin but also to document the extent to which these snails may already be experiencing stress, whether it be from increased temperature, pollution, deliberate exposure to molluscicidal chemicals, or even pharmaceuticals (Boisseaux et al. 2017). How this will ultimately affect disease transmission is hard to predict, but the weight of evidence indicates that every change has a cost, and if snails are simultaneously taxed by too many challenges (temperature, pollution, expensive immune response to parasites, direct harmful effects of parasites), then snails exposed to digeneans might die at a higher rate, one that is faster than the abilities of the digeneans to complete their complex developmental programs within snails, thus diminishing the numbers of patent infections and lowering transmission.

Land Snails Among the most endangered of all molluscs are the many species of terrestrial gastropods with restricted geographic ranges, such as the 4000+ species endemic to the Pacific Islands (excluding New Guinea and New Zealand), approximately half of which are estimated to have gone extinct in recent times (Lydeard et al. 2004). Habitat destruction, agriculture, and introductions of alien plants and carnivorous snails and flatworms have all had major impacts. Climate change is bound to further exacerbate the situation (Nicolai et al. 2017). Given the small and declining numbers in endemic populations of island land snails remaining in the wild, captive breeding programs have been established. The example of the apparent extinction of *Partula turgida* from the Society Island of Raiatea is both instructive and disturbing. The last specimens seen in the wild were collected in 1991 for captive propagation but, after increasing in numbers, the captive population then crashed. Necropsy of five snails, including the very last individual of the species to die, revealed the presence of a disseminated infection by a microsporidian of the genus *Steinhausia* (Cunningham and Daszak 1998). The origin, exact identity, and host specificity of the microsporidian species are unknown, and it too may have gone extinct with its snail host. As noted by the authors, this represents an example where extinction of a land snail species could be attributed to an infectious disease. It raises the possibility that a similar fate could await other molluscs maintained under artificial conditions where other opportunistic pathogens could be unwittingly introduced, especially if several species are maintained in shared habitats. The greatest threats come from generalist pathogens with broad host species ranges that might be maintained in the environment by widespread host species (Fisher et al. 2012). Also, small populations may be particularly prone to infectious disease (McCallum and Dobson 1995). This raises the possibility that one of the proximal factors driving other endangered species to extinction may be immune competence and prior experience with pathogen exposure. It could easily escape attention if this were the case, and it must be said that virtually nothing is known of the immune systems of any of the endangered gastropods, so one productive line of investigation might be to gain some perspective on how their immune systems function and whether particular immune or stress-related features could be used to monitor their health in both captive and wild situations.

Opportunities and Challenges for the Future

We reiterate that this is a most exciting time to be a comparative immunologist: the tools and prospects for significant advancements have never been better and the needs from both basic and applied points of view never greater. Below, we attempt to feature some of the opportunities and challenges posed for those interested in the study of molluscan immunobiology.

For Most Lineages of Molluscs, We Know Almost Nothing Not surprisingly, only for a small number of species of economic or medical significance have we achieved any depth of understanding of the structure of the molluscan immune system, with deep functional understanding still lagging quite far behind. These model systems will continue to lead the way and will eventually illuminate much of the essence of molluscan immunobiology. But the numbers of papers published or sequences generated for many molluscan groups is very modest (Table 2), so there are exciting opportunities awaiting those with interest in comparative approaches. One need is simply to obtain genomic and transcriptomic data for representatives of the major lineages of molluscs, with the goal of piecing together the basic architecture and gene representation of the molluscan immunome. Certainly the goal of achieving a grand overview of molluscan immunobiology, and learning how it has been sculpted among and within molluscan classes, should be highly rewarding. As an additional bonus, from a more complete molecular representation of immune genes among the major groups of molluscs will come insights that should shed light on unresolved phylogenetic relationships among these groups.

Molluscs, with Their Diverse Lifestyles, Pose Exciting Fundamental Immunological Questions The many different molluscan lifestyles provide a wonderful opportunity to explore how malleable and inventive the innate immune system can be. One of the unique opportunities presented by some molluscs is their extreme longevity. For example, the marine bivalve *Arctica islandica* can live for

Table 2 Indicated for major molluscan lineages are the numbers of papers published as compiled from Google Scholar or PubMed, and the number of expressed sequence tags available in GenBank

Class	Search phrase	Google Scholar[a]	PubMed	ESTs[b]
Caudofoveata	caudofovea[a] AND immun[a]	0	0	0
Solenogastres	solenogastr[a] AND immun[a]	0	0	0
Monoplacophora	monoplacopho[a] AND immun[a]	1	0	0
Polyplacophora	polyplacopho[a] AND immun[a]	5	1	1548
Scaphopoda	scaphopod[a] AND immun[a]	249	0	0
Bivalvia	bivalv[b] AND immun[a]	18,400	469	382,612
Gastropoda	gastropod[a] AND immun[a]	13,600	207	647,770
Cephalopoda	cephalopod[a] AND immun[a]	5660	31	114,034

[a]The numbers include frequent irrelevant hits

[b]Expressed sequence tags (ESTs), as of 2016, compiled from GenBank by Schultz and Adema (2017)

more than 500 years and the freshwater unionoid *Margaritifera margaritifera* can live for more than 200 years. These molluscs are well suited for immunologically oriented gerontological research and offer many avenues of study, ranging from how they manage DNA repair and oxidative stress (Ridgway et al. 2014) to prevention of, and recovery from, infections. Little has yet been done to explore the rudiments of immune functioning in long-lived molluscs. Do they just happen to live in stable and relatively pathogen-free environments that allow them to avoid the hazardous consequences of stress or infection (especially in combination) such that the "standard issue model" of the bivalve immune system suffices? Or do they have extra dimensions to their immune systems that favor longevity? If ever there were invertebrates for which some form of immunological memory might prove advantageous, exceptionally long-lived bivalve species would seem to qualify. For such molluscs, the initiation and continued support of long-term studies to gain some appreciation for how they quietly go about outliving us all would be very much in order, particularly given that among the long-lived bivalves are many unionoid species imperiled by habitat loss.

Another fundamental immunological questions posed by molluscs is: how do some gastropods and bivalves accommodate massive monospecific populations of obligate symbionts, including bacteria and dinoflagellates, without compromising their ability to fight off other kinds of pathogens? To what extent do their obligate symbionts assist the host mollusc in defending against attack by other microbes (see Desriac et al. (2014) for an interesting potential application involving bivalves)? The same question might even be asked of unambiguous parasites such as digenetic trematodes; do they too contribute to the defense of the "parasite–host unit" to ensure their own survival? As another example, what happens to the immune system in those molluscs that adopt parasitism, including that of other molluscs? Do parasitic molluscs forego immunity altogether and depend on their hosts for protection, and what kinds of gene family expansions, modifications, or deletions are seen to enable their parasitic lifestyle when the major group from which they have emerged is overwhelmingly free living in lifestyle? Have gene families we might ordinarily not associate with immune or stress responses been co-opted to function in infectivity or protection from immune attack? This is but a small sample of the kinds of basic questions that can be asked of molluscs and that should be of interest to many biologists.

A Diversity of Experimental Approaches Is Needed to Better Understand Immune System Function The genomic, transcriptomic, and proteomic studies now forthcoming continue to build an enormously important structural framework with which we can reassess all aspects of molluscan immunobiology. They have also accentuated the need for more tools to ascertain the functional relevance of the many candidate immune factors postulated to be relevant to protection of molluscs from infection. Here we briefly discuss a few needs.

There is a multitude of ways in which immortal cell lines have facilitated efforts to document components of immune and other processes. Consequently, many

substantive yet unsuccessful efforts have been made to develop cell lines from various noninsect invertebrates (Bayne 1998; Rinkevich 2011). Yet, among all lophotrochozoans, just a single immortal cell line exists (Yoshino et al. 2013a). Derived from embryonic *B. glabrata*, the *Bge* cell line (Hansen 1976) has enabled a variety of studies aimed at unraveling immune pathways in *B. glabrata* (Coustau et al. 2003; Humphries and Yoshino 2006; Yoshino et al. 2008). In the absence of such tools, in vitro experiments have been forced to be done using short-term primary culture systems, from which much has been learned. However, without self-sustaining cell lines, progress in virology (and many other aspects of the biology of these animals) has been slowed.

We still lack basic information on some of the more dynamic aspects of molluscan immunobiology. How long do hemocytes live, especially in long-lived species? We have a growing appreciation that functional specialization occurs among hemocytes (Pila et al. 2017), but how do these cells differ from one another? How specialized are they with respect to what they make and secrete? And how many different functional groups of hemocytes are present? Access to single hemocyte transcriptomes—an approach that has opened new vistas in mammalian immunology (Papalexi and Satija 2017)—offers a way forward here, as does continued and expanded use of cell-sorting technologies, which often have to be modified to accommodate the tendency of molluscan hemocytes to clump together in vitro (Chen and Bayne 1995).

There has been increased emphasis placed on the importance of microbiomes in influencing all aspects of their host's biology, including their immune competence. In some cases, as with molluscs with obvious symbiotic partnerships, the importance of the microbiome seems obvious. But additional study is needed to determine if the importance of the microbiome has been underappreciated or overhyped in other molluscs. A more detailed understanding of the contributions of individual species within the microbiome for how they might educate or manipulate the host's immune system, and the nature of the metabolic contributions they make to either stimulate or augment the host mollusc's immune system, would help us to fully appreciate their role in molluscan immunobiology.

Another pressing need is development of robust reverse genetics approaches to enable functional characterization of particular genes. Studies exploiting small RNA oligonucleotides to interfere with gene expression have explored the role of FREP3 (Hanington et al. 2010, 2012), a TLR (Pila et al. 2016c), granulin (Pila et al. 2016b), and *grctm6* (Allan et al. 2017) in the resistance of *B. glabrata* to *Echinostoma paraensei* or *S. mansoni*. Insofar as these studies have achieved sufficient knockdown of the candidate genes to permit full development of *S. mansoni* in a strain noted for its full resistance to infection, or to increase cercariae production in susceptible snails, siRNAs have proven to be very useful tools. But one of the shortcomings of siRNA oligonucleotides is that their effects are transient; they do not provide a permanent and total knockdown to further facilitate interpretation of gene function.

The rate of advance of functional characterization will pick up considerably when clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) technology can be made effective in molluscs. At the time of

writing, we know of only one paper (Perry and Henry 2015) that has reported use of CRISPR/Cas9 methodology in a mollusc, in this case using a guide RNA to facilitate knock-in of a donor segment containing an *mCherry*-encoding sequence into the β-catenin gene in the marine caenogastropod *Crepidula fornicata*. This application was intended to facilitate in vivo monitoring of β-catenin expression during development.

We are aware that multiple lab groups are actively pursuing pragmatic ways to use CRISPR/Cas in molluscan studies and, as has been developed with model organisms such as *Drosophila* and zebrafish (e.g., Port et al. (2014)), the availability of Cas9-expressing transgenic lines and associated guide RNA expression plasmids of molluscs would mark a major step forward in eventual characterization of individual molluscan immune genes. Such lines could potentially be used for loss-of-function insertions or deletions of candidate genes or for precise modifications to generate alternative functional versions of candidate genes. Yet another important use of CRISPR/Cas technology is to take a page from the mosquito playbook (Gantz et al. 2015; Alphey 2016) and couple it with gene drives such that a genetic modification can be driven into populations as a means to effect a desired change such as increased thermal or disease resistance in oysters or heightened levels of resistance to schistosome infections in snails. Very exciting opportunities lie ahead in this realm.

We Don't Know What We Don't Know—Our Minds Must Remain Open for Unknown or Unusual Paradigms Modern approaches for discovery of immune factors most often rely on identification of transcripts that are upregulated following infection, followed by assessment of their degree of homology with what is in existing databases. Factors with homology to known immune genes in other systems then become prime candidates for further study. As one caveat to consider here, are levels of transcripts really a faithful indicator of how much actual translated protein is then produced? Plant immunology has often proven instructive for invertebrate immunologists, and some recent studies in *Arabidopsis* suggest that translation is tightly regulated and that transcription and translation are poorly correlated (Xu et al. 2017). This study emphasizes the importance of signaling properties of the mRNA molecule in facilitating subsequent translation. Small noncoding RNAs occur in molluscs, are also likely to influence mRNA stability and translation, and may have profound effects on antivirus (Chen et al. 2014) and antiparasite responses (Rosani et al. 2016; Queiroz et al. 2017). As of now, this field is wide open for study.

Additionally, many molluscan genes, including ones that are highly responsive following exposure to infection, either have unknown functions or fall within functional groups that seem irrelevant or peripheral to defense, and so garner little attention. Yet such genes may be of critical importance and may simply have functional roles we have yet to connect with defense. One recent example from our own work is the GTPase of the immune-associated proteins, or GIMAPs, that were found to be upregulated in the APO of *B. glabrata* following immune stimulation (Zhang et al. 2016) and were also responsive in *B. pfeifferi* following exposure to *S. mansoni* in an Illumina-based transcriptome study (Buddenborg et al. 2017). GIMAPs have

since been identified as another expanded gene family in oysters and responded to infection by downregulation, possibly involved in promoting hemocyte survival by negatively regulating apoptosis (McDowell et al. 2016). GIMAPs are known to play a role in plant defense but, given their patchy distribution among both protostomes and deuterostomes, we may easily have overlooked their importance in those animals in whey do occur (McDowell et al. 2016). In general, there is an important role to be played for alternative approaches—proteomics, epigenetic modifiers, and transcriptional control by small RNAs of host or parasite origin all come to mind, but other categories of molecules should not be ruled out—to identify nonsuspect molecules in locations highly relevant to defense that provide a needed and distinctive way to identify new immune candidates. The discovery of novel AMPs from *Crossostrea* gills, using biochemical approaches (Seo et al. 2013), is an example.

Other approaches that help us discover and implicate the "unknown unknowns" in immunity are genetic studies involving selective breeding for disease resistance, quantitative trait locus (QTL) mapping, candidate-based association studies, and genome-wide association studies (Guo et al. 2015). One example is provided by the work of Blouin and colleagues (Tennessen et al. 2015a, b; Allan et al. 2017) in identifying genomic regions in *B. glabrata* that are associated with resistance to *S. mansoni*. This has enabled them to identify a resistance complex in snails from Guadeloupe, in which there are several predicted genes that may be associated with resistance. This complex includes several genes that would not normally catch the eye of an immunologist but in which the predicted protein structures reveal a hypothetical basis for immune functions. Consider too the example provided by association of a gene (*CvSI-1*) encoding a serine protease inhibitor with resistance of the oyster *C. virginica* to *P. marinus* infection. CvSI-1 was implicated in inhibiting *P. marinus* proteases and slowing its in vitro growth, and oysters selected for resistance to *P. marinus* expressed higher levels of CvSI-1 (LaPeyre et al. 2010). A study based on survival of groups of oysters upon challenge with *P. marinus* showed that survival was associated with a variant form of *CvSI-1* but even more so with a 25-bp deletion in the associated promoter that favored enhanced expression of *CvSI-1* (He et al. 2012). The point here is that resistance limiting dermo infection in *C. virginica* may hinge on the presence of a novel allele but even more importantly on the level of transcription, something that easily could have escaped attention without linkage studies (Nikapitiya et al. 2014). Understanding the underlying basis for how molluscs achieve "resistance" to infection with viruses, cellular pathogens, or even transmissible neoplasias is one important way in which molluscan immunologists can contribute practically to the preservation of endangered mollusc species!

As another example of a "wild card," to go along with the unexpected discovery of transmissible neoplasia in marine bivalves, Liscovitch-Brauer et al. (2017) recently reported that RNA editing is particularly common in behaviorally advanced coleoid cephalopods, especially in nervous system molecules affecting excitability and neuronal morphology. This process greatly increases transcriptome plasticity and protein diversity. The authors noted other potential uses for robust RNA editing as mediated by ADAR (adenosine deaminases acting on RNA) enzymes, such as in innate immune processes, though this remains to be shown. RNA editing has been

implicated as a mechanism of diversification of immune proteins in sea urchins (Ghosh et al. 2010). A study of mRNAs in the cephalopod nervous system may well lead to totally unforeseen new insights into molluscan immunobiology.

Molluscan Conservation Immunology Molluscs have suffered extinction events that have been disproportionately large relative to those of other animal groups, and there are many reasons to be concerned that molluscan extinctions will continue. Particular groups have been singled out for concern, among them unionoid bivalves and endemic land snails from the Pacific Islands. Although threats from pathogens may not rank highly on the long list of concerns for these imperiled molluscs, when the synergistic roles of stress and infection are acknowledged, the importance of infection may be seen to be greater than has been realized. An infectious disease may quietly apply the coup de grace for some mollusc species without us even knowing it. So, it hardly seems irresponsible to advocate for a better understanding of the structure and function of the immune systems of endangered molluscs, starting with next-generation genomic and transcriptomic studies. These require very little source material. Particularly useful would be comparisons of how the immunomes of thriving unionoid or terrestrial gastropod species compare with those of imperiled close relatives. Also of interest are studies to determine if loss of genetic diversity at key loci has disproportionate impacts on susceptibility to infection, which might help to explain why captive remnants of some species might be vulnerable and how the noble efforts of captive breeding programs can be further enhanced.

The plight of rare and endangered molluscs fits into a broader global narrative regarding the impact of changing environments on molluscs of all kinds. The consequences of these changes are unpredictable, and increased funding is needed to continually monitor important populations of commercial bivalves for signs of distress or outbreaks of epidemics, including newly emerging and unexpected threats. With respect to molluscan disease vectors, such as the snails that transmit human schistosomiasis so pervasively in sub-Saharan Africa, new efforts are needed to monitor snails in prominent transmission foci for signs of thermal or other kinds of stress that might herald shifts in immune-mediated susceptibility/resistance to infection, or that might combine with the biotic stress of infection itself to limit the period of time during which snails can continue to support parasite development, with direct effects on the production of human-infective larval stages (Kalinda et al. 2017).

Some Final Projections and Reflections Many worthy questions pertaining to molluscan immunobiology remain to be addressed. Some are briefly recounted here as additional opportunities for enlightening research. For instance, to what extent have different molluscan classes arrived at distinctive defense solutions? As a specific example, a clear role for phenoloxidase-dependent melanization in the process of killing pathogens within hemocytes has been documented for bivalves (Raftos et al. 2014). But no similar claim has been made for other molluscs. However, a recent study using refined procedures has documented hemolymph phenoloxidase

activity attributed to a multi-copper oxidase gene family member (laccase) in *B. glabrata*. This activity appears to be associated with plasma rather than hemocytes and activity diminishes late in the course of schistosome infection (Le Clec'h et al. 2016). If phenoloxidase is relevant in the gastropod immune arsenal, then—although this is a simplistic extrapolation—one might suspect the possibility that more heavily melanized lines of a given species might be more able than their less melanized relatives to kill parasites. As it happens, there appears to be no correlation, at least in the *B. glabrata–S. mansoni* system (Allegretti et al. 2009). The reasonable inference is that the phenoloxidase system is either a minor component of gastropod immune killing or does not contribute to killing. How different will the responses of molluscs from different classes prove to be, and why?

Fungal diseases have rarely been reported in molluscs (Cheng 1967), so their antifungal defenses are presumed to be highly effective and warrant investigation. "Omic" studies have revealed probable genes encoding putative receptors for β-1,3-glucans (Zhang et al. 2010), but the spectrum of genes, proteins, and other components with which molluscs deal with recognized fungal intruders remains mysterious.

The role of epigenetic modification of both parasites and molluscan hosts in affecting outcomes of infection is also a topic worthy of future study. It has already been shown that epigenetic modification of *S. mansoni* miracidia can modify, and in some cases improve, their infection success in *B. glabrata* (Cosseau et al. 2010; Fneich et al. 2016). Production by *S. mansoni* of more variants of polymorphic mucins following exposure to trichostatin A (TSA) was identified as the factor increasing compatibility with snail defenses. Epigenetic modifications (phosphoacetylation) of DNA in neurons in the pedal ganglia of the gastropod *Pomacea canaliculata* have been reported following infection (Ottaviani et al. 2013), raising the possibility that immunologically relevant epigenetic changes occur in molluscs too (Ittiprasert et al. 2015). The discovery, functional characterization, and impacts of molluscan viruses and fungal pathogens of molluscs—and the nature of the molluscan response to these agents—all still largely comprise a black box, in part because of the lack of certain tools such as stable cell lines, but progress is beginning to emerge on this front, especially for bivalves (Allam and Raftos 2015; He et al. 2015; Green et al. 2015; Huang et al. 2017).

As we conclude this chapter, we come to the happy realization that molluscan immunobiology is a burgeoning field, one that is difficult to concisely summarize, and one with many healthy growth nodes that offer a wealth of opportunity to interested investigators. The array of technical and conceptual approaches is powerful and dazzling. We have two final observations, the first being that much of molluscan biodiversity is imperiled in the Anthropocene epoch in which we live, and a fundamental understanding of immunology can provide valuable input in many different contexts, ranging from countering the effects of unexpected and emerging diseases in commercially important species to helping preserve precious remnant populations of endangered molluscs. Lastly, as powerful and entrancing as big-data approaches are for solving complex problems and moving us forward, we also wish

to accentuate the immense value of learning the natural histories of molluscs—including understanding the opportunities and constraints posed by their environments—and to encourage appreciation of their histories, their unique body plans, and their diversity, including that exemplified by their immune systems.

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Echinodermata: The Complex Immune System in Echinoderms

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Echinoderm Life History and Phylogeny

Echinoderms are benthic marine invertebrates living in communities ranging from shallow nearshore waters to the abyssal depths. Often members of this phylum are top predators or herbivores that shape and/or control the ecological characteristics of

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their habitats. Five classes are defined within echinoderms: Crinoidea (sea lilies and feather stars), Ophiuroidea (brittle stars), Asteroidea (sea stars and sea daisies), Holothuroidea (sea cucumbers), and Echinoidea (sea urchins and sand dollars) (Fig. 1a–e). As a consequence of rapid divergence that occurred shortly after the origin of the echinoderm phyla, which emerged an estimated 570 million years ago (Pisani et al. 2012), the phylogenetic relationships among the classes have been difficult to establish, and vary depending on the data set and phylogenetic methods (Fig. 1f) (Janies et al. 2011). Crinoids are unequivocally the basal group, whereas the monophyly of asteroids and ophiuroids remains in debate. The Echinodermata and Hemichordata phyla constitute the Ambulacraria, which is the basal group within the

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deuterostome lineage (Blair and Hedges 2005) and sister to the chordates (Fig. 1g). The nearly 8000 extant echinoderms (from the Greek, meaning "spiny skin") share several aspects of life cycle and body plan. Most echinoderm species develop indirectly through a dispersal-stage planktonic larva, which exhibits bilateral symmetry and swims and feeds through the activities of surface cilia and the ciliary band. Metamorphosis occurs when the larva descends to the benthos from the littoral zone and everts the adult rudiment into a juvenile with five spines and five tube feet. Most adults share the characteristics of a radial body plan with pentameral symmetry, a rigid calcite endoskeleton, and a water vascular system that functions as a hydraulic mechanism for tube foot extension and locomotion, sensory activity (including responses to light/dark (Ullrich-Lüter et al. 2011), and food capture (Hyman 1955).

Early Evidence for Echinoderm Immune Responses

Allograft Rejection Defines an Innate Immune System in Echinoderms

For centuries, the prevailing theory was that animals without backbones lacked immune systems. Invertebrates infected with pathogens either recovered or were deleted from the gene pool, and populations remained at steady state as a consequence of a large number of offspring. When this assumption was first challenged, the most straightforward strategy to identify immune responses in echinoderms was to use allograft rejection experiments designed to determine whether individuals could differentiate between self and nonself. When skin allografts and autografts were transplanted among individuals of the sea star *Dermasterias imbricata*, allografts were always rejected, whereas autografts were accepted and healed into the grafted site (Fig. 2a, b) (Hildemann and Dix 1972; Karp and Hildemann 1976).

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Similar experiments using body wall transplants demonstrated that nonself-recognition mechanisms are also present in the white sea urchin *Lytechinus pictus* (Coffaro and Hinegardner 1977). Although the kinetics of the second-set allograft rejections are significantly faster than the first-set rejections in this species, the kinetics of first-set versus third-party rejections are identical, suggesting a lack of specific immune memory (Fig. 2c) (Smith and Davidson 1992). This demonstrated that echinoderms have an immune system that is efficient for host protection, but that it functions with innate mechanisms in the absence of adaptive capabilities.

Foreign Particles Are Cleared Rapidly from the Coelomic Cavity

The use of allograft rejection to demonstrate immune capabilities in echinoderms, albeit effective, is an artificial experimental approach. Unlike the well-established systems in colonial tunicates and hydroids (Nydam and DeTomaso 2011; Rosengarten and Nicotra 2011; Taketa and DeTomaso 2015), natural allografts do not occur in echinoderms. As an alternative approach to assess nonself recognition capacity, echinoderms have been evaluated for their abilities to clear foreign particles and cells injected into their coelomic cavity. A wide range of particles and molecules have been employed in these studies, including foreign proteins, T4 bacteriophage, carbon particles, carmine, Sephadex, and latex beads, which are all cleared effectively (reviewed in Smith and Davidson (1994)). In response to injection with sheep red blood cells (RBCs), the sea cucumber *Holothuroidea polii* clears these cells within 8 days, which correlates with the appearance of dark brown bodies in the coelomic cavity (Canicatti and D'Ancona 1989). These bodies are consistent with aggregates of hemoglobin from the RBCs encapsulated by coelomocytes that have activated phenoloxidase activity and melanin production. In the sea urchin *Strongylocentrotus nudus*, intracoelomic injection of sheep RBCs is followed by the production of reactive oxygen intermediates (Ito et al. 1992). Furthermore, the rate of RBC phagocytosis is increased as a consequence of preincubation or opsonization with *Strongylocentrotus nudus* coelomic fluid (CF). Echinoderms also respond to foreign cells from other species within the phylum, and the injection of coelomocytes from the sea urchin *Arbacia punctulata* into the sea star *Asterias forbesi* results in the swift clearance of the sea urchin cells and corresponds to a transient decrease in the sea star coelomocytes (Reinisch and Bang 1971). A more

Fig. 1 (continued) *Patiria miniata*. (**e**) The Ophiuroidea are represented by the brittle star *Amphiura filiformis*. (Reprinted from Arnone et al. (2015).) (**f**) There are two possible relationships among the echinoderm classes. Two total evidence trees include nuclear sequences (18S RNA, 28S RNA, H3 histone genes), mitochondrial sequences (12S RNA, 16S RNA, tRNA cluster, cytochrome c oxidase 1), and morphological characters. The left tree is the result of direct optimization, whereas the tree on the right results from the 50% majority rule in MRBAYES. Both trees are modified from Janies et al. (2011) to show the differences in the relationships among the classes. (**g**) A simplified phylogenetic tree of the Deuterostomia shows the relationships between the Ambulacraria (which include the Echinodermata) and the Chordata. The Protostomia are indicated as the outgroup

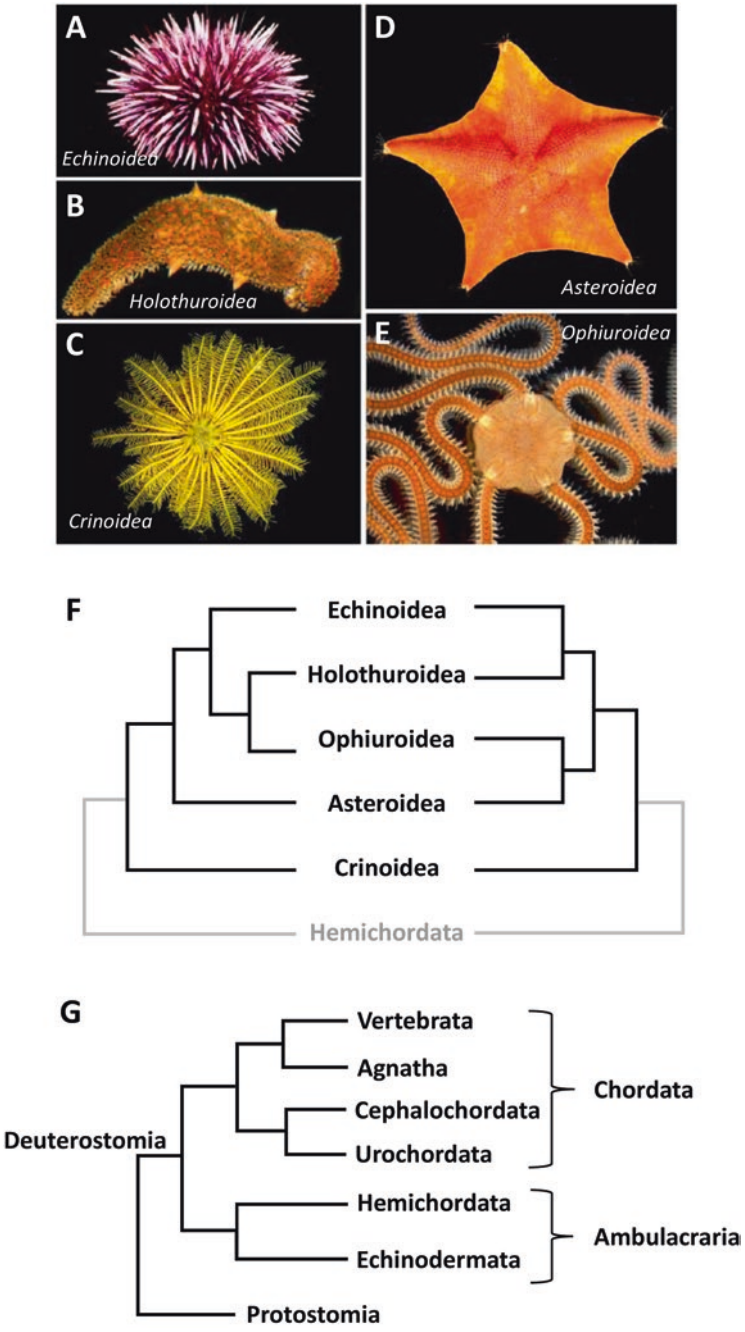

Fig. 1 *Five classes of the Echinodermata*. (**a**–**e**) Species from each of the five classes of echinoderms are shown. (**a**) The Echinoidea comprise sea urchins and sand dollars, and are represented by the purple sea urchin, *Strongylocentrotus purpuratus*. (**b**) The Holothuroidea are represented by the sea cucumber *Parastichopus parvimensis*. (**c**) The Crinoidea are represented by the sea lily *Oxycomanthus intermedius*. (**d**) The Asteroidea comprise sea stars and sea daisies, and are represented by the bat star,

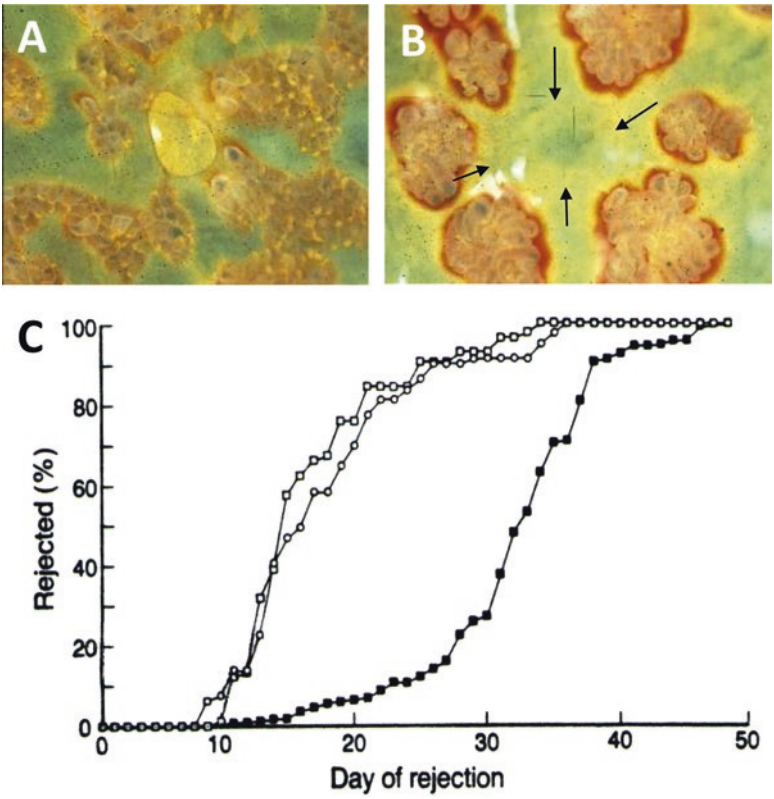

Fig. 2 *Tissue transplantation establishes echinoderm immune activity as innate in character*. (**a**) Allograft rejection in the sea star *Dermasterias imbricata*. Skin transplantation shows rejecting allograft tissue that is lighter in color than the normal green color of the sea star skin. (Modified from Karp and Hildemann (1976).) (**b**) Autograft fusion in *D. imbricata*. The control autograft shows fused tissue that is the same color as normal sea star skin. The edges of the accepted graft are indicated by arrows. The reddish areas on the surface of the sea star are normal dermal papillae. (Modified from Karp and Hildemann (1976).) (**c**) Allograft rejection of body wall transplants in the sea urchin *Lytechinus pictus* demonstrates innate immune function in echinoderms. About half of the first-set allografts (black boxes) are rejected by 35 days, whereas about half of the second-set allografts (open squares) are rejected by 15 days. However, the rejection kinetics for the third-party allografts (open circles) are the same as those for the second-set allografts, indicating a lack of specific immune memory. (Reprinted from Smith and Davidson (1992), with permission from Elsevier)

immunologically relevant approach to characterize the immune response, however, is to assess the ability of echinoderms to clear pathogenic bacteria from the coelomic cavity. Injections of unidentified marine bacteria or a freshwater fish pathogen into the coelomic cavity of the purple sea urchin, *Strongylocentrotus purpuratus*, results in clearance of 90–99% of the microbes within 3–6 h (Yui and Bayne 1983). Similarly, the congeneric *Strongylocentrotus droebachiensis* clears the echinoid

pathogen *Vibrio anguillarum* in 10 h (Plytyzc and Sejelid 1993). In both species, clearance of injected microbes is accompanied by a transient decrease in the numbers of coelomocytes in the coelomic cavity. Together, these results suggest that the transient decrease in the coelomocyte concentration in the CF in response to injections of foreign cells is a measurable parameter of phagocytosis and encapsulation, and illustrates that coelomocytes in echinoderms are the primary mediators of immune response.

Diseases of Adult Echinoderms

Mass Mortality Events in Echinoderms

The presence of an active immune system in echinoderms is evident from diseases that manifest as symptoms and death of large populations. However, the survival of the phylum in general (and the recovery from diseases by certain species in particular) is based on their immunological responses to those diseases. Although echinoderms do not generally succumb to bacteria that are injected experimentally, echinoderm diseases in nature have led to mass die-offs, which have been recorded for coastal species in shallow habitats and are often associated with sudden and dramatic ecological fluctuations. Overarching forces that tend to drive fluctuations in marine habitats stem from natural events such as hurricanes (which detach warm-core rings from the Gulf Stream and move warm water into the cold coastal waters of the northwestern Atlantic) and El Niño phases (which push warm water up and down the North American Pacific coast) (Scheibling and Hennigar 1997; Burge et al. 2014). Environmental fluctuations such as the quick changes in water temperature and salinity that are induced by these forces likely increase stress and reduce echinoderm resistance to waterborne pathogens, which leads to disease outbreaks and mass mortality events (reviewed in Burge et al. (2014); Jurgens et al. (2015)). Perhaps the greatest threat to the health of the coastal benthic community is the impact of increased human populations near coastlines, tourism, and commercial overfishing (Blois et al. 2013; Moritz and Agudo 2013; Norris et al. 2013; Stocker et al. 2013). Continued anthropogenic global climate change will result in extinctions, reduced species diversity, and drastic changes in ecosystems (Blois et al. 2013; Moritz and Agudo 2013; Norris et al. 2013), as well as an increased likelihood of disease outbreaks (Burge et al. 2014; Harvell et al. 1999).

Numerous disease-causing microorganisms have been isolated from echinoderms that maintain either pathogenic or symbiotic interactions with their hosts. These agents include bacteria, fungi and yeast, viruses, and parasites including amoebas, ciliates, cyanophytes, flagellates, apicomplexans, haplosporidians, algae, mesozoans, sponges, cnidarians, entoprocts, nematodes, turbellarians, trematodes, annelids, polychaetes, gastropods, bivalves, barnacles, crustaceans, amphipods, copepods, pycnogonids, tardigrades, bryozoans, and pearl fish (Turton and Wardlaw

1987; Jangoux 1990; Stein and Halvorsen 1998; Gudenkauf et al. 2014). Although these pathogens and parasites have been isolated from various echinoderms, few have been linked to a specific disease phenotype (e.g., spotted gonad disease in the sea urchin *Strongylocentrotus intermedius* (Shimizu 1994)) or have been verified experimentally as the pathogen of a mass mortality event. Thus, most of the reported mass mortalities are due to unknown or unobserved agents (reviewed in Lawrence (1996); Schultz (2016)).

The largest and most widespread report of a massive mortality event, and for which the pathogen remains unknown, occurred in populations of the long-spined black sea urchin, *Diadema antillarum*, in the Caribbean and Western Pacific near Central America. This species suffered a sudden massive die-off in 1982–1984 with repeated die-offs in 1985 and 1991–2, resulting in population declines ranging from 85% to 100% in localities across the entire Caribbean (Bak et al. 1984; Lessios et al. 1984; Lessios 1988; Moses and Bonem 2001; Hughes et al. 1985). The only populations of *D. antillarum* that were unaffected by this event were located outside the Caribbean, in the eastern Atlantic Ocean (e.g., in the Canary and Portuguese Islands (Lessios 1988)). No other Caribbean sea urchin species were disturbed during this time, suggesting that the causative agent infected *D. antillarum* specifically (Lessios et al. 1984). Although the involvement of pollution and global warming could not be excluded, early reports of these events speculated about a pathogenic cause (Liddell and Ohlhorst 1986) and a waterborne pathogenic causative agent was supported by the path of infection, which followed surface water currents (Lessios 1988). Both viral (Gudenkauf et al. 2014) and microbial (Bauer and Agerter 1987) pathogens were proposed; however, isolates from *D. antillarum* resulted in many microbial organisms, of which any might have been the lethal pathogen (Bauer and Agerter 1994). Mass die-offs of an apex herbivore have an immediate and significant impact on the ecology of coral reefs, which shift from predominantly coral to algal cover. This outcome followed predictions from experimental exclusion of *D. antillarum* from a reef patch, which released algal growth control from herbivory, so corals were outcompeted and smothered (Sammarco 1980). On the larger format of the Caribbean basin, removal of the top herbivore resulted in drastic ecological changes in the reef communities and fish populations (Lessios 1988; Carpenter 1988, 1990; Robertson 1991). As the populations of *D. antillarum* slowly recover, the species has become a key indicator for the recovery of reef communities because they promote the recruitment of new reef-building corals (Ogden et al. 1973; Edmunds and Carpenter 2001) and continued ecological changes in the reefs.

Bald Sea Urchin Disease

A few echinoderm disease outbreaks have been linked to specific pathogens (Becker et al. 2008). One example is the bald sea urchin disease, a bacterial infection that affects several species of sea urchins (e.g., *Mesocentrotus franciscanus*, *S. purpuratus*, and *Paracentrotus lividus)* (Pearse et al. 1977; Maes and Jangoux 1984; Becker et al. 2007; Johnson 1970). (For a revision to the strongylocentrotid family, see

Kober and Bernardi (2013)). A resurgence of bald sea urchin disease was noted in *S. purpuratus* near Santa Barbara, California, during the winter of 2016–2017, coincident with an El Niño event (Pierre and Smith, personal observations, 2016). The disease is characterized by lesions typically associated with injuries or abrasions on the lateral and oral surfaces of the animal. Subsequent surface infections show loss of spines and other appendages, including tube feet and pedicellariae, and appear as bare (or bald) patches of exposed test that may include partial destruction of the upper test layer (Fig. 3a). In some cases, the lesions are associated with a green discoloration of the affected area (Fig. 3b) (Johnson 1970; Maes and Jangoux 1984). Infiltration of immune cells (see section "Echinoderm Immunity Is Mediated by Coelomocytes") into the affected area followed by tissue regeneration and recovery may occur if the infection covers <30% of the animal surface (Jangoux 1990). Bald sea urchin disease is considered to be a consequence of infection with *Vibrio anguillarum* and/or *Aeromonas salmonicida*, because isolates from diseased animals can reproduce the disease in healthy purple sea urchins (*S. purpuratus*) (Gilles and Pearse 1986). Molecular analyses of bacteria isolated from disease lesions in the sea urchin *P. lividus* show an enrichment of α- and γ-proteobacteria (*Vibrio* and *Aeromonas*, respectively) as well as *Cytophagales*, *Flavobacteraceae*, and *Bacteriodetes* groups (Becker et al. 2008). Although the symptoms of bald sea urchin disease are similar in different echinoid species, the microbial basis of the disease is likely complex and infection with different microbes may manifest similar symptoms.

Echinoderm Paramoebiasis

The amoeba *Paramoeba invadens* has been isolated from the CF of sea urchins. This protozoan causes epizootic outbreaks of paramoebiasis disease in the green sea urchin *S. droebachiensis* resulting in mass mortality events along the Atlantic coast of Nova Scotia, which are associated with warm-core rings from hurricanes (Jones 1985; Scheibling and Hennigar 1997; Miller and Colodey 1983; Scheibling et al. 2010). The disease can be transferred to healthy sea urchins by immersion in seawater containing *P. invadens* (Jellett et al. 1988) or by injection of the amoeba into the coelomic cavity (Jones et al. 1985; Jones and Scheibling 1985). Symptoms of the disease appear 10–15 days after exposure as muscle necrosis leading to loss of tube foot function, substrate detachment, mouth gaping, spine loss, and tissue discoloration (Jellett et al. 1988). Infected animals exhibit a decreased coelomocyte concentration in the CF and an increased protein concentration, which corresponds to an impaired coelomocyte-mediated clotting response.

Viral and Bacterial Diseases of Sea Cucumbers

Diseases of edible, maricultured echinoderms result in significant losses to farmers and prompt investigations into causative agents of these diseases. The sea cucumber

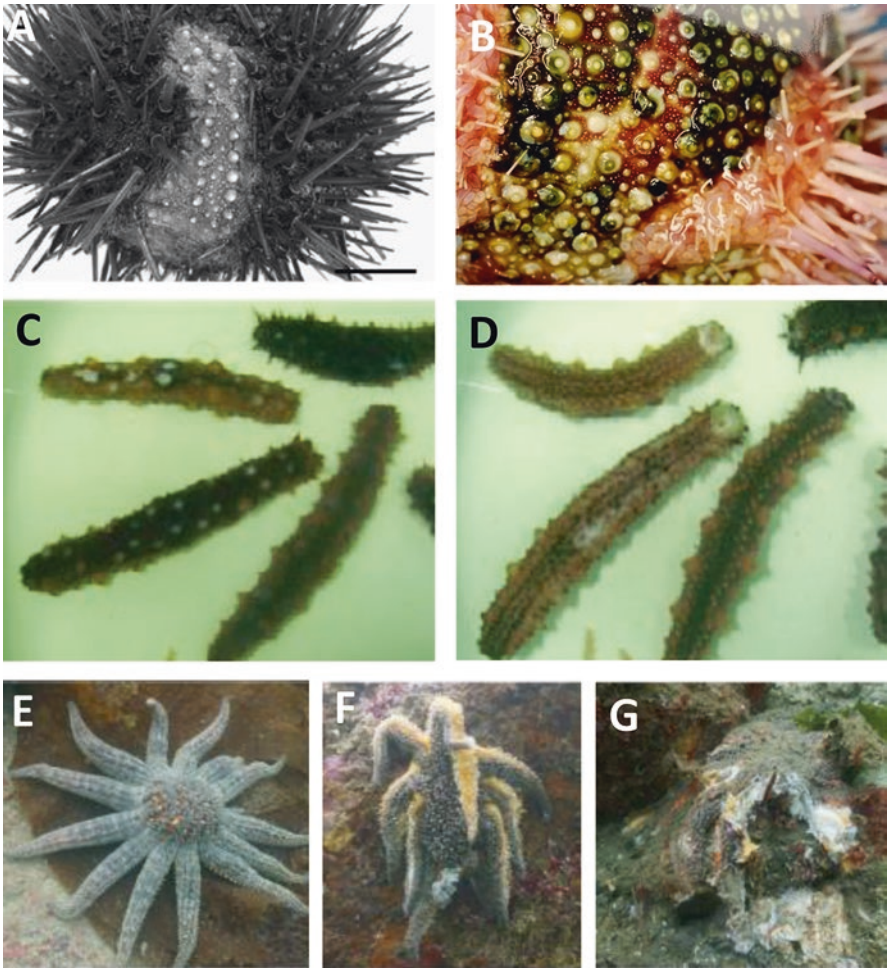

Fig. 3 *Diseases of echinoderms*. (**a**) Bald sea urchin disease in the sea urchin *Paracentrotus lividus* is characterized by spine loss and, in advanced cases, loss of ectoderm (*scale bar* = 1 cm). (Reprinted from Becker et al. (2008), with permission from Elsevier.) (**b**) Bald sea urchin disease in the sea urchin *Echinus esculentus* shows spine loss and discoloration of the exposed test. (Image courtesy of Dr. Anne Böttger.) (**c**, **d**) Skin ulceration and peristome tumescence syndrome virus (SUPTSV) infection in sea cucumbers. (Reprinted from Liu et al. (2010a), with permission from Elsevier.) (**c**) Skin ulcers are apparent on the dorsal side of the sea cucumber *Apostichopus japonicus* after immersion in seawater with SUPTSV. (**d**) Ulcers from SUPTSV infection on the ventral side surround the mouth. (**e**, **f**) Progression of sea star wasting disease (SSWD) in the sunflower star, *Pycnopodia helianthoides*. (Reprinted from Schultz et al. (2016), with minor modifications to image sizes following restrictions cited in https://creativecommons.org/licenses/by/4.0/legalcode.) (**e**) A healthy sunflower star. (**f**) An infected sunflower star shows loss of turgor, body wall disintegration, and exposure of internal organs. (**g**) A lethal infection of a sunflower star results in disintegration with the appearance of skeletal ossicles on the substrate

Apostichopus japonicus is subject to two diseases with distinct symptoms: a skin ulceration disease and a peristome tumescence disease. In mariculture facilities in China, these have triggered mass mortalities, which typically occur during the winter (Deng et al. 2009; Liu et al. 2010a). Skin ulceration disease is characterized by lesions around the mouth and/or cloacal openings, which can spread across the entire animal (Fig. 3c, d), and it is associated with cessation of feeding, head shaking, mouth sensitivity, and general atrophy. Peristome tumescence disease is characterized by ulceration of the integument muscle (Wang et al. 2005; Liu et al. 2010a). Although the cause for both diseases was believed to be bacterial (Wang et al. 2005; Zhang et al. 2006; Deng et al. 2009), repeated tissue sampling from intestine and tentacles of diseased animals identified the pathogen as a spherical enveloped virus of 100–250 nm, which causes both diseases and was subsequently called skin ulceration and peristome tumescence syndrome virus (SUPTSV) (Liu et al. 2010a). Two bacterial species, *Pseudoalteromonas tetraodonis* and *Pseudoalteromonas* sp., were also isolated from infected tissue samples that produced similar but different disease symptoms. Early symptoms of the bacterial infection in *A. japonicus* show loss of tentacle activity plus ulcers on the dorsal skin and abdominal parapodia, which increase in size and number and eventually merge. The differences between the diseases indicate that the bacterial pathogens primarily cause ulceration of the skin, whereas SUPTSV initially targets cellular function in the tentacles and peristome. Although each of the pathogens induces symptoms with overlapping characteristics, the likelihood of mixed infection under natural or aquaculture conditions is high, in which both types of early symptoms could develop rapidly from multiple pathogens (Liu et al. 2010a).

Sea Star Wasting Disease

The first observed outbreak of sea star wasting disease (SSWD) was in 1977–1978 in the Gulf of California and affected the predatory sun star *Heliaster kubiniji* (Dungan et al. 1982). Between 1978 and 1998, repeated outbreaks of SSWD caused mass mortalities of multiple species of asteroids in the California Channel Islands (Engle et al. 1994; Eckert et al. 1999; Blanchette et al. 2005). The ochre sea star, *Pisaster ochraceus*, at two different sites on Vancouver Island, British Columbia, also showed evidence of SSWD (Bates et al. 2009). Although SSWD primarily affects asteroids, symptoms can also be displayed by sea urchins (*S. purpuratus*, *M. franciscanus*, and *Lytechinus pictus*) and brittle stars (*Ophioplocus esmarki* and *Ophiopteris papillosa*) (Eckert et al. 1999). SSWD outbreaks tend to coincide with warmer waters that occur during El Niño events (Engle et al. 1994; Eckert et al. 1999; Blanchette et al. 2005). The presence and severity of SSWD and its association with temperature can be replicated experimentally by maintaining sea stars at different temperatures (Bates et al. 2009).

An unprecedented SSWD epidemic in early 2013 killed millions of asteroids of up to 20 species along the eastern Pacific coastline of North America (Hewson et al. 2014; Stokstad 2014). Symptoms include necrotic lesions, body wall edema and dermal inflammation, loss of body turgor and lethargy, behavior changes, limb curling and autotomy, and death, which appears as a rapid degradation or "melting" of the animal, leaving behind the skeletal elements on the substrate (Fig. 3e–g) (Hewson et al. 2014). The absence of bacterial and eukaryote pathogens in lesions from infected animals suggested that the causative agent for SSWD might be a virus. Consistent with this prediction, filtered seawater (0.22 μm filters) collected from tanks containing infected sea stars or similarly filtered tissue supernatants from infected animals cause SSWD in healthy animals (Hewson et al. 2014; Fuess et al. 2015). Massively parallel sequencing of tissues isolated from infected versus healthy sea stars revealed that the most common sequence in infected sea stars is that of a densovirus (Parvoviridae), subsequently termed sea star–associated densovirus (SSaDV). Densoviruses bind to a transferrin receptor that is expressed on coelomocytes in many echinoderm species, which may be the basis for the multispecies epidemic. Analysis of museum specimens also identified SSaDV in tissues collected in 1942 (prior to any reports of wasting disease), suggesting that the virus has been present in the environment for a long time, that it may have a nonasteroid reservoir, and that environmental changes or other stressors either to asteroids or the virus may have initiated the epidemic (Hewson et al. 2014). This suggestion is in accordance with the 2013 summary report from the Intergovernmental Panel on Climate Change, reporting new prediction models for continued emissions of greenhouse gases that will lead to global climate change throughout the twenty-first century and likely result in an increasing incidence of disease outbreaks and mass mortalities with less time for recovery (Burge et al. 2014). Thus, understanding the functions of the innate immune responses to pathogens in echinoderms continues to be important because many serve as keystone species for the coastlines of our world.

Immunogenomics: Immune Genes Encoded in Echinoderm Genomes

Genomes from species within most echinoderm classes (Fig. 1) have been sequenced, including sea urchins (Echinoidea; *S. purpuratus* and *Lytechinus variegatus*), brittle stars (Ophiuroidea; *Ophiothrix spiculata* and *Ophionereis fasciata*), sea stars (Asteroidea; *Patiria miniata* and *Patiriella regularis*), and sea cucumbers (Holothuroidea; *Parastichopus parvimensis* and *Australostichopus mollis*) (Sodergren et al. 2006; Cameron et al. 2009; Long et al. 2016) (see also www.echinobase.org). Of these, the purple sea urchin, *S. purpuratus*, was the first large marine invertebrate organism to have its genome sequenced (Sodergren et al. 2006). Through a community-wide collaboration, the annotation of many of the approximately 23,000 gene models encoded in this 814-Mb sequence was accomplished with an emphasis on molecules involved in several aspects of the life history (see *Developmental Biology*, volume 1, 2006). Some of the most striking results to

emerge from the analysis were homologues of genes that function in either immune cell development or immune response (Hibino et al. 2006). Comprehensive surveys for these homologues relied on both primary sequence similarity (e.g., BLAST-based searches) and a domain-based strategy to identify more distantly related molecules (see Buckley and Rast (2011)). In total, over 1000 immune genes fall into the following categories: pattern recognition receptors (PRRs) and other immune receptors, intracellular signaling, transcription factors, cytokines and growth factors, and immune effector genes, as well as genes involved in coagulation and the complement system, and homologues of genes that function in the vertebrate adaptive immune system (Table 1) (Hibino et al. 2006; Rast et al. 2006). Many of these genes (e.g., the complement genes, the *SpRAG*-like gene cluster, and the *SpTransformer* [formerly *Sp185/333*] effector gene family) are described elsewhere in this chapter. This analysis complemented previous studies of the echinoderm immune response and unexpectedly revealed that the system is complex, sophisticated, robust, and likely highly flexible for detecting and responding to a wide range of potential pathogens in the marine environment.

Pattern Recognition Receptors

An exceptional example of the complexity of the echinoderm immune system is the significant expansion of gene families encoding PRRs, specifically the Toll-like receptors (*SpTLR*s; 253 genes), NOD-like receptors (*SpNLR*s; >200 genes), and scavenger receptors containing multiple scavenger receptor cysteine-rich domains (*SpSRCR*; 1095 domains in 218 genes) (Table 1) (Rast et al. 2006; Hibino et al. 2006; Rast and Messier-Solek 2008; Messier-Solek et al. 2010; Buckley and Rast 2015). This set of sea urchin immune genes contrasts with homologous repertoires in the well-characterized vertebrate (e.g., mammalian) and protostome (e.g., *Drosophila* and *C. elegans*) systems, which typically harbor 5–20 genes in each of these families. Genes within the expanded *SpTLR* multigene family encode transmembrane proteins with a cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain that mediates signaling, and a ligand-binding ectodomain consisting of a series of leucine-rich repeats (LRRs) capped by specialized N-terminal and C-terminal LRRs (Rast et al. 2006; Hibino et al. 2006; Buckley and Rast 2012, 2015; Messier-Solek et al. 2010). The *SpTLR* genes form 11 subfamilies that exhibit differential expression patterns in larval and adult tissues, although some are primarily expressed at sites of immune interaction (e.g., coelomocytes and the gut). Genes within several of the subfamilies are subject to significant levels of diversifying selection. In some subfamilies, specific residues under positive selection are predicted to cluster spatially within the ectodomain, which is consistent with the speculation that these regions may be important for binding the pathogen or PAMP and for dimerization, as in other systems (Choe et al. 2005; Sackton et al. 2007). The *SpTLR* genes are intronless, clustered within the genome, and characterized by numerous insertion/deletion events. Of the 194 complete *SpTLR* genes, 127 encode a full-length SpTLR protein, while the remaining 59 (30.1%) are predicted to be pseudogenes based on

Table 1 The immune gene repertoire encoded in the *Strongylocentrotus purpuratus* genome sequence[a]

Gene	Function in other systems	Copy number
A. Immune genes		
Pattern recognition receptors		
TLRs	Extracellular pathogen recognition	253
NLRs	Intracellular pathogen recognition	203
SRCR domain–containing proteins	Extracellular or secreted pathogen recognition	1095 domains; 218 gene models
The complement system		
C3/4/5	Central components of the complement pathways	2
Thioester-containing protein		5
Mannose-binding lectin	Activates the lectin pathway	1
C1q	Activates the classical pathway	4
Ficolin	Activates the lectin pathway	46[b]
C2/Factor B	Involved in alternative activation pathway	3
CD59	Inhibitor of the membrane attack complex	4
Immune effector genes		
Macpf	Similar to proteins in terminal complement pathway; kills target cells by creating pores in the membranes	21
SpTrf	Not present in other systems, antipathogen activity	15–50
Cytokines		
IL-17	Induces inflammation and cell migration; maintains barrier integrity	35
MIF	Regulates inflammatory responses	9
B. Transcription factors		
Hematopoietic factors		*Vertebrate orthologues*
bHLH factors	SpScl	Scl, TAL-2, Lyl-1
	SpId	Id1, Id2, Id3, Id5
	SpE-protein	HEB, E2A, Id-2
GATA factors	SpGata1/2/3	Gata-1, Gata-2, Gata-3
	SpGata4/5/6	Gata-4, Gata-5, Gata-6
Homeobox factor	SpNot	None
Other	SpGcm	None; homologous to gcm in *Drosophila melanogaster*

IL interleukin, *MIF* macrophage migration inhibitory factor, *NLRs* Nod-like receptors, *SRCR* scavenger receptor, cysteine-rich, *TLRs* Toll-like receptors

[a]Gene numbers in this table are compiled from Hibino et al. (2006), Buckley et al. (2017), Buckley and Rast (2012, 2015), Buckley et al. (2008a), and Oren et al. (2016a)

[b]The ficolin genes include all gene models that encode fibrinogen domains, some with coiled coil domains but none with N-terminal collagen domains (Hibino et al. 2006)

frameshifts or premature stop codons. The high levels of diversity within the *SpTLR* genes, their structural similarities to TLR proteins in other organisms, and their expression in immune-related tissues suggest important pathogen detection functions within the immune system.

The evolutionary strategy of gene family expansion to generate PRR diversity is not limited to the purple sea urchin. The estimated sizes of the *TLR* gene family in two other strongylocentrotid species are similar to that of *S. purpuratus*: there are 276 *TLR* genes in *Mesocentrotus franciscanus*, 238 in *S. fragilis*, and 68 in *L. variegatus* (Buckley and Rast 2012). Outside the echinoderms, similarly large *TLR* gene families are present in in other invertebrate deuterostomes (e.g., *Branchiostoma floridae* (Holland et al. 2008)) and several lophotrochozoan species (Davidson et al. 2008; Zhang et al. 2015). The expansions may provide invaluable benefits to these animals by increasing the potential microbial recognition capacity. This is relevant for host defense and also to shape a beneficial microbiota, particularly given observations that normal commensal microbes can become opportunistic pathogens under stress conditions (e.g., bald sea urchin disease).

The Transcriptional Response to Sea Star Wasting Disease

In addition to genome sequences, transcriptomic data are increasingly available from echinoderms in various states of immune challenge. These strategies have been used not only to identify echinoderm pathogens (e.g., SSDaV) but also to characterize the host response to the pathogen. When the coelomocyte transcriptome from the sunflower star, *Pycnopodia helianthoides*, infected with SSWD (Fig. 3f, g) is compared with that from the bat star, *Patiria miniata*, and the purple sea urchin, *S. purpuratus*, 52% and 26% (respectively) of the encoded proteins can be predicted (Fuess et al. 2015). Sunflower star coelomocyte gene expression is particularly high for genes involved in complement pathways (see section "The Complement System"), including homologues of C3, factor B, ficolins, and properdin, which are likely important for opsonization to augment phagocytosis. Sunflower stars also upregulate members of TLR signaling pathways (e.g., MyD88 and NFκB), cytokines (e.g., the IL-6 receptor and IL-17), and genes that encode proteins involved in melanin/prophenyloxidase activation, arachidonic acid metabolism that may mediate phagocytosis, inflammation, pain, and chemotaxis. Concurrently, increases in expression of genes encoding proteins involved in extracellular matrix remodeling such as proteases and collagenases is consistent with significant changes in the connective tissues and animal morphology exemplified by "melting" (Fig. 3g). These transcriptomic data highlight aspects of the echinoderm immune response that are complex and are both novel within this phyla and more broadly conserved among animals.

SpRAG1L and *SpRAG2L* Expression and Function in Sea Urchins

The complex array of pattern recognition receptors in the *S. purpuratus* genome suggests that sea urchins have a sophisticated system of pathogen detection that differs from that in jawed vertebrates (Hibino et al. 2006). The jawed vertebrates rely heavily on the protection provided by the adaptive immune system, a central hallmark of which is the antigen receptor genes immunoglobulin (Ig) and T-cell receptor (TCR), which exist as gene segments in a nonfunctional configuration in the germline. Their assembly into functional Ig and TCR genes in developing lymphocytes is mediated by an enzyme complex consisting of the products of the recombination activating genes 1 and 2 (*RAG1* and *RAG2*) (reviewed in Gellert (2002)). These genes were originally identified exclusively in the genomes of jawed vertebrates and thus were intimately linked to the presence of diversifying Ig and TCR genes and the presence of an adaptive arm of the immune system (reviewed in Schatz (2004)). However, the *S. purpuratus* genome includes a pair of genes with striking similarities to vertebrate *RAG* genes on the basis of the locus structure, as well as the deduced amino acid sequence and domain structure of the encoded proteins (Fig. 4a) (Fugmann et al. 2006). Consequently, these genes are called *SpRAG1L* (*SpRAG1-Like*) and *SpRAG2L*, respectively. Their transcripts are predominantly expressed in coelomocytes in the adult sea urchin and, just like their vertebrate counterparts, these genes are always coexpressed (Fugmann et al. 2006). Furthermore, the encoded SpRAG1L and SpRAG2L proteins form complexes with each other and with selected vertebrate RAG1 and RAG2 proteins. SpRAG1L has low but clearly detectable recombinase activity on an artificial vertebrate recombination substrate when ectopically expressed in murine 3T3 cells (Carmona et al. 2016). Given that the DNA binding domain of SpRAG1L is one of the least conserved regions of the protein, it is likely that higher activity levels would be detected on the cognate but yet unidentified DNA target of the SpRAG1L/SpRAG2L complex in the sea urchin genome. In addition, SpRAG2L binds specifically to histones that are methylated on the fourth lysine in histone H3 (H3K4me2/3), which mirrors interactions observed for mammalian RAG2 (Wilson et al. 2008). Finally, homologues of the *SpRAG1L* and *SpRAG2L* genes are present in the genomes of other echinoderms, including the sea urchin *L. variegatus*, the bat star, *P. miniata*, and the brittle star *Ophiothrix spiculata* (Fig. 4b) (Kapitonov and Koonin 2015; KM Buckley, JP Rast, and SD Fugmann unpublished data, 2015). Together, with the recent discovery of a *RAG1/RAG2-like* gene pair (likely a transposable element) in the amphioxus *Branchiostoma belcheri* (Huang et al. 2016), these observations suggest that an ancestral *RAG1/RAG2* gene pair was present in the genome of the last common deuterostome ancestor prior to the emergence of the adaptive immune system in jawed vertebrates. While the possibility exists that the encoded proteins are involved in gene rearrangements, their functions in echinoderm immunity remain to be elucidated.

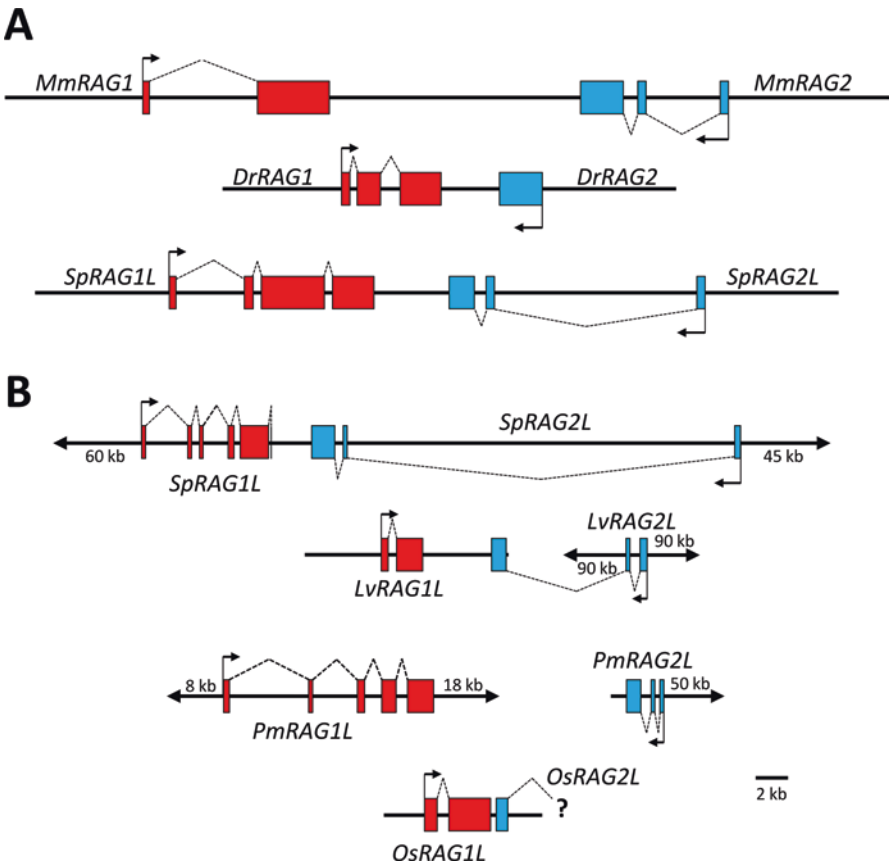

Fig. 4 *SpRAGL genes in echinoderms*. (**a**) *SpRAG-like* (*SpRAGL*) genes in the purple sea urchin, *Strongylocentrotus purpuratus*, are clustered and in the same tail-to-tail orientation as *RAG* genes in the mouse *Mus musculus* (*MmRAG*) and the zebrafish, *Danio rerio* (*DrRAG*). The exons of the *RAG1* and *RAG2* homologues are shown in red and blue, respectively. (Modified from Fugmann et al. (2006) and reprinted with permission, copyright 2006, National Academy of Sciences, USA.) (**b**) Genomic loci of *RAG1L* and *RAG2L* genes in echinoderms. Scaffolds containing the genomic loci of *SpRAG1L* and *SpRAG2L* from the sea urchin *Strongylocentrotus purpuratus* and *LvRAG1L* and *LvRAG2L* from *Lytechinus variegatus* are shown to scale with those from the sea star *Patiria miniata* (*PmRAG1L* and *PmRAG2L*) and the brittle star *Ophiothrix spiculata* (*OsRAG1L* and *OsRAG2L*). Arrowheads indicate the parts of the genomic scaffolds that have been omitted for clarity, and the sizes of these omitted regions are indicated. The putative transcription start site for each gene is marked with a bent arrow. Note that the *OsRAG2L* gene is incomplete in the publicly available draft version of the *Ophiothrix spiculata* genome (9/2016). Information to generate this figure was derived from the respective genome sequences at www.echinobase.org

Echinoderm Immunity Is Mediated by Coelomocytes

The ability of echinoderms to reject allografts, clear foreign cells and particles from the CF, and survive infection from a variety of pathogen agents all indicate the importance of the collection of cells within the CF that are known as coelomocytes. Echinoderm coelomocytes are highly heterogeneous, with a wide range of cell types and sizes that have been identified (Kindred 1924; Boolootian and Giese 1958; Hetzel 1963; Endean 1966; Smith 1981; Smith and Davidson 1992; Chia and Xing 1996; Gross et al. 1999; Ramírez-Gómez and García-Arrarás 2010; Smith et al. 2010). The description of coelomocytes presented here adopts a simplified classification scheme based on selected previous reviews (Ramírez-Gómez and García-Arrarás 2010; Pinsino and Matranga 2015). Cell categories are based on morphological criteria and classified into at least six cell types, although not all six have been identified in all classes or species. Coelomocytes are defined as phagocytes (also called leukocytes), spherule cells (also called spherulocytes, amoebocytes, morula cells, or granulocytes), vibratile cells, hemocytes, progenitor cells, and crystal cells, of which some are more restricted in their phylogenetic distribution (Smith 1981). In general, coelomocyte types are relatively conserved among sea urchin (Echinoidea) and sea star (Asteroidea) species and less conserved among different species of sea cucumbers (Holothuroidea). Although the original work to identify and characterize cell types was done using transmitted bright field light microscopy with multiple histological staining techniques, more recent reports have employed fluorescence microscopy to differentiate coelomocyte subpopulations by cellular cytoskeletal structure and protein localization, as well as electron microscopy to identify ultrastructural characteristics. Some functions of coelomocyte subsets are becoming better defined, while the functions and activities of other types remain poorly understood. In the section on "Immune Cells in Adult Echinoderms", the six coelomocyte types are discussed as they relate to their presence and function in sea urchins, sea cucumbers, and sea stars. Note that relatively little is known about coelomocytes in the brittle stars (Ophiuroidea) and sea lilies (Crinoidea).

Immune Cells in Adult Echinoderms

Phagocytes

Phagocytes are often the most abundant coelomocytes in echinoderms, particularly in sea urchins and sea stars. Their functional characteristics are associated with phagocytosis and/or encapsulation of foreign invaders, allograft rejection, and cytolytic and cytotoxic responses (Gross et al. 1999), in addition to the expression and secretion of antimicrobial peptides (AMPs) (Li et al. 2010a, b; 2014a) (see section "Echinoderm AMPs"). They can undergo shape changes from a petaloid/bladder form to a filopodial form and can self-aggregate, which is involved with CF clotting and syncytia formation (Kindred 1924; Boolootian and Giese 1959; Edds 1977; Majeske et al. 2013b). Sea urchin phagocytes range in size from 20 to 50 μm; they are typically the most abundant coelomocyte type and make up approximately 40–80% of the total cells found in the CF of sea urchins, depending on the species (Fig. 5a–d) (Bertheussen

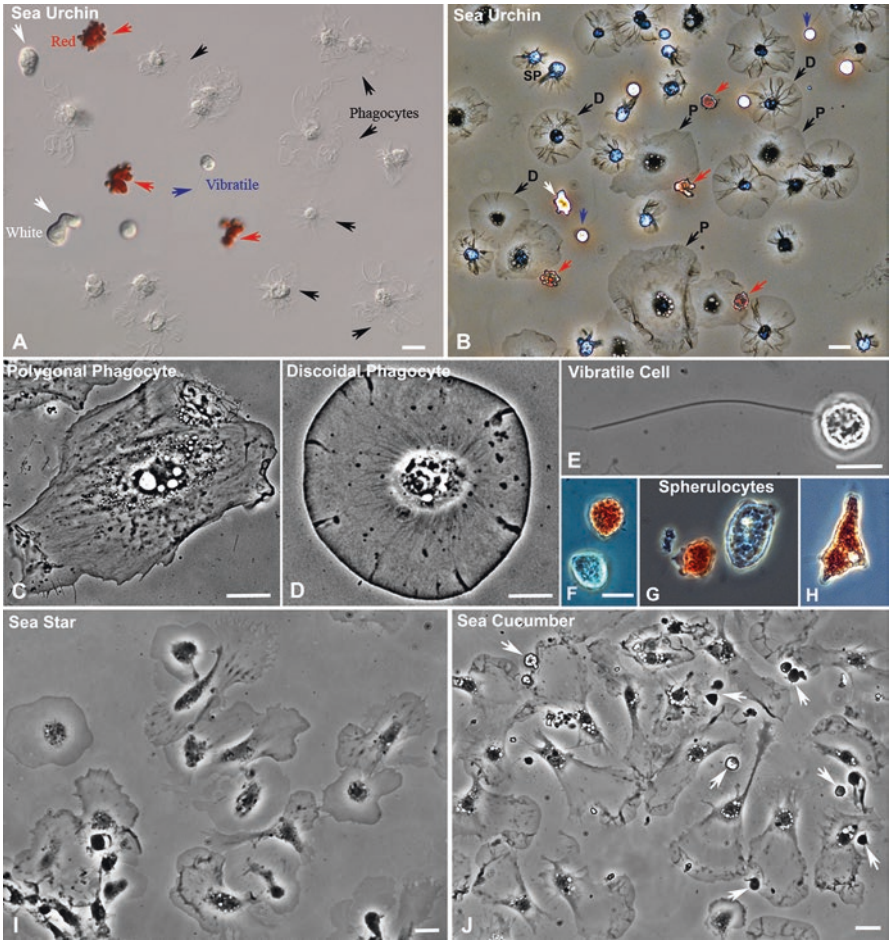

Fig. 5 *Echinoderm coelomocytes*. Live coelomocytes collected from sea urchins (**a**–**h**), a sea star (**i**), and a sea cucumber (**j**). Cells from the sea urchins *Paracentrotus lividus* (**a**: DIC imaging) and *Strongylocentrotus droebachiensis* (**b**–**h**: phase contrast imaging) include two types of large phagocytes. (**a**, **b**) Black arrows indicate phagocytes, red arrows indicate red spherule cells, white arrows indicate white or colorless spherule cells, and blue arrows indicate vibratile cells with single flagellae. (**b**) Phagocytes are indicated as discoidal phagocytes (D), polygonal phagocytes (P), and small phagocytes (SP). The red and colorless spherule cells and the vibratile cells are indicated as in panel (**a**). (**c**–**e**) High-resolution phase contrast imaging of large phagocytes (**c**, **d**) emphasizes the difference in the cytoskeletal and organellar organization in the polygonal cells (**c**) compared with the discoidal cells (**d**) and demonstrates the presence of the cytoplasmic granules and flagellum of a vibratile cell (**e**). (**f**–**h**) Red and colorless spherule cells in the process of spreading on glass and progressing from a spherical shape (**f**) to a more amoeboid shape (**g**, **h**). (**i**) Coelomocytes from the sea star *Asterias forbesi* are only phagocytes, which often group together into aggregates. (**j**) Coelomocytes from the sea cucumber *Sclerodactyla briareus* include numerous phagocytes with broad lamellipodial edges, as well as a variety of other cell types, which may include spherule cells, hemocytes, and/or progenitor cells (white arrows). The scale bars are 5 μm in panel (**a**) and 10 μm in all other panels. The magnifications in panels (**f**–**h**) are equivalent

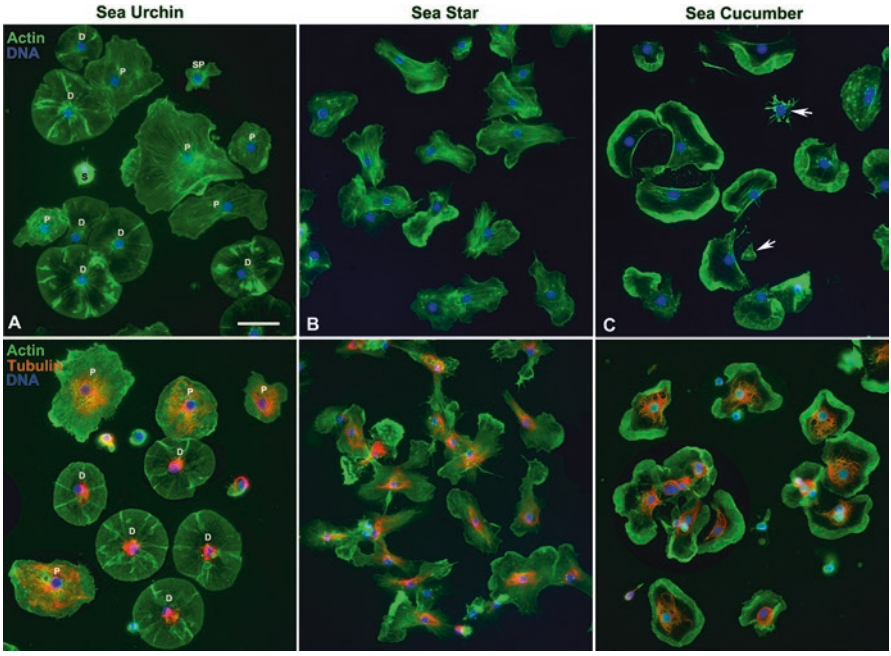

Fig. 6 *Phagocyte morphology varies among species from different echinoderm classes*. All species have phagocytes that stain for actin filaments (green), microtubules (red), and DNA (blue). (**a**, **d**) Large phagocytes from the sea urchin *Strongylocentrotus droebachiensis* are discriminated as polygonal (P) and discoidal (D) by actin staining and cytoskeletal morphology. A small phagocyte (SP) and a white or red spherule cell (S) are also shown. (**b**, **e**) Phagocytes from the sea star *Asterias forbesi* are primarily cells with an elaborate actin cytoskeleton, which is most similar to sea urchin polygonal phagocytes. Other distinct morphological subtypes of phagocytes are not obvious. (**c**, **f**) Phagocytes from the sea cucumber *Sclerodactyla briareus* have phagocytes with strong labeling of a dense actin network in the lamellipodial region at the cellular edge, and many have similar organization of the actin cytoskeleton. Some nonphagocyte cells are present (white arrows). The scale bars are 20 μm. The magnifications in panels (**a–c)** and panels (**d–f)** are equivalent

and Seljelid 1978; Jellett et al. 1988; Smith et al. 2010). Large phagocytes in suspension include a subset of cells that appear with veils of cytoplasm and a central nucleus, which are often referred to as bladder or petaloid cells (Figs. 5b, d and 6a, d) (Edds 1977; Henson et al. 1999). When spread on glass, they become discoidal in shape, which is the result of uniform spreading of the actin-rich lamellipodial cytoskeleton associated with a radial array of actin bundles (Figs. 5b, d and 6a, d) (Henson et al. 1999). A second subset of large phagocytes in sea urchins appear polygonal in shape when spread on a substrate, where they display a cytoskeleton with elongated actin bundles oriented parallel to the long axis of the cell that are reminiscent of actin stress fibers in mammalian tissue culture cells (Figs. 5a, c and 6a). These two cell types also differ in nuclear morphology, cytoplasmic organelle distribution, microtubule cytoskeleton and motor proteins, the extent of actin-mediated centripetal flow, and associated phagocytosis (Figs. 5b–d and 6a, d) (Edds 1993; Henson et al. 1992, 1999).

However, both types of large phagocytes undergo a transformation to a filopodial morphology in vivo as part of the clotting process in response to wounding of the animal, or as an in vitro response to osmotic shock or elevation in intracellular calcium (Smith 1981; Henson and Schatten 1983; Edds 1993; Henson et al. 1992, 1999; Chia and Xing 1996). In addition to the large phagocytes, there is a population of small phagocytes, the abundance of which appears dependent on the immunological activation status of a given individual sea urchin (Figs. 5b and 6a) (Gross et al. 2000; Brockton et al. 2008). A subset of these cells stain intensely for the sea urchin immune response proteins of the SpTransformer family (see section "The *SpTransformer* Gene Family in Euechinoids") (Brockton et al. 2008).

Phagocytes in sea stars (Figs. 5i and 6b, e) constitute up to 95% of the coelomocytes, which also undergo petaloid to filopodial shape changes (Coteur et al. 2002a; Pinsino et al. 2007) and show differences in size and granularity (Coteur et al. 2002a). Four subsets have been tentatively defined on the basis of morphology and size: small (10–15%), large (5%), and vesiculate (70–75%) phagocytes, and cells with short pseudopodia (10–15%) (Jangoux and Vanden Bossche 1975). There may be subpopulations of sea star phagocytes that differ in their level of phagocytic activity, their ability to undergo immunomodulation, and their relative aggregation as part of a CF clotting process (Kanungo 1982; Coteur et al. 2002a, b). Live cell imaging (Fig. 5i) for actin and microtubule staining (Fig. 6b, e) indicates that sea star phagocytes have variable morphology and are often found as aggregates, and that sea stars do not appear to have an equivalent of the distinct phagocyte subtypes that are present in sea urchins.

In sea cucumbers, phagocytes make up about one third of the coelomocytes in the CF in some species and can appear in either petaloid or filopodial morphologies similar to phagocytes in sea urchins, although clear distinctions among phagocyte types are not evident (Figs. 5j and 6c, f) (Hetzel 1963; Chia and Xing 1996; Eliseikina and Magarlamov 2002; Xing et al. 2008; Ramírez-Gómez and García-Arrarás 2010). Cells in the petaloid or bladder morphology exhibit extensive motility of their cytoplasmic veils and are highly phagocytic, and the process of phagocytosis has been linked to the transformation from the petaloid to the filopodial morphology (Chia and Xing 1996). Cytoskeletal staining of settled sea cucumber phagocytes shows broad lamellipodial regions with the characteristic dendritic actin network consistent with extensive centripetal motility, as well as centralized microtubule arrays (Fig. 6c, f). Phagocytes in sea cucumbers contribute to cell aggregation and clotting, and make up about two thirds of the cells found in early stages of aggregation (Taguchi et al. 2016).

Spherule Cells

Spherule cells (also called amoebocytes) are rounded or ovoid shaped (8–20 μm in diameter) in suspension with a small nucleus that has condensed chromatin, and large cytoplasmic granules containing mucopolysaccharides and protein (Fontaine and Lambert 1977; Canicatti and D'Ancona 1989; Smith 1981; Chia and Xing 1996; Pinsino and Matranga 2015). Functions assigned to this coelomocyte type include antibacterial activity, inflammation, wound healing, encapsulation, graft

rejection, and cytotoxic activity (reviewed in Gross et al. (1999)). Sea urchins typically contain red and colorless (also called white) spherule cells (Fig. 5a, b, f–h) that make up 5–40% of the total coelomocyte population (Ramírez-Gómez and García-Arrarás 2010; Smith et al. 2010), depending on the species as well as the pathophysiological condition, which varies among animals. The spherule cells are round in suspension but once in contact with a substrate they exhibit actin-based amoeboid-like motility and can extend elongate processes (Figs. 5a, b, f–h and 6a). The red spherule cells have a distinct pigmentation due to the chemical echinochrome A within the cytoplasmic granules, which is a naphthaquinone double-ring structure that forms peroxide in the presence of extracellular concentrations of calcium (Perry and Epel 1981) and exhibits antibacterial activity against a variety of marine and nonmarine microbes (Service and Wardlaw 1984). It is produced from the activities of a distinctive set of enzymes that are expressed in larval pigment cells (see section "Pigment Cells") and red spherule cells in adults (Calestani et al. 2003). Colorless spherule cells (also called white spherule cells) in sea urchins display cytotoxic activity against mammalian cells in vitro, an activity that is increased by the presence of phagocytes (Arizza et al. 2007). The spherule cell type in sea cucumbers (Fig. 5j) appears to be particularly variable with multiple subtypes, defined in the literature on the basis of color (colorless, green, or yellow), presence of granules, reaction to histological and histochemical stains, and/or appearance in transmission electron micrographs (reviewed by Chia and Xing (1996)), although there is no general agreement on a standardized nomenclature for these subtypes. Like sea urchin spherule cells, these cells in sea cucumbers exhibit pseudopodia-based amoeboid-like motility (Hetzel 1963; Fontaine and Lambert 1977). In sea stars, red and colorless spherule cells have been reported only as a relatively rare coelomocyte type (Pinsino et al. 2007).

Vibratile Cells

Vibratile cells are spherical and 5–10 μm in diameter, with an irregular nucleus and large cytoplasmic granules, and they are distinctive because of their single long flagellum. They are highly motile, which has been suggested to assist in circulation of the CF (Smith 1981; Chia and Xing 1996). Vibratile cells degranulate during the CF clotting process, may be a source of some clotting proteins, and may also function in hemostasis (Chia and Xing 1996). In sea urchins, vibratile cells are relatively abundant, making up 8–20% of the total population in the CF (Fig. 5a, b, e) (Vethamany and Fung 1972; Arizza et al. 2007; Jellett et al. 1988; Matranga et al. 2006). In live preparations of freshly withdrawn CF, these cells have also been observed in sea cucumbers (Eliseikina and Magarlamov 2002), although they are rarely reported for sea stars (Pinsino et al. 2007). In general, vibratile cells are not consistently present in all echinoderm classes and their functions are not understood.

Hemocytes

Hemocytes are relatively large (10–23 μm) coelomocytes that are biconcave or spherical with a round nucleus and hemoglobin in their cytoplasm (Hetzel 1963; Smith 1981; Chia and Xing 1996). They are prominent in some species of sea cucumbers, have been preliminarily reported in some sea stars, and are thought to

function in oxygen transport (Hetzel 1963; Eliseikina and Magarlamov 2002; Pinsino et al. 2007).

Progenitor Cells

Progenitor cells, also called lymphocytes, are small, spherical cells of 2–8 μm in diameter, with large round nuclei, prominent nucleoli, and a thin rim of minimal cytoplasm. These cells show morphological similarities to small lymphocytes in vertebrates (Smith 1981) and have been hypothesized to be stem cells from which other coelomocytes are derived, although this hypothesis has not been tested. They are the predominant coelomocyte type in several sea cucumber species (Eliseikina and Magarlamov 2002; Taguchi et al. 2016), although their presence in sea urchins and sea stars has not been well documented and, if present, they may not be found in the CF.

Crystal Cells

Crystal cells are rhomboid shaped and 2–24 μm in length, with a crescent-shaped heterochromatic nucleus and a rectangular vacuole (Chia and Xing 1996; Eliseikina and Magarlamov 2002). These cells appear to be restricted to sea cucumbers, in which they are a rare coelomocyte type, making up fewer than 0.5% of the total coelomocyte population. Structurally similar cells have been noted within the CF that have a central vacuole containing small crystal-like structures, which appear to become crystal cells in live preparations under conditions of increasing osmotic pressure (Eliseikina and Magarlamov 2002). The function of crystal cells is unknown.

Immune Cells in Larval Sea Urchins

Most echinoderm species develop through an intermediate larval stage, which lives for several weeks to months prior to metamorphosis into a juvenile form. That these free-swimming, feeding larvae survive in microbe-rich seawater for this length of time suggests that even in this morphologically simple life stage, echinoderms rely on robust immune systems. The immune cells of echinoderm larvae were originally discovered by Metchnikoff on the basis of his experiments inserting rose prickles into the blastocoel of a sea star larva (Metchnikoff 1893). He observed migrations of mesenchymal cells toward the foreign surface and its subsequent encapsulation. Although this was the first demonstration of phagocytosis and encapsulation in an echinoderm (or in any animal), in the century since Metchnikoff's landmark work, which earned him the 1908 Nobel Prize in Physiology or Medicine, mesodermal immune cells have been classified for many different species of echinoderm larvae (Furukawa et al. 2009; Hibino et al. 2006; Ho et al. 2016; Kominami et al. 2001; Silva 2000). Collectively, larval immunocytes share several similarities with adult coelomocytes, yet they also exhibit some key differences.

Pigment Cells

Pigment cells in the larval sea urchin *S. purpuratus* (Fig. 7a1) are granular, stellate cells, which migrate through the blastocoel and embed within or are closely apposed to the larval ectoderm (Krupke et al. 2016), and by 10 days post fertilization (dpf),

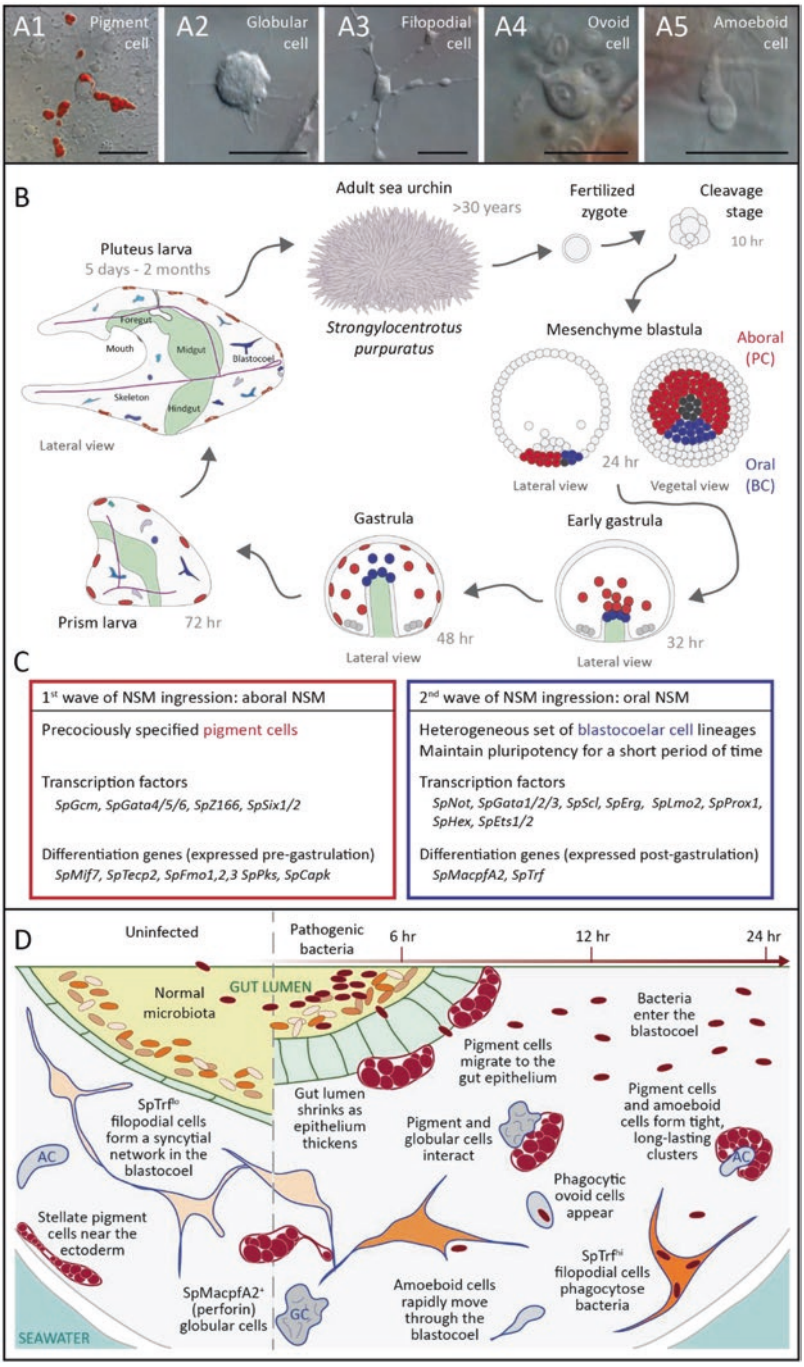

Fig. 7 *Purple sea urchin larvae have mesodermally derived immune cell types that respond rapidly to gut infection*. (**a**) The purple sea urchin larva contains five types of immune cells. Pigment cells (a1) are present in the ectoderm and have stellate morphology in their resting state.

the larvae have approximately 80 pigment cells. These cells have two to four pseudopodia, which constantly move within the plane of the ectoderm (Gibson and Burke 1987; Ho et al. 2016) and are filled with ~40 small, red granules (1–2 μm in diameter). The red pigment is due to echinochrome A, which is also present in the adult red spherule cells (see section "Spherule Cells") that mediate wound healing and antibacterial responses, and these two cell types are likely homologous. Larval pigment cells express a suite of genes that are involved in several aspects of the immune response (Table 1). These include genes encoding the transcription factor c-rel (Ransick and Davidson 2012), the cytokine macrophage migration inhibitory-like factor 7 (SpMif7; also known as dopachrome tautomerase (SpDopT) (Rast et al. 2002; Ho et al. 2016)), a thioester-containing protein (SpTecp2), the scavenger receptor SpSRCR142 (Ho et al. 2016), and polyketide synthase (SpPKS), an enzyme involved in echinochrome biosynthesis (Calestani et al. 2003). In vertebrates, SRCRs are involved in pathogen recognition and clearance, and adult coelomocytes also express a complex and dynamic array of *SpSRCR* genes (Pancer 2000). Whole-mount in situ hybridization (WMISH) shows that while both *SpSRCR142* and *SpTecp2* are expressed in cells that also express *SpPKS*, conversely cells that are *SpPKS* negative do not express *SpSRCR142* or *SpTecp2*, suggesting that pigment

Fig. 7 (continued) Four types of blastocoelar cells are classified on the basis of morphology and behavior: globular cells (a2), a subset of filopodial cells that form a network within the blastocoel (a3), ovoid cells (a4), and amoeboid cells (a5). The scale bars are 20 μm. (**b**) The purple sea urchin has a biphasic life history. The life cycle times shown apply to *S. purpuratus*. Fertilized zygotes undergo rapid cleavage to form a mesenchyme blastula by 24 h post fertilization (hpf). The mesoderm is partitioned along the oral (blue)–aboral (red) axis and contains precursors of pigment cells (PC) and blastocoelar cells (BC). Pigment cell precursors in the aboral mesoderm ingress into the blastocoel at the onset of gastrulation at ~30 hpf. Expression of *SpGata1/2/3* and *SpScl* homologues mark the oral mesoderm, which gives rise to several blastocoelar cell types that ingress late in gastrulation (>42 hpf). Larvae initiate feeding and contain a tripartite gut (foregut, midgut, and hindgut), as well as a calcite skeleton. Pigment cells are often found apposed to the ectoderm. The blastocoel contains several morphologically distinct types of immunocytes illustrated in panel (**a**). The sizes of the adult sea urchin and the cleavage stages are not drawn to scale. (**c**) Larval immunocytes are patterned from two fields of mesoderm. Pigment cell (red box) and blastocoelar cell (blue box) precursors reside in the aboral and oral region of the mesoderm, respectively (a schema of the vegetal view of blastula-stage mesoderm is shown in panel (**b**)). The relevant gene expression for each cell type is listed within each box. For a more detailed gene regulatory network of immunocyte patterning, see Fig. 8. (**d**) An organism-wide larval response to gut-associated bacterial perturbation occurs among different cells and tissues. Larvae fed high concentrations of the marine bacteria *Vibrio diazotrophicus* and imaged with time-lapse microscopy over the course of the infection exhibit a reproducible and reversible cellular response across the entire animal. A timeline of the larval response is shown above the graphic summary of the changes that occur in the gut morphology, cell behavior, and gene expression levels. In uninfected conditions, dendritic pigment cells are apposed to the ectoderm and low levels of *SpTrf* (formerly known as *Sp185/333*) gene expression are evident in select filopodial cells. *Vibrio diazotrophicus* exposure induces a robust thickening of the gut epithelium and elicits cell migrations. Pigment cells and amoeboid cells migrate to the gut within 24 h of exposure to *Vibrio*, and upregulation of *SpTrf* occurs in subsets of filopodial cells. As bacterial cells escape from the gut into the blastocoel, they are phagocytosed by SpTrf-positive ($SpTrf^+$) cells. AC amoeboid cell, GC globular cell. (Modified from Ho et al. (2016) and reprinted with permission from the Nature Publishing Group)

cells may be more heterogeneous than previously appreciated. Mature pigment cells express a battery of genes that imply various cellular functions. These include the single copy gene encoding the ephrin homologue (*Eph*), which facilitates cellular trafficking to the ectoderm (Krupke et al. 2016), the gene encoding the multidrug resistance transporter ABCC5a (Shipp et al. 2015), and genes involved in metabolism, including *cyclin-dependent AMP kinase* (*SpCAPK*; Rast et al. (2002)), sulfotransferase (*SpSult*), and several members of a flavin-monoxygenase family (*SpFmo1*, *−2*, *and −3*, and *−a*).

Blastocoelar Cells

Blastocoelar cells in the larval sea urchin are a mixed population of cells that are present in the blastocoel and exhibit several distinct morphologies, behaviors, and functions (Fig. 7a2–5), particularly with regard to immune function (Ho et al. 2016). Blastocoelar cells are phagocytic (Silva 2000), exhibit immune surveillance–like behavior, and participate in cell–cell interactions during the course of an immune response. Four distinct types of blastocoelar cells have been described and are termed globular, filopodial, ovoid, and amoeboid cells (Ho et al. 2016).

Globular Cells

Globular cells are large (10–15 μm), vessicular, and relatively slow moving (2 μm/min) (Fig. 7a2). Several of these cells wander in the blastocoel while others cluster in the tips of larval arms and at the aboral apex of the body. Globular cells exhibit surveillance-like behavior by extending short pseudopodial projections. They express *SpMacpfA2*, a perforin-like gene, which is a member of the multigene family that encodes proteins characterized by conserved membrane attack complex/perforin (MACPF) domains (Table 1). MACPF domains are also present in vertebrate perforins (lytic molecules secreted onto target cells by NK cells and T killer cells), in *Mpeg1* (a macrophage-specific gene conserved in fish and tetrapods), and in the complement proteins C6–C9 (which are members of the terminal pathway (Anderluh et al. 2014)), leading to the speculation that these cells may have killer activity.

Filopodial Cells

Filopodial cells are small cells (5–7 μm in diameter) that form a syncytial network within the blastocoel (Fig. 7a3) (Tamboline and Burke 1992). These cells extend two to five long, branching filopodia (10–50 μm) that span the blastocoel and connect the filopodial cells with the gut, epidermis, and skeletal rods. Although filopodial cells show little net movement within larvae, the nucleus and cytoplasm move extensively along the cellular processes. At the late gastrula stage (50 h post fertilization (hpf)) there are 10–15 filopodial cells per embryo in *S. purpuratus*, and by 72 hpf there are 20 in the early (prism) larval stage (Tamboline and Burke 1992). In response to immune challenge, subsets of filopodial cells express the immune effector *SpTransformer* gene family (see section "The *SpTransformer* Gene Family in Euechinoids") and are able to phagocytose bacteria in the blastocoel (Ho et al. 2016).

Ovoid Cells

Ovoid cells are oval-shaped cells measuring approximately 10–15 μm along their long axis (Fig. 7a4). They are motile, granular cells, which likely emerge rapidly from the filopodial networks, and appear at the sites of microbes within the blastocoel (Ho et al. 2016). When present, they are very efficient phagocytes.

Amoeboid Cells

Larvae (10 dpf) have two to five motile amoeboid cells (Fig. 7a5) within the blastocoel, which are medium-sized (5–10 μm), comma shaped, rapidly motile (5 μm/min), and morphologically similar to colorless spherule cells in the adult. Amoeboid cells traffic rapidly between the gut epithelium and the larval ectoderm, and maintain interactions with several other cell types. This activity is attenuated during immune responses in infected larvae, such that their migration rate slows (< 2 μm/min) as they interact with other cell types. Their most frequent and longest interactions are with pigment cells, which are dynamic and can last several hours.

Immune Cells in Larval Sea Stars

Immune cell morphology and behavior in asteroid larvae are similar to those in echinoid larvae. In the sea star *Patiria pectinifera*, most mesenchyme cells in bipinnaria larvae (4 dpf), which account for ~1% of the total larval cell number, are distributed beneath the body wall and move randomly, forming a dynamic network structure (Furukawa et al. 2009). This distributed pattern is effective for phagocytosis of both cellular constituents released from the ectodermal cells and foreign substances that access the blastocoel through the body wall. On the basis of their strong phagocytic activity and the characteristics of the larval immune cells, the mesenchyme cells are functionally equivalent to blastocoelar cells in larval echinoids. However, differences in size and morphology are not observed for the sea star cells, and no functional subgroups of cells have been defined.

Larval Immune Cell Development

The development of larval immune cells is best characterized in the purple sea urchin, *S. purpuratus* (Gibson and Burke 1985, 1987; Hibino et al. 2006; Ho et al. 2016; Krupke et al. 2016; Materna and Davidson 2012; Materna et al. 2013; Ransick and Davidson 2006, 2012; Schrankel et al. 2016; Solek et al. 2013; Tamboline and Burke 1992) and in the Japanese sea urchin *Hemicentrotus pulcherrimus* (Kominami 2000; Kominami et al. 2001; Shoguchi et al. 2002; Tokuoka et al. 2002; Kominami and Takata 2003; Katow 2004; Ohguro et al. 2011). Fate-mapping experiments show that two lineages of larval immune cells develop from the asymmetric allocation of a one-cell-thick ring of nonskeletal mesodermal (NSM) cells, which are patterned in early embryogenesis (Fig. 7b) (Kominami and Takata 2003; Ruffins

and Ettensohn 1996). The first is the precociously specified pigment cell lineage, which arises early in gastrulation (Fig. 7b, c). The second lineage develops into the heterogeneous blastocoelar cell population (Gibson and Burke 1985; Tamboline and Burke 1992; Hibino et al. 2006; Ho et al. 2016). Pigment cells express lineage-specific markers by 20 hpf, whereas the blastocoelar cell precursors remain undifferentiated for several hours (Fig. 7b, c). They do not express terminal differentiation markers until 48 hpf or later, when they differentiate into globular, filopodial, amoeboid, or ovoid cells (see section "Immune Cells in Larval Sea Urchins") (Solek et al. 2013; Schrankel et al. 2016).

Immune cell development in echinoderm larvae has illuminated a regulatory heritage that is shared across phyla (Schrankel et al. 2016; Solek et al. 2013). These processes are largely controlled by a suite of transcription factors that regulate hematopoiesis on the basis of their homology to proteins in vertebrates and *Drosophila*. Analysis of the *S. purpuratus* genome identified representatives of each major transcription factor family. As echinoderms diverged prior to the several rounds of whole-genome duplication within the vertebrate lineage, most of these factors are encoded by single gene orthologues (Table 1). Interactions among these proteins are described using well-characterized gene regulatory network (GRN) models (Fig. 8).

Gene Regulatory Networks that Control Larval Immune Cell Differentiation

The first step in larval immune cell differentiation is specification of a ring of NSM cells in the mesenchyme blastula, which are positioned at the point at which invagination will occur during gastrulation (Fig. 7b). NSM cell specification is initiated when Delta/Notch (D/N) signaling (Materna and Davidson 2012; Sherwood and McClay 1999; Sweet et al. 2002) activates expression of the gene encoding the transcription factor glial cells missing (*SpGcm*; Ransick and Davidson 2006). The NSM ring is segregated along the oral–aboral axis (OA; also termed the dorsal–ventral axis) in a process that involves the reciprocal activities of Nodal and BMP2/4 signaling (Duboc et al. 2010; Lapraz et al. 2015). Cells within the oral side of the NSM ring develop into the blastocoelar cell lineages, whereas pigment cell precursors form in the aboral two thirds of the ring (Fig. 7b). Following the early activation of *SpGcm* throughout the NSM, its expression resolves to the aboral side, where it specifies the pigment cell lineage in concert with the transcription factor SpGata4/5/6 (also known as Gatae (Lee and Davidson 2004; Materna et al. 2013)). Together, SpGcm and SpGata4/5/6 activate downstream differentiation gene batteries (Calestani et al. 2003; Calestani and Rogers 2010), and perturbation of either factor results in "albino" larvae that lack pigment cells.

The regulatory state of the oral NSM is initiated by the homeobox factor SpNot, which works synergistically with D/N signaling to activate genes involved in blastocoelar cell specification (Fig. 8) (Materna et al. 2013; Materna and Davidson 2012). Upon development to the mesenchyme blastula stage, blastocoelar cells

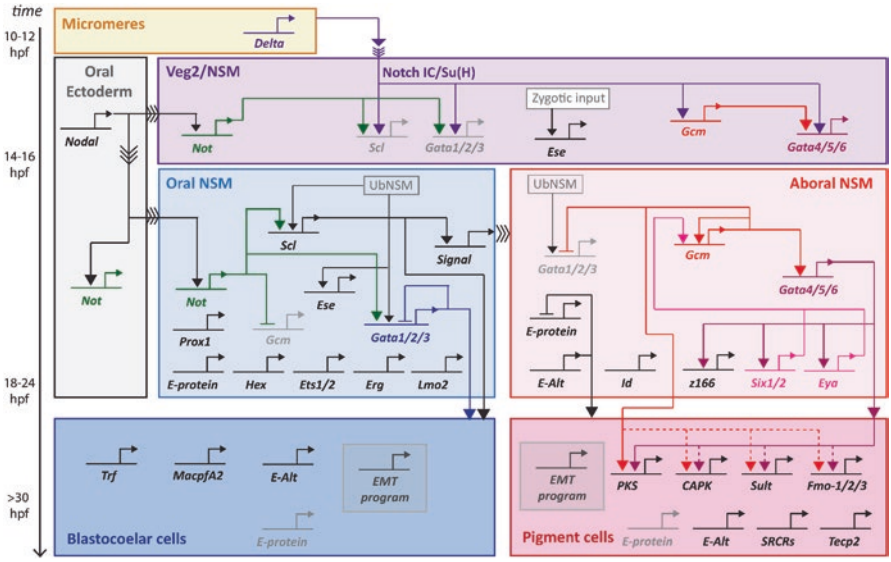

Fig. 8 *A gene regulatory network model describing larval immune cell differentiation in the purple sea urchin (S. purpuratus) embryo*. Larval immune cells develop from a ring of mesodermal precursor cells over the course of about 24 h. An approximate time scale is shown on the right. Colored boxes indicate regulatory states in tissues or cell types. Regulatory interactions are indicated by arrows (indicating gene activation) or crossbars (indicating gene repression). Signal transduction events among regulatory states are shown as chevrons. Genes shown in colors are transcriptionally active; genes in gray are not expressed. Interactions between regulatory genes and downstream terminal target genes are indicated by connecting lines. The nonskeletal mesoderm (NSM) territory is initially specified following Delta/Notch (D/N) signaling from the micromeres, which activates *SpGcm* and *SpGata4/5/6* expression in a coherent feed-forward loop. Nodal signaling from the oral ectoderm subsequently activates *SpNot* expression in the NSM by 16 hpf. After 18 hpf, parallel inputs from D/N and SpNot activate *SpGata1/2/3* and *SpScl* expression throughout the NSM, which is spatially variable and low. SpNot represses *SpGcm* in the oral NSM after 18 hpf and enriches *SpGata1/2/3* and *SpScl* expression in these cells. A ubiquitous NSM activator (UbNSM) and an unknown SpScl-dependent mechanism maintain expression of the oral NSM transcription factors. The aboral NSM regulatory state is specified when SpGcm and SpGata4/5/6 restrict *SpGata1/2/3* expression and also induce directly a terminal differentiation battery encoding pigment synthesis genes (dotted lines indicate predicted inputs that have not been verified experimentally). Proper expression of *SpGata1/2/3* and *SpScl* in the NSM are required for immunocyte epithelial-mesenchymal transition (EMT) and potential activation of downstream markers in blastocoelar cells, such as the expression of an alternative isoform of the *SpE-protein* gene (*SpE-Alt*). SpE-Alt activity negatively regulates expression of the canonical *SpE-protein* isoform within developing immunocytes. (This GRN model integrates data from Ransick and Davidson (2006), Duboc et al. (2010), Materna et al. (2010), Materna and Davidson (2012), Ransick and Davidson (2012), Materna et al. (2013), Solek et al. (2013), and Schrankel et al. (2016). A complete GRN describing endomesoderm development can be found at http://sugp.caltech.edu/endomes/)

express a suite of genes encoding transcription factors including *SpErg* (Rizzo et al. 2006), *SpGata1/2/3* (Davidson et al. 2002; Duboc et al. 2010; Solek et al. 2013), *SpLmo2* (Duboc et al. 2010; Solek et al. 2013), and *SpScl* (Duboc et al. 2010; Solek et al. 2013). The expression levels of *SpGata1/2/3*, *SpScl*, and *SpErg* peak shortly

after the mesenchyme blastula stage, after which they are sharply downregulated (Solek et al. 2013). Notably, these transcription factors are also expressed in adult coelomocytes (Pancer et al. 1999). The usage of SpGata, SpScl, and SpErg transcription factors in the sea urchin reflects deeply conserved regulation of deuterostome immune cell development. In zebrafish and tetrapods, gata1/2/3 and scl factors are also activated by Notch and BMP during hematopoietic stem cell emergence (Davidson and Zon 2004; Kim et al. 2014; Oren et al. 2005; Walmsley et al. 2002). In vertebrate hematopoietic stem cells, *gata-2*, *scl*, and *fli-1* genes are molecularly connected in a bistable positive feedback loop that maintains a self-renewing, pluripotent state, and immune cell differentiation occurs only upon the downregulation of *gata-2* (Narula et al. 2010, 2013; Pimanda et al. 2007). Similarly, in sea urchin larvae, immune gene markers are not expressed until several hours after *SpGata1/2/3* and *SpScl* are downregulated (Fig. 8).

In vertebrates, gata1/2/3, scl, and lmo2 factors form multifactor transcriptional complexes that regulate various stages of hematopoiesis (Wilson et al. 2010), and their activities are modulated by the E-protein basic helix-loop-helix (bHLH) factors (E2A, E2–2, and HEB) (reviewed in De Pooter (2010) and Kee (2009)). Similar interactions are believed to occur in the development of sea urchin larval immune cells. The *S. purpuratus* genome sequence contains a single orthologue encoding these bHLH factors, known as *SpE-protein*, which is expressed as two isoforms: a longer, canonical form (SpE-Can) and a truncated, alternative form (SpE-Alt), which is generated from a secondary transcriptional start site and is homologous with alternative transcripts in vertebrate *HEB* and *E2-2* (Skerjanc et al. 1996; Wang et al. 2006). *SpE-Can* is expressed ubiquitously in the early embryo (Howard-Ashby et al. 2006; Schrankel et al. 2016; Solek et al. 2013). *SpE-Alt* expression initiates in the aboral NSM and expands to blastocoelar cells once they initiate ingression from the vegetal plate and immune marker expression (Fig. 8). Perturbation of *SpE-Alt* expression leads to diminished migratory activity of both pigment and blastocoelar cells, and abrogates terminal marker expression in blastocoelar cells. Furthermore, the two isoforms exhibit mutually exclusive expression patterns; as *SpE-Alt* expression increases in immunocytes after gastrulation, *SpE-Can* transcription is diminished in these cells. This affect requires SpE-Alt activity, which highlights a novel regulatory connection in E-protein biology.

Among echinoderm species, some aspects of larval immune cell development are highly conserved whereas others exhibit considerable variation. The best example of this is the absence of pigment cells outside the echinoid lineage. At the molecular level, however, in the asteroid *P. miniata*, orthologues of *SpEts1/2* and *SpGata1/2/3* are coexpressed in mesodermal progenitor cells during the blastula stage (McCauley et al. 2010). Subsets of these cells migrate and behave similarly to sea urchin larval blastocoelar cells.

The Immune Response in Echinoderm Larvae

The immune cells of echinoderm larvae display a variety of immune behaviors, which were originally discovered by Metchnikoff on the basis of his experiment of inserting rose prickles into the blastocoel of a larval sea star (Metchnikoff 1893).

Since Metchnikoff's landmark work that earned him the Nobel Prize in 1908, mesoderm-derived immune cells have been classified across many different species of echinoderm larvae (Furukawa et al. 2009; Hibino et al. 2006; Ho et al. 2016; Kominami et al. 2001; Silva 2000). More recent descriptions that duplicated the Metchnikoff experiment, but at an earlier point in embryonic development of the sea star *Patiria pectinifera* and the sea urchin *Lytechinus variegatus*, have defined the stage at which the embryos become competent for phagocytosis (Silva 2000; Furukawa, 2009). When foreign particles are injected into the blastocoel at the mid-gastrula stage of the sea star, they are quickly phagocytosed by the mesenchyme cells that are released from the tip of the archenteron (R. Furukawa, unpublished observation, 2009). This is in agreement with the description by Metchnikoff a century ago. When the yeast *Saccharomyces cerevisiae* is injected into the blastocoel at the hatched blastula stage, it is not phagocytosed by the mesenchyme cells until mid-gastrula, which indicates the onset of abilities not only for phagocytosis but also for detection of nonself. These results indicate that phagoytosis begins at the same developmental stage for both sea stars and sea urchins.

The Immune Response in Sea Star Larvae

In the bipinnaria larvae of the sea star *Patiria pectinifera*, the distribution pattern of the mesenchyme cells within the blastocoel is effective for phagocytosis of both cellular constituents released from the ectodermal cells and foreign substances that access the blastocoel through the body wall (Furukawa et al. 2009). Although larval immune cells phagocytose foreign particles injected into the blastocoel within 2 h, when small amounts of small foreign particles (≤~10 μm in diameter) are injected into the blastocoel, individual cells in the blastocoel readily phagocytose the particles. When larger amounts of foreign materials are injected, or when particle sizes are large relative to the mesenchyme cell sizes, multiple cells converge on the site from within the blastocoel and undergo cell–cell fusion to form a syncytial multinucleated giant cell that encapsulates the aggregated particles. Similar syncytial formations have also been noted for adult phagocytes in vitro (Majeske et al. 2013b). The number of recruited immune cells is dependent on the amount and size of the foreign substance (Furukawa et al. 2009), indicating that the process of clearing the blastocoel is strictly regulated by the larval immune system.

The immunoreactive migration and encapsulation of foreign particles by mesenchyme cells in the sea star *P. pectinifera* are regulated by two macrophage migration inhibitory factors (MIFs): *Ap*MIF2 and *Ap*MIF1 (Furukawa et al. 2016). (The names of these proteins and those described below have the prefix *Ap*, reflecting their description relative to the genus name of *Asterina* rather than *Patiria*; this change was made on the basis of the revision of the Asterinidae (O'Laughlin and Waters 2004).) *Ap*MIF1 and *Ap*MIF2 act sequentially first to stimulate and then to inhibit chemotactic activity, respectively, and thereby coordinate the regulated recruitment of the appropriate numbers of mesenchyme cells during the immune response. During this chemotactic migration, a member of the dedicator of cytokinesis 1 (DOCK180; ~180 kDa) superfamily in the sea star *P. pectinifera*, *Ap*DOCK,

regulates F-actin organization at the leading edge of lamellipodia in mesenchyme cells (Furukawa et al. 2012a). Actin organization under control of *Ap*DOCK is also essential for the persistence of encapsulation. Perturbation of *Ap*DOCK results in imperfect lamellipodial formation and in deficient membrane ruffling at the leading edge of mesenchyme cells. These immune effectors are evolutionally conserved, and homologues are present in the sea urchin genome sequence. Therefore, the immune cell behaviors of chemotaxis, cytoskeletal modifications, and syncytia formation in the sea urchin may also be regulated by the orthologues of MIF and DOCK.

The Immune Response in Sea Urchin Larvae

After exposure to the marine bacteria *Vibrio diazotrophicus*, which are ingested as a potential food source, sea urchin larvae initiate a system-wide suite of stereotypic cellular and transcriptional responses (Ho et al. 2016). Within 6 h of bacterial exposure, the gut epithelium thickens, reducing significantly the luminal volume of the midgut (Fig. 7d) (Buckley et al. 2017). By 12 h post exposure (hpe), pigment cells in both the ectoderm and blastocoel migrate more rapidly, whereas amoeboid cell velocity decreases. After 24 h, pigment cells accumulate at the gut epithelium, although some of this cellular activity may be in response to *Vibrio* bacteria that penetrate the gut epithelium and enter the blastocoel.

Coincident with this cellular response, larvae exhibit a series of transcriptional changes in both the gut and the peripheral immune cells. Surveys of gene activity indicate that of the >1000 genes annotated with immune function (Table 1) (Hibino et al. 2006), 200 exhibit a greater than threefold change in expression in the course of the larval response to contact with *Vibrio*. These genes encode immune receptors, intercellular signaling molecules, signal mediators, transcription factors, and effector molecules (K. M. Buckley and J. P. Rast, unpublished data, 2015). Embryos express the complement component *SpC3* and transcript levels from this gene increase in response to bacterial contact (Shah et al. 2003), which may suggest an opsonic function for the SpC3 protein similar to that shown in adult sea urchins (Clow et al. 2004). There are several genes in the sea urchin genome sequence encoding thioester-containing proteins that may have complement function in opsonizing pathogens. Thioester-containing protein 2 (*SpTecp2*) is expressed in larval pigment cells, where it is slightly upregulated in response to *Vibrio* infection (Ho et al. 2016). Conversely, macrophage inhibitory factor 7 (*SpMif7*) is quickly downregulated in pigment cells in response to *Vibrio*. Homologues of Mif factors act as cytokines in other systems and they may have similar functions in infected larvae. Finally, the immune effector gene family *SpTransformer* (reviewed in Ghosh et al. (2010) and Smith and Lun (2017); see also section "The *SpTransformer* Gene Family in Euechinoids") is upregulated in filopodial blastocoelar immune cells by 24 h after *Vibrio* exposure (Ho et al. 2016).

In the course of the larval immune response to *Vibrio diazotrophicus*, the most strongly activated genes belong to two families of interleukin-17 (IL-17) homologues (Buckley et al. 2017). IL-17 cytokines are of central importance in the vertebrate immune response, where they are expressed in lymphocytes as well as in

epithelial barrier cells (Korn et al. 2009; Song et al. 2011). The *S. purpuratus* genome encodes 35 *IL-17* genes (Table 1), which form ten subfamilies based on sequence similarity. Genes within two of these families (*SpIL-1-1* and *SpIL17-4*) are upregulated in larvae within 2–4 hpe to *Vibrio* and expression is largely attenuated within 24 h. Analyses using whole-mount in situ hybridization and BAC-based fluorescent reporter protein constructs indicate that expression of these transcripts is restricted to gut epithelial cells in infected larvae. To assess the functions of these cytokines in the larval immune response, SpIL-17 signaling was perturbed by interference with correct splicing of its receptor *SpIL-17R1* (Buckley et al. 2017). Larvae subject to this perturbation exhibit decreased transcription of several genes associated with IL-17 in vertebrates (*tnfaip3*, *nfkbiz*, *cebpα*, and *cebpγ*) in response to immune challenge. Notably, expression of the *SpIL17-4* genes is also reduced, which hints at potential feedback mechanisms among these factors. Finally, homologues of the *IL-17* genes are present in the genomes of four additional sea urchin species (including the cidaroid *Eucidaris tribuloides*), as well as the asteroid *Patiria miniata* (Buckley et al. 2017), suggesting an ancient and conserved function for these cytokines within echinoderm immunity. Together, these data point to a complex larval immune response including both features that are novel to the echinoderm lineage and those that were present in the last common deuterostome ancestor.

Immune Response Genes and Proteins in Echinoderms

SRCR Genes and Proteins

The *S. purpuratus* genome sequence has nearly 1100 regions that encode SRCR domains. Similarly expanded *SRCR* repertoires are present in the genome sequences of the sea urchin *L. variegatus* and the sea star *Patiria miniata*, suggesting that this feature is common in echinoderms (Buckley and Rast 2015). Proteins containing multiple SRCR domains (termed SRCR proteins; see section "Pattern Recognition Receptors") are involved in the innate immune system of metazoan animals (Sarrias et al. 2004). The diversity of the sea urchin *SpSRCR* repertoire was predicted from a transcriptional analysis of coelomocytes prior to the genome sequencing project (Pancer 2000, 2001). Analysis of coelomocyte transcripts by northern blot shows that individual sea urchins express unique patterns of *SpSRCR* transcripts, and genome blots confirmed that this intraspecific variation was consistent with variations in the *SpSRCR* gene family (Pancer 2000). Furthermore, expression profiles exhibit dynamic shifts after immune challenge. This level of polymorphism in the population of *S. purpuratus* and the variability in expression of these genes in coelomocytes suggest not only complex functions of the encoded proteins but a complex system to control expression of this multigene family.

Although the function of SpSRCR proteins in the immune system is unknown, ApSRCR1 acts as a bacterial opsonin in both the larval and adult stages of the sea star *Patiria pectinifera* (Furukawa et al. 2012b). The *Ap*SRCR1 protein has nine SRCR domains, one short consensus repeat (SCR), one transmembrane region, and a very

short cytoplasmic tail. It is localized to cytoplasmic vesicles in larval mesenchyme cells and adult coelomocytes. When bacteria invade the larval blastocoel or the coelomic cavity of the adult, *ApSRCR1* gene expression is upregulated and the extracellular region of the *Ap*SRCR1 protein is secreted into the larval blastocoel or the adult coelomic cavity. This fragment binds to and aggregates bacteria and promotes phagocytosis by the larval and adult immune cells. The SpSRCR7.2 protein in the sea urchin *S. purpuratus* has a similar structure (with seven SRCR domains, an SCR, and a transmembrane region (Pancer 2000)) and may have similar activities to *Ap*SRCR1. The diverse structure of the *SRCR* gene family within and among species from different echinoderm classes and the dynamic expression patterns among individuals are consistent with important innate immune response functions for host protection.

The *SpTransformer* Gene Family in Euechinoids

The purple sea urchin, *Strongylocentrotus purpuratus*, responds to a variety of immune challenges, such as marine bacteria and PAMPs, with a rapid upregulation of the *Sp185/333* gene family (Rast et al. 2000; Nair et al. 2005; Terwilliger et al. 2006, 2007). Because no sequence similarity is apparent within species from other phyla, other echinoderm classes, or the cidaroid family of echinoids, the transcripts were named on the basis of matches to one full-length cDNA sequence (DD185) (Rast et al. 2000) and one partial cDNA sequence (EST333) (Smith et al. 1996) from sea urchin coelomocytes. The original name was meant to avoid implications for protein function, which were unknown and could not be predicted on the basis of the sequence. However, analysis of one recombinant protein has elucidated its structure and function (see section "SpTrf Protein Functions"), which has provided the opportunity for a name change to *SpTransformer* (*SpTrf*) (Lun et al. 2016, 2017a, b) (for reviews, see Ghosh et al. (2010), Smith (2012), and Smith and Lun (2017)). The name *SpTrf* will be used hereafter in this chapter.

The *SpTrf* cDNA sequences are remarkably diverse and characterized by large blocks of shared sequences called *elements*, which are present in mosaics and result in a variety of *element patterns* (Fig. 9) (Terwilliger et al. 2006, 2007). *SpTrf* genes are composed of two exons, of which the second exon is characterized by six types of repeats: a series of two to four imperfect tandem repeats near the 5′ end of the second exon, as well as five types of interspersed repeats located toward the 3′ end (Buckley and Smith 2007). In addition to the variability among the element patterns, genes of identical sequence are not shared among animals, indicating the level of diversity within the population (Buckley and Smith 2007). The sequence diversity of the genes and transcripts, as well as their upregulation in response to challenge (Rast et al. 2000; Nair et al. 2005; Terwilliger et al. 2007), predict that these genes are involved in the sea urchin immune response.

SpTrf Genes Are Clustered in the Genome

The *S. purpuratus* genome sequence (v4.2) contains six tandem *SpTrf* genes that are clustered in a single locus. However, the sequence diversity of genes isolated from

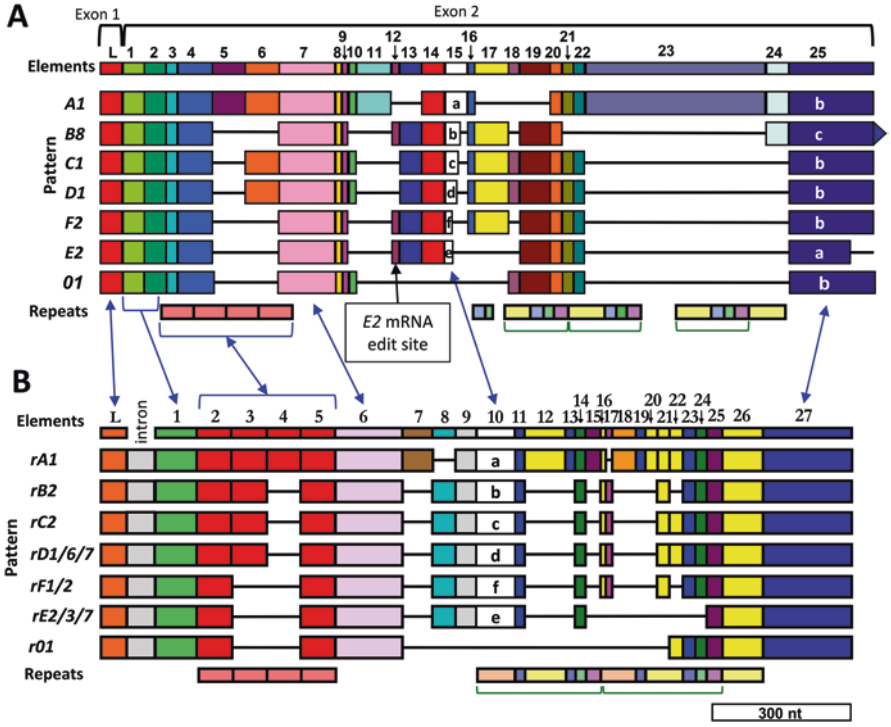

Fig. 9 *The SpTrf transcript sequences are diverse*. (**a**) The cDNA-based alignment of message sequences is based on Terwilliger et al. (2006, 2007). Blocks of sequences, called elements, are shown as colored rectangles, and large gaps are indicated by horizontal black lines. Gene exons are indicated at the top. The leader (L) is encoded by exon 1, and the elements that are present in the mature protein are encoded by exon 2. The names of element patterns (listed to the left) are based on the sequence of element 15, which is highly diverse for both sequence and length. Different versions of element 15 are associated with different sets of elements that appear as element patterns. A common edit site in the *E2* transcripts (indicated) encodes a truncated protein lacking the histidine-rich region. Element 25a, b, and c correlate with 1, 2, and 3 stop codons. (**b**) The repeat-based alignment is based on gene sequences reported by Buckley and Smith (2007), in which the edges of the elements correspond, where possible, with the edges of the repeats. The intron (~400 nt) is indicated as gray boxes and is not shown to scale. For both alignments, all possible elements are shown at the top, and repeats within the coding regions are shown in different colors at the bottom. Green brackets surround subsets of interspersed repeats that are duplicated. Blue arrows between the two alignments indicate corresponding regions. (Reprinted from Smith and Lun (2017))

the genomic DNA from individual animals (Buckley and Smith 2007) plus estimates from bacterial artificial chromosome (BAC) library screens suggest that the gene copy number in single genomes may be much higher (see Buckley et al. (2008a), reviewed in Smith (2012) and Smith and Lun (2017)). To clarify this discrepancy, inserts of several BAC clones (from the library that was the basis for the sea urchin genome assembly) were sequenced and analyzed (Miller et al. 2010; Oren et al. 2016a). The BACs contain 15 *SpTrf* genes with a typical structure of two exons; they are flanked by short tandem repeats (STRs) and are distributed in three

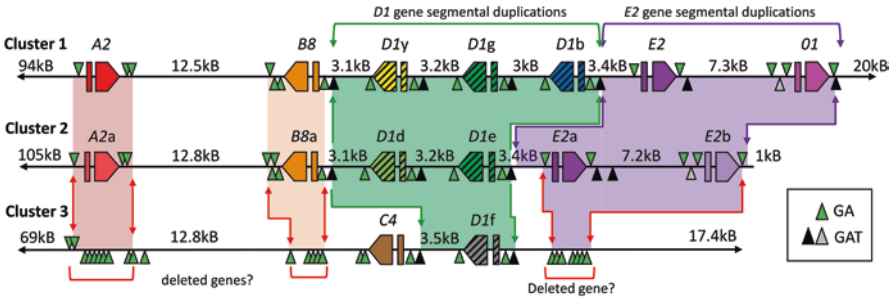

Fig. 10 *The SpTrf genes are arranged in three genomic clusters at two loci*. The genes within clusters 1, 2, and 3 range in size from 1170 to 1894 nt and are spaced apart by 3.0–12.8 kB. All genes have two exons, as indicated by the rectangle (first exon) and pentagon (second exon), which also indicates gene orientation. Element patterns (see Fig. 9) are listed above each gene; however, those with the same element pattern do not necessarily have identical sequences. All genes are surrounded by short tandem repeats (STRs; green triangles) of GA dinucleotides. Long stretches of GA STRs of up to 3 kB may be the remnants of deleted regions (red arrows), including deleted genes in cluster 3 (red brackets). Segmental duplications that include *D1* genes (green shading *and* green arrows) and *E2* genes (plus the *01* gene) (purple shading and purple arrows) are flanked by GAT STRs (black triangles indicate >35 repeats, gray triangles indicate 4–17 repeats). Clusters 1 and 2 are likely allelic on the basis of matches in the flanking regions outside the gene clusters, even though the numbers of genes do not match within locus I. The flanking regions outside cluster 3 indicate that it is positioned separately at locus II. (Reprinted from Smith and Lun (2017))

clusters of seven, six, and two genes (Fig. 10). Clusters 1 and 2 are likely allelic, indicating that the sequenced genome may contain only two *SpTrf* loci. The scaffold in the assembled genome harboring *SpTrf* genes is a hybrid sequence of allelic clusters 1 and 2, which is likely a consequence of the repetitive nature of the *SpTrf* region (Oren et al. 2016a).

Among the three clusters, the genes have similar orientations, intergenic spacing, and relative positioning (Fig. 10) (Miller et al. 2010; Oren et al. 2016a). Furthermore, all of the *SpTrf* genes are associated with GA STRs and some with GAT STRs located in intergenic regions at strategic positions on both sides of each gene and at the edges of segmental duplications (Miller et al. 2010; Oren et al. 2016a). This repetitive structure may contribute to gene diversification by promoting genomic instability, as has been noted in other systems (Pearson et al. 2005; Thys et al. 2014), perhaps through *SpTrf* gene deletion and by blocking the progression of sequence homogenization of clustered genes from gene conversion (Miller et al. 2010; Oren et al. 2016b). In addition to the short STRs that flank all genes, cluster 3 has two long stretches of GA STRs that correlate with the relative positions of genes in clusters 1 and 2 (Fig. 10). These long STRs may be remnant signatures of genes deleted from cluster 2 as a consequence of repeat-mediated genomic instability (Oren et al. 2016a). On the other hand, shorter GAT STRs flank five segmental duplications that appear in tandem in the three gene clusters and include nearly identical genes. The structure of the *SpTrf* family—including the modular element patterns, the repeats within and near the genes, and the gene

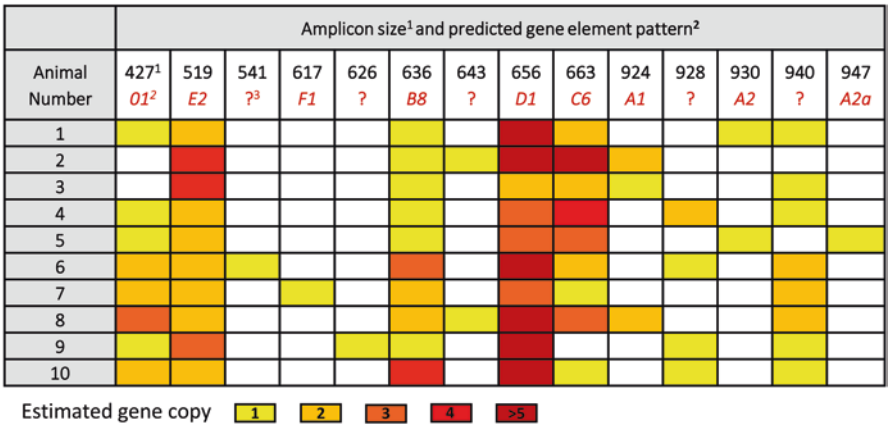

Fig. 11 *The SpTrf gene repertoire is different among individual sea urchins. SpTrf* gene profiles of ten sea urchins are shown. Element patterns from alleles are predicted from the amplicon length for the second exon based on fragment analysis on an ABI 3130 capillary sequencer (Oren et al. 2016a). Each sea urchin shows a unique gene repertoire. Genes with the *D1* element pattern (see Fig. 9 for element patterns) are present in all ten animals, in agreement with gene sequencing (Buckley and Smith 2007). Estimates of copy numbers for each allele are based on the allele frequencies in each animal under the assumption that the least abundant amplicon per animal represents a single copy gene. [1]Amplicon size is based on primers that amplify the second exon (Buckley and Smith 2007). [2]Element pattern prediction is based on amplicon sizes that match to known gene sequences (Buckley and Smith 2007). [3]Question marks indicate amplicon sizes that do not correlate with a known element pattern length. (Modified from Oren et al. (2016a))

clustering—likely contribute to genomic instability, which may underpin the exceptional diversity of this family.

The structure and gene content of the *SpTrf* family may be resolved for the individual sea urchin for which the genome was sequenced; however, the diversity of the family in terms of gene sequence and presence among different animals is extremely high (Buckley and Smith 2007). Analysis of the *SpTrf* gene repertoire in ten different individuals shows significant differences, and the family in each genome may be unique, with some genes common in the population and others more rare (Fig. 11) (Oren et al. 2016a). While the diversification mechanisms promoting this characteristic have not been determined, bioinformatic predictions of the *SpTrf* gene sequences suggest the involvement of gene deletion, duplication, recombination, and conversion (Buckley and Smith 2007; Miller et al. 2010; Oren et al. 2016a). In other families of clustered genes, these processes often result in pseudogenes for rapidly diversifying immune gene families (Oren et al. 2016b), as is exemplified by the clustered sea urchin *SpTLR* family (see section "Immunogenomics: Immune Genes Encoded in Echinoderm Genomes") (Buckley and Rast 2012). Consequently, it is unusual that no *SpTrf* gene fragments and only one pseudogene (likely a retroposon) have been identified from 198 gene sequences, suggesting that the diversification process may be highly regulated (Oren et al. 2016a).

SpTrf gene expression is rapidly upregulated in adult coelomocytes in response to immune challenge. Notably, however, analysis of transcript prevalence reveals that individual coelomocytes contain transcripts of a single *SpTrf* sequence and likely express a single *SpTrf* gene (Majeske et al. 2014). This restricted expression may be regulated on the basis of detection of specific pathogens, which may reflect the usage of a complex pathogen detection mechanism such as the large *SpTLR* gene family (Buckley and Rast 2012) (see also section "Immunogenomics: Immune Genes Encoded in Echinoderm Genomes"). It remains unclear whether a single *SpTrf* gene is expressed per cell or if all but one of the genes are actively repressed. The structure and diversity of the *SpTrf* gene family, variability of the family among individual sea urchins, and restricted expression patterns in individual cells indicate that this system is dynamic, flexible, highly sophisticated, and functions to maintain the survival of *S. purpuratus* in the microbe-rich marine environment.

SpTrf Protein Diversity and Expression

The predicted structure of the SpTrf proteins has an N-terminal hydrophobic signal sequence, which is likely cleaved during protein processing. The mature, full-length protein, which is encoded by the second exon, has a glycine-rich region with an arginine, glycine, aspartic acid (RGD) motif; a histidine-rich region; and a C-terminal region (Fig. 12a) (Terwilliger et al. 2006). All full-length proteins characterized to date lack cysteines. The sequence diversity of SpTrf proteins is a consequence of the mosaic element patterns, as well as mRNA editing, which may result in single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) leading to missense sequence and early stop codons that expand the predicted size range of proteins (4–55 kDa) (Buckley et al. 2008b; Terwilliger et al. 2006, 2007). An individual sea urchin may express up to 260 different SpTrf protein variants according to analysis by two-dimensional (2D) western blots (Dheilly et al. 2009), which is significantly more than the ~50 estimated genes. Native SpTrf (natSpTrf) proteins isolated from individual sea urchins are unexpectedly larger than the protein size prediction, implying that the natSpTrf protein variants multimerize to form nondenaturable, high molecular weight protein complexes (Brockton et al. 2008; Dheilly et al. 2009). Repertoires of full-length natSpTrf proteins differ among individual sea urchins after multiple immune challenges with the same or different bacterial species (Sherman et al. 2015).

Fig. 12 (continued) and a C-terminal region (gray). (**b**) A small phagocyte has SpTrf proteins on the surface and associated with small vesicles within the cell. (**c**) A polygonal phagocyte has SpTrf proteins within vesicles in the cytoplasm. (**d**) A minority of discoidal phagocytes have SpTrf proteins in very small perinuclear vesicles. (**e**) Red spherule cells are negative for SpTrf proteins. (**f**) Vibratile cells are negative for SpTrf proteins. (**g**) A cross-section of gut from *S. purpuratus* shows SpTrf-positive cells within the columnar epithelium. The gut lumen is at the top of the image and the coelomic space is at the bottom. (**h**) The axial organ has many SpTrf-positive cells. Images (**b**) and (**d**–**g**) are captured from fluorescence microscopy, and images (**c**) and (**h**) are from confocal microscopy. The scale bars are 10 μm in images (**b**–**f**) and 100 μm in images (**g**) and (**h**). (Reprinted from Smith and Lun (2017))

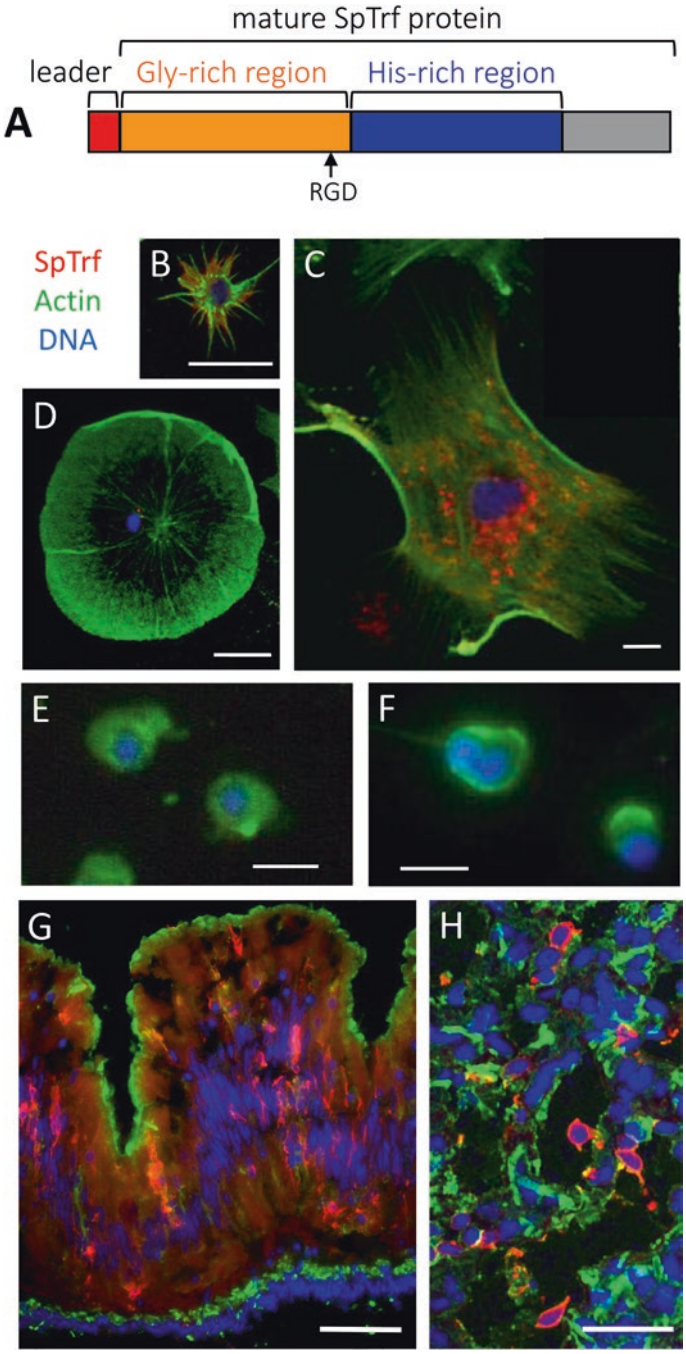

Fig. 12 *SpTrf proteins are expressed in phagocytes.* (**a**) The standard SpTrf protein structure is composed of a leader (red), which is likely cleaved during processing, and a mature protein with a glycine-rich region (orange) with an arginine, glycine, aspartic acid (RGD) motif, a histidine-rich region (blue),

The amino acid sequences of the SpTrf proteins do not predict any obvious structural or functional features. Yet the nucleotide diversity of the genes and transcripts, the swift expression in response to immune challenge (Nair et al. 2005; Terwilliger et al. 2006, 2007; Buckley and Smith 2007), mRNA editing (Buckley et al. 2008b), and variable protein repertoires among animals (Dheilly et al. 2009; Sherman et al. 2015) suggest important immune activities. Most immunoquiescent (IQ) sea urchins (those maintained in recirculating, closed aquaria with downregulated immune activity) show decreased or no expression of the *SpTrf* genes (Nair et al. 2005; Terwilliger et al. 2007). Transcripts isolated from IQ animals tend to encode truncated SpTrf proteins that lack the histidine-rich region as a consequence of mRNA editing (Figs. 9a and 12a) (see Buckley et al. (2008b) and Sherman et al. (2015), reviewed in Smith and Lun (2017)). Edited transcripts encoding truncated proteins missing the histidine-rich region are elevated in IQ sea urchins, whereas after an immune challenge, transcripts encoding full-length SpTrf proteins increase. This suggests not only regulation for at least some of the transcript edit sites but also that the truncated proteins may have surveillance functions based on their presence prior to an immune response, and that after challenge, the histidine-rich region may have pathogen-binding activity.

The SpTrf proteins are expressed by the phagocytes where they are associated with membranes of perinuclear vesicles in discoidal, polygonal, and small phagocytes, and are present on the surface of the plasma membrane of small phagocytes (Fig. 12b–f) (see Brockton et al. (2008), Dheilly et al. (2011a), and Majeske et al. (2014), reviewed in Smith and Lun (2017)). The SpTrf proteins are also expressed in all major organs in adult sea urchins, including the axial organ, pharynx, esophagus, intestine, and gonad (Fig. 12g, h) (Majeske et al. 2013a). SpTrf protein expression increases in most of these organs after an immune challenge; however, gene expression, SpTrf protein content, and numbers of SpTrf-positive cells increase significantly only in the axial organ (Table 2), suggesting that it may be an organ with immune functions. Although it is not known whether SpTrf expression in the adult organs is specific to phagocytes that have infiltrated the adult tissues, the implication from *SpTrf* gene expression in larvae, which is restricted to the blastocoelar cells (Fig. 7d) (Ho et al. 2016), suggests that expression and production of SpTrf proteins in the adult is also restricted to the phagocytes.

Table 2 SpTrf responses to challenge with LPS

Tissue	mRNA	SpTrf protein	SpTrf$^+$ cells
Pharynx	–[a]	+	–
Esophagus	–	+	–
Intestine	+[b]	+	–
Gonad	–	–	–
Axial organ	+	+	+

This table is modified from Majeske et al. (2013a)

[a]–, decreased expression of genes or proteins, or decreases in SpTrf-positive (SpTrf$^+$) cells

[b]+, increased expression of genes or proteins, or increases in SpTrf$^+$ cells

SpTrf Protein Functions

The diversity and expression patterns of the SpTrf proteins suggested antipathogen activities. Binding activities of the natSpTrf proteins from different sea urchins exhibit variable and diverse binding to Gram-positive and Gram-negative bacteria, sheep red blood cells (SRBCs), and insect cells from the lepidopteran *Spodoptera frugiperda* (Sherman et al. 2015; Lun et al. 2016). Detailed functional analysis of these proteins is complicated by their sequence diversity, complex multimerization patterns, and potential interactions with other proteins. Thus, a recombinant protein called rSpTrf-E1 (formerly rSp0032) was generated for functional analyses (see Fig. 9a for the E1 element pattern), in addition to recombinant fragments of rSpTrf-E1 spanning the complete glycine-rich region (rGly-rich fragment), the C-terminal portion of the glycine region (rC-Gly), and the histidine-rich region (rHis-rich fragment) (Lun et al. 2016). rSpTrf-E1 binds specifically and tightly to *Vibrio diazotrophicus* and baker's yeast (*Saccharomyces cerevisiae*) but does not bind to two Gram-positive *Bacillus* species (Fig. 13a). The rGly-rich and rHis-rich fragments bind to *Vibrio* and yeast, and also to *Bacillus*, suggesting that interactions among these two regions in the full-length protein may underlie the binding specificities in the full-length protein. The rC-Gly fragment multimerizes upon isolation, indicating that this region may mediate multimerization for all SpTrf proteins (Lun et al. 2016). rSpTrf-E1 binds to flagellin from *Vibrio diazotrophicus* (Table 3) and from *Salmonella typhimurium*, LPS from *Escherichia coli*, and β-1,3-glucan from *Saccharomyces* but does not bind to peptidoglycan (PGN) from *Bacillus* subtilis (Fig. 13a). Competition assays indicate that rSpTrf-E1 binding to LPS, flagellin, and β-1,3-glucan is specific and irreversible (Fig. 13b).

The outcome of rSpTrf-E1 binding to several different PAMPs suggests the possibility of structural changes in the protein that allow interactions with different binding targets (Lun et al. 2016). Bioinformatic prediction from amino acid sequences indicates that most if not all SpTrf proteins, including rSpTrf-E1 and HeTrf sequences (from *Heliocidaris erythrogramma*; see section "*HeTransformer* Genes in *Heliocidaris erythrogramma*"), have a hydrophobic α helical leader and that the mature proteins are entirely hydrophilic and intrinsically disordered proteins (IDPs) (Fig. 13c). The flexibility of IDPs allows conformational plasticity of proteins to adopt different conformations upon encountering a range of binding targets (Uversky 2010). Circular dichroism (CD) confirms that rSpTrf-E1 is intrinsically disordered and is capable of undergoing structural transformations to mostly α helical in the presence of sodium dodecyl sulfate (SDS) (de Latour et al. 2010) and 2,2,2-trifluorethanol (TFE) (Table 4) (Lun et al. 2017a). LPS also induces a conformational transformation of rSpTrf-E1 from disorder to mostly α helical. Although the rGly-rich and rHis-rich fragments are also predicted to be IDPs, CD analysis shows that they are partially α helical, they intensify their α helical content in SDS, and they unexpectedly transform to β strands in TFE (Table 4). These fragments demonstrate opposite structural transformation in the presence of LPS, in which the rGly-rich fragment transforms from α helical to β strand and the rHis-rich fragment intensifies its α helical content. These results predict unusual

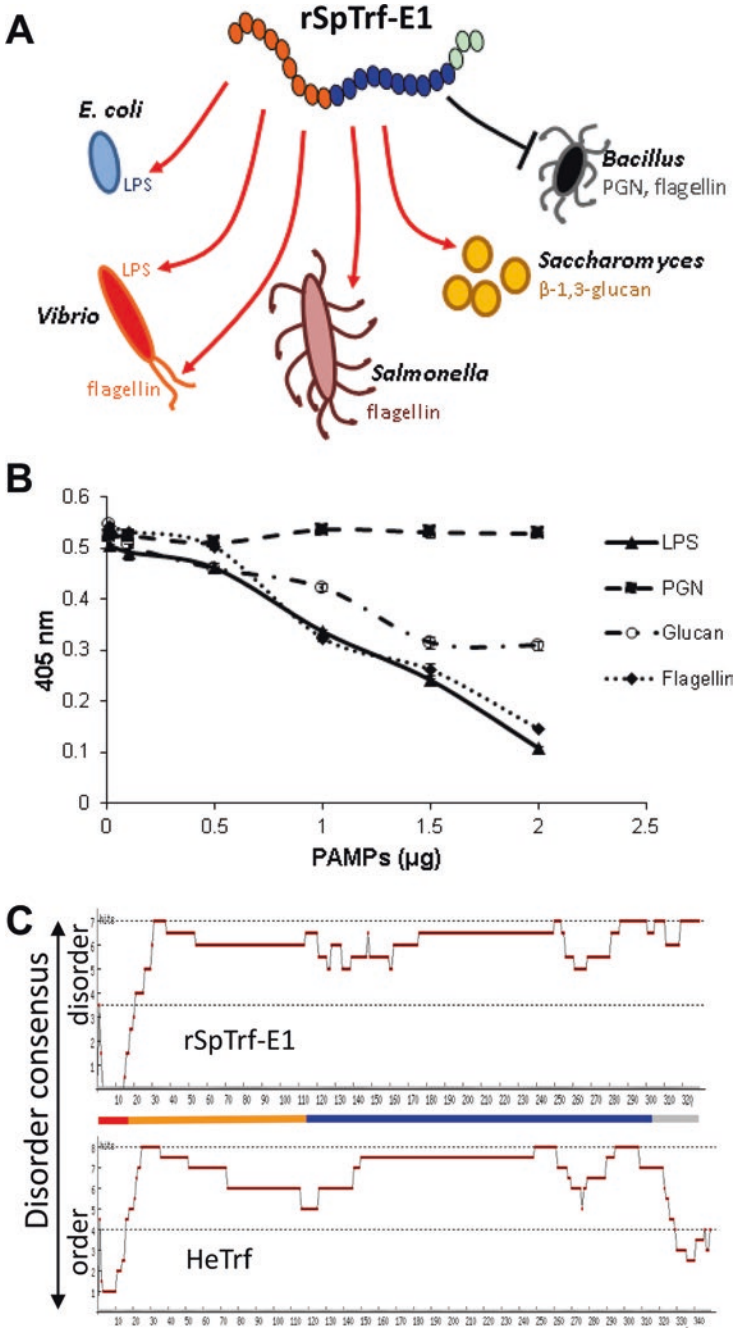

Fig. 13 *rSpTrf-E1 has multitasking binding activity, and Trf proteins are intrinsically disordered.* (**a**) rSpTrf-E1 is shown as an unfolded disordered protein. (Republished from Smith and Lun (2016), with minor revisions.) The colors correlate with protein regions in Fig. 12a. *Red arrows* indicate binding targets of rSpTrf-E1. The black line indicates that rSpTrf-E1 does not bind to *Bacillus*

Table 3 rSpTrf-E1 binds to *Vibrio* flagellin

Sample	MW	Peptide sequences identified[b]	Significant matches	Match; GenBank accession number
rSpTrf-E1 and *Vibrio*[a]	60 kDa	IAETTSFGGNK DDAAGLQISNR	Flagellin B OS; *Vibrio anguillarum*	Q56702
		SQILSQASSSILAQAK IAETTSFGGNK DDAAGLQISNR	Polar flagellin B/D OS; *Vibrio parahaemolyticus*	Q56572
		GDGEEETDAAQQIGDGLGGR RGDGEEETDAAQQIGDGLGGR	SpTrf protein	NP_001073016.1

This table is modified from Lun et al. (2016)

[a]*Vibrio diazotrophicus* was incubated with rSpTrf-E1 and washed, and proteins were separated by SDS-PAGE. Bands that correlated in size to SpTrf-positive bands on a parallel western blot were evaluated by MS

[b]rSpTrf-E1 is ~37 kDa and flagellin is 45–50 kDa; however, the SpTrf-positive band of 60 kDa includes sequences of both rSpTrf-E1 and flagellin

conformational plasticity for the sea urchin SpTrf protein family, perhaps leading to flexible binding activity with a wide range of foreign targets.

The Trf proteins associate with membranes of perinuclear vesicles and the plasma membranes of phagocytes (Brockton et al. 2008; Majeske et al. 2014; Dheilly et al. 2011a), and this association is unexpected because they lack transmembrane domains and glycophosphatidylinositol linkages (Terwilliger et al. 2006, 2007). However, rSpTrf-E1 and the rGly-rich and rHis-rich fragments all bind to phosphatidic acid (PA), which is a small cone-shaped phospholipid with a single phosphate as the head group (Lun et al. 2017b). In the presence of PA, rSpTrf-E1 transforms from an IDP to mostly α helical (Table 4). When rSpTrf-E1 binds to synthetic liposomes composed of 10% PA and 90% phosphatidylcholine (PC), it induces membrane curvature in liposomes, which is associated with PA clustering and changes in liposome shape in the form of budding, invagination, and fusion (Fig. 14a, b). These morphological changes may result from the positively charged amino acids in rSpTrf-E1 binding to the negatively charged phosphate head group of PA, followed by rSpTrf-E1 multimerization; this causes PA clustering (Fig. 14c–h), which is known to induce membrane curvature (Zimmerberg and Kozlov 2006). rSpTrf-E1 and the rHis-rich fragment cause leakage of luminal

Fig. 13 (continued) species or to peptidoglycan (PGN). No binding to flagellin from *Bacillus* is assumed, but this has not been tested. (**b**) rSpTrf binds tightly and specifically to multiple PAMPs. (Reprinted from Lun et al. 2016.) When LPS, β-1,3-glucan (glucan), flagellin, or PGN are preincubated with rSpTrf-E1, LPS reduces rSpTrf-E1 binding to LPS bound to an ELISA well. Glucan and flagellin also complete and reduce rSpTrf-E1 binding to LPS. Preincubation with PGN does not interfere with rSpTrf-E1 binding to LPS. (**c**) rSpTrf-E1 (GenBank accession number DQ183168, from Terwilliger et al. (2006)) and an HeTrf protein (GenBank accession number AFK91970, from Roth et al. (2014)) of similar sizes are predicted to be disordered with an N-terminal α helical leader on the basis of the Disorder Prediction Meta-Server (DisMeta, http://www-nmr.cabm.rutgers.edu/bioinformatics/disorder/). The y axis indicates the confidence level of the disorder consensus based on the outcome of the seven or eight predictor programs for each protein. A represenation of rSpTrf-E1 is shown between the graphs, and colors indicate regions of the protein, as in Fig. 12a

Table 4 rSpTrf-E1 and the recombinant fragments change secondary structure in response to buffer additives and binding targets

	Secondary structure in different reagents[b]				
Protein structure	PO_4	SDS	TFE	LPS	PA
rSpTrf-E1	Disordered	α helical	α helical	α helical	α helical
% α helical	1–2%[c]	79%	95.1%	78.5%	71.8%
Helix tightness[a]		0.59	1	0.66	0.7
rGly-rich fragment	α helical	α helical	β strand	β strand	ND
% α helical	15–17%	75.1%	NA[d]	NA	
Helix tightness		0.78	NA	NA	
rHis-rich fragment	α helical	α helical	β strand	α helical	ND
% α helical	19–30%	70.7%	46.2%	72.8%	
Helix tightness		0.76	NA	0.78	

Reprinted from Smith and Lun (2017)

[a]Helix tightness is estimated from the *R* value obtained from circular dichroism spectra and is used to infer the width of an α helical twist. A standard helix has an *R* value of 1. A 3_{10} helix has an *R* value of 0.4, which has a smaller diameter and is longer for a similar number of amino acids (Vieira-Pires and Morais-Cabral 2010; Lun et al. 2017a).

[b]PO_4, phosphate buffer (10 mM, pH 7.4); SDS, sodium dodecyl sulfate; TFE, 2,2,2-trifluoroethanol; LPS, lipopolysaccharide from *Escherichia coli*; PA, phosphatidic acid in the form of small vesicles.

[c]The percentage of the secondary structure for either the α helix or β strand is deconvoluted from the CD spectra using the DichroWeb online server (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml; Whitmore and Wallace 2004, 2008).

[d]N/A, not applicable. The deconvolution to calculate the β strand percentage is not feasible for this sample (Lun et al. 2017a).

ND, not done.

material from 10% PA liposomes, whereas dimerized rSpTrf-E1 and the rGly-rich fragment do not, suggesting that the histidine-rich region of rSpTrf-E1 is required for this activity (Fig. 15). The lack of activity by dimerized rSpTrf-E1 suggests that multimerization of natSpTrf proteins may be an intrinsic control mechanism to limit their activities in vivo.

Association with negatively charged lipids may be a possible mechanism for binding of natSpTrf proteins to membranes (Brockton et al. 2008; Dheilly et al. 2011a), and content leakage may indicate cytotoxic activities upon binding to pathogens. Although the underlying binding mechanisms remain elusive, the multitasking binding ability and intrinsically disordered nature of rSpTrf-E1 provide an initial insight into how this novel immune protein family may bind to a wide range of pathogens and other foreign targets. The natSpTrf proteins may opsonize a broad array of pathogens and, given the many isoforms that are likely present in individual sea urchins, each may have slightly different but overlapping binding activities and serve as a very effective immune response system in euechinoids.

HeTransformer Genes in *Heliocidaris erythrogramma*

Structure of the *HeTrf* Transcripts and Genes

Outside of investigations of the *SpTrf* family in *S. purpuratus*, the most extensive characterization of the *Trf* family has been reported for the sea urchin *Heliocidaris*

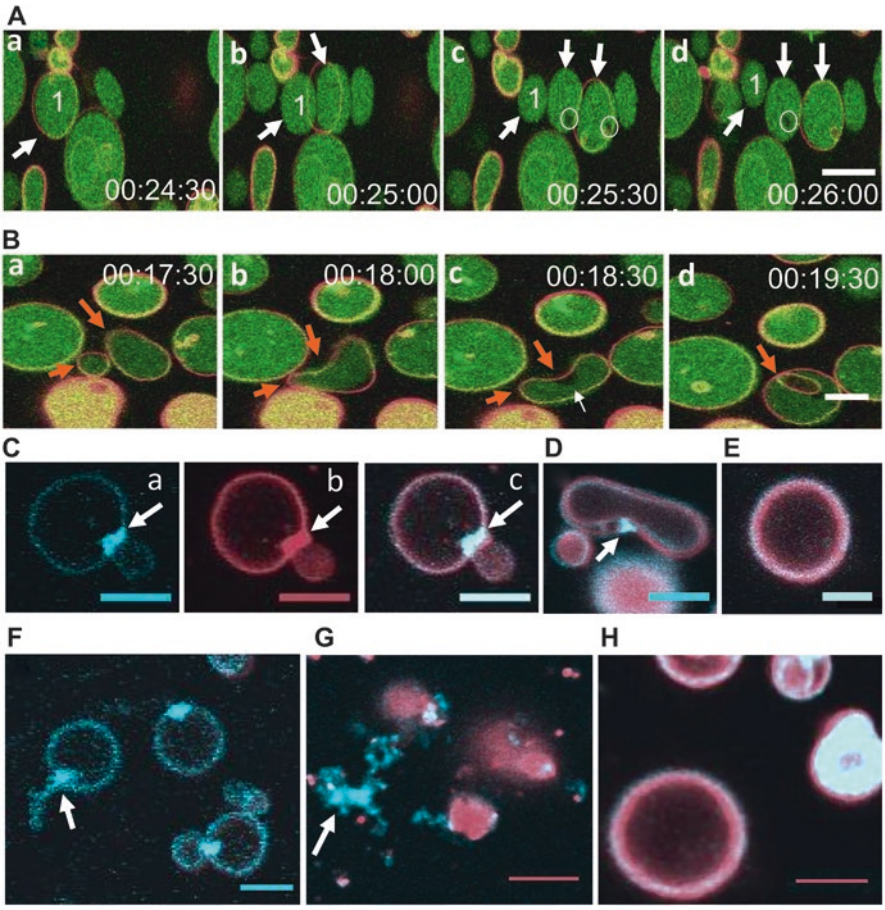

Fig. 14 *rSpTrf-E1 alters liposome membranes*. Liposomes (10% PA; 90% PC) are visualized by dextran labeled with AlexaFluor® 488 (dextran-488; green) in the lumen and the lipophilic dye DiD (red) in the membrane. (**a**) Incubation of liposomes with rSpTrf-E1 results in budding. Liposome 1 buds into three liposomes (white arrows) over 30 s. Dextran-488 leakage is suggested by black spaces within some of the liposomes (white circles in a**c** and a**d**). (**b**) Two liposomes of different sizes fuse in the presence of rSpTrf-E1 (b**a**–**b**, orange arrows). The fused liposome has a dark region in the lumen (**c**, white arrow) near the concave region of the membrane. This liposome invaginates at the site of the concave membrane and generates an internal vesicle that does not contain dextran-488 (b**d**, orange arrow). The confocal image capture was set for 30-s intervals as indicated for (**a**) and (**b**). (**c**) Clusters of blue-labeled phosphatidic acid (blue-PA; arrow) are present at sites of contacting membranes for two liposomes after incubation for 20 min with rSpTrf-E1. The membrane is labeled with the lipophilic dye DiD (red). (c**a**) blue-PA; (c**b**) red DiD; (c**c**) merge. (**d**) A cluster of blue-PA (arrow) is present at the convex curve of a liposome membrane after incubation with rSpTrf-E1 for 20 min. This image is a merge of red and blue channels. (**e**) Blue-PA is evenly distributed in a control liposome after 20 min in the absence of rSpTrf-E1. The image is a merge of the blue-PA and DiD (red). (**f**) Clusters of blue-PA (blue channel only) are present in liposome membranes incubated with rSpTrf-E1 for 20 min. In one liposome, the blue-PA cluster appears to be partially extracted from the membrane (arrow). (**g**) After 2 h of incubation with rSpTrf-E1, blue-PA appears as disordered clusters that are separated from the liposomes (arrow). (**h**) In the absence of rSpTrf-E1, liposomes show an even distribution of blue-PA that remains in the membrane after 2 h. (All images are captured by confocal microscopy. The scale bars are 10 μm.) (Modified from Smith and Lun (2017))

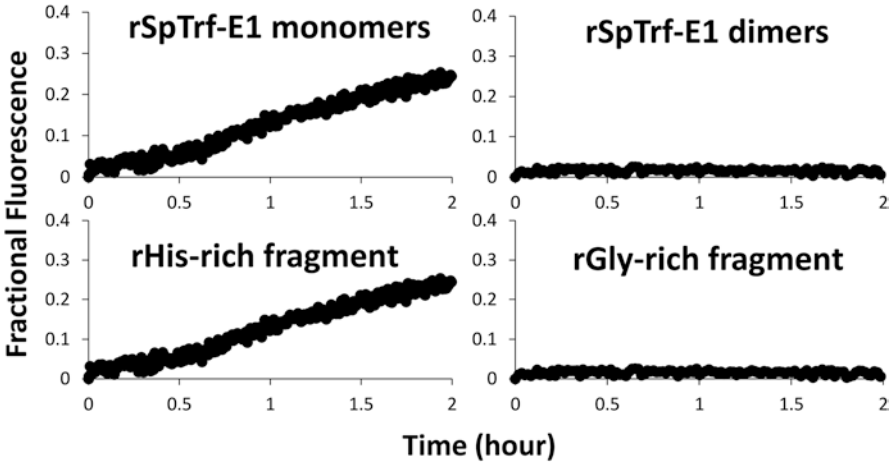

Fig. 15 *rSpTrf-E1 and the rHis-rich fragment induce leakage of luminal contents from liposomes*. Liposomes composed of 10% PA and loaded with 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS; fluorescent dye) plus p-xylene-Bis-pyridinium bromide (DPX; quencher) are incubated for 2 h with recombinant proteins. Luminal leakage separates ANTS from DPX by dilution into the buffer, and fluorescence is recorded relative to control liposomes that are lysed to measure leakage equivalent to 100% (fractional fluorescence). Dye leakage from liposomes is detected only with monomeric rSpTrf-E1 or the rHis-rich fragment, whereas neither the dimerized rSpTrf-E1 nor the rGly-rich fragment induce leakage. (Modified from Smith and Lun (2017))

erythrogramma, which lives in the southern hemisphere (Dheilly et al. 2011a; Roth et al. 2014). The *HeTrf* genes are closely related to those in *S. purpuratus*, with a conserved overall structure of two exons with substantial levels of sequence homology (Roth et al. 2014). Both *Trf* families have mosaic patterns of elements in transcripts and genes although the introns are different (Fig. 16a, b). The sequences and numbers of elements differ slightly among the *HeTrf* and *SpTrf* sequences (compare Figs. 16a and 9), but the structure of the transcripts and genes are generally similar (Terwilliger et al. 2006, 2007; Buckley and Smith 2007; Roth et al. 2014). Sequence diversity in the *HeTrf* sequences is largely based on element pattern differences, but SNPs, indels, and a variety of repeats also contribute (Roth et al. 2014). Sequence alignments indicate that negative selection against codon diversification is common in both families, with significant negative selection apparent in 4.7% of *HeTrf* codons and 11% of *SpTrf* codons. In contrast, codons subject to positive selection are more rare (2.5% in *HeTrf* and 2.6% in *SpTrf*). These differences likely result from differential selection pressures from variable versus stable binding targets in the relative habitats of these two sea urchin species.

HeTrf Proteins: Diversity and Cellular Localization

The Trf protein repertoires of both species show significant differences among individual animals and significant changes in response to immunological challenges (Dheilly et al. 2009; 2011a; Sherman et al. 2015). The HeTrf and SpTrf proteins are

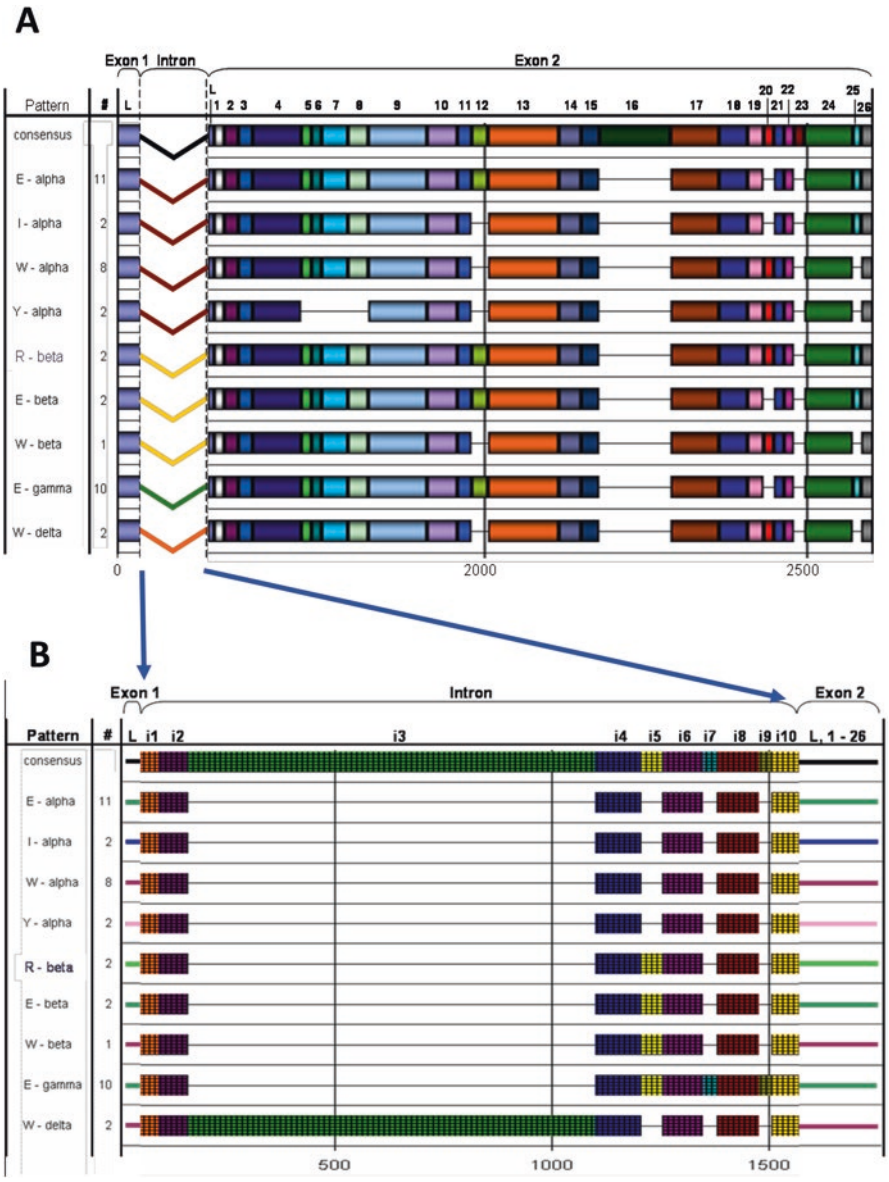

Fig. 16 *An alignment of HeTrf gene sequences identifies 39 unique element patterns.* (**a**) Exon element patterns. The two exons are separated by an intron that is positioned within the region encoding the leader (L). Nine combinations of exon and intron elements of 29 element patterns are shown. All possible elements are shown at the top as a consensus pattern. The numbers (#) of genes with unique nucleotide sequences that comprise a certain element pattern are shown in the second column. (**b**) An expanded area in (**a**) shows the regions that encode the leader and the intron. The introns align optimally after the insertion of large gaps that define ten intron elements and four intron element patterns that are designated as alpha, beta, gamma, and delta. Individual intron elements are termed i1 to i10 and are shown as blocks of different colors with hatched patterns. Exons are shown as horizontal lines that flank the intron elements, with different colors representing different exon element patterns. (Reprinted from Roth et al. (2014))

similar, with a hydrophobic leader, glycine-rich and histidine-rich regions, and several potential N- and O-linked glycosylation sites. However, most HeTrf sequences lack the RGD motif near the intersection of the glycine-rich and histidine-rich regions (Fig. 12a) (Roth et al. 2014). Despite differences in element patterns (compare Figs. 9 and 16a), the protein families have some well-conserved blocks of predicted amino acid sequence with identities of up to 100%. The HeTrf proteins exhibit a diverse range of molecular weights and isoelectric points, which is similar to that of SpTrf proteins (Brockton et al. 2008; Dheilly et al. 2009; Roth et al. 2014; Sherman et al. 2015). Under strong reducing conditions, both HeTrf and SpTrf proteins have far larger apparent molecular weights than predicted from transcripts, which may reflect a combination of posttranslational modifications and multimerization.

The HeTrf proteins are predominantly associated with the cell surface and perinuclear vesicles of coelomocytes (Roth et al. 2014), and associate directly with the membranes of vesicles originating from the trans face of the Golgi apparatus, later fusing with the plasma membrane (Dheilly et al. 2011a). HeTrf proteins are also expressed by the rare fusiform cells of *H. erythrogramma*, a cell type that is absent in *S. purpuratus* (Dheilly et al. 2011a). Most significantly, the HeTrf proteins associate with what appear to be phagosomes containing ingested bacteria within cells found in the gut epithelium (Dheilly et al. 2011a). The location of the HeTrf-positive cells in the gut is in agreement with gut-associated SpTrf-positive cells (Fig. 12g) (Majeske et al. 2013a). These observations suggest that HeTrf proteins may be involved in the opsonization of microbes that have invaded the gut epithelium (see section "The Immune Response in Sea Urchin Larvae" for larval responses to *Vibrio* infection in the gut).

Phylogenetic Differences in the Two Families of *Trf* Genes

Phylogenetic analysis of *HeTrf* and *SpTrf* transcripts indicates that the sequences cluster into two separate, species-specific clades (Fig. 17), suggesting that the two Trf families evolved independently after the genera diverged about 35 million years ago (Palumbi and Lessios 2005; Roth et al. 2014). This divergence may be a consequence of the different life history traits in these two sea urchins, in which *S. purpuratus* develops via a relatively long-lived larval stage that feeds in the plankton, whereas *H. erythrogramma* produces relatively few large eggs that develop directly into juveniles (Laegdsgaard et al. 1991; Palumbi and Lessios 2005). However, it is also likely that the divergence between the two *Trf* gene families is the result of pathogen pressure not only for the larval *S. purpuratus* in the water column but also for the adults living in different habitats and feeding on different macroalgae and substrate biofilms. This is expected for gene families encoding proteins that interact with the environment and are under pressure from pathogens. Swift diversification of the *SpTrf* gene family has been predicted not only from the gene clusters and the associated repeats but also as an advantage in the arms race with pathogens (Smith and Coscia 2016).

The Complement System

The vertebrate complement system is composed of more than 50 proteins (Volanakis 1998) functioning in three proteolytic activation cascades that converge to cleave

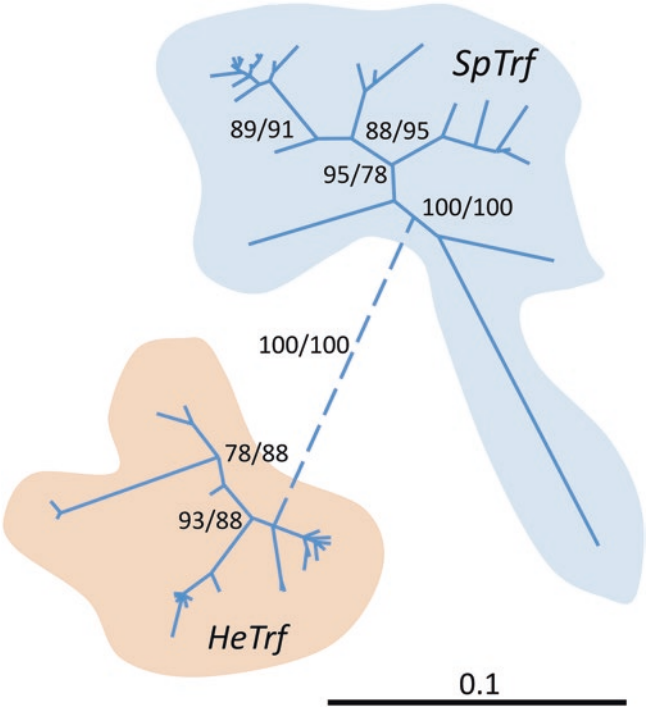

Fig. 17 *The Trf transcript sequences from Strongylocentrotus purpuratus and Heliocidaris erythrogramma cluster separately*. The phylogenetic dendrogram shows that *SpTrf* (blue) and *HeTrf* (orange) sequences form two distinct clades. Each branch represents a single distinct *SpTrf* or *HeTrf* sequence. The central branch (dashed line) separating the two clades is shortened for display purposes and reflects a corrected genetic distance of 0.27949 that is supported by bootstrap values of 100/100. A subset of bootstrap values are shown that indicate values for neighbor joining and maximum parsimony analyses (NJ/MP). All nodes with bootstrap values of <50 are collapsed. The scale bar indicates the corrected genetic distance. (Revised from Roth et al. (2014))

and activate the central component of complement, C3, which has an important opsonization function and is required to activate the terminal or lytic pathway. The classical and lectin activation pathways are triggered by the recognition of nonself antigens or PAMPs, which initiate a cascade of serine proteases that activate the effector complement proteins. The alternative activation pathway is constitutively activated by the C3 "tick-over" mechanism in which the thioester site is spontaneously activated and exposed (Tomlinson 1993). The central component, C3, functions in all pathways, and once it is cleaved, the C3a fragment exerts proinflammatory functions by interacting with receptors on self cells and the larger C3b fragment with the activated thioester site binds to foreign cell surfaces. Furthermore, cleavage of C3 to C3b initiates the positive feedback loop of the alternative pathway that amplifies the production of cleaved and activated C3 fragments to augment opsonization of a binding target, which increases immune responsiveness and effectiveness (Hugli 1990). Many complement components are secreted as zymogens

(inactive precursors), which are activated by cleavage to fragments with effector functions. In addition, there are numerous proteins with regulatory and inhibitory activity to control the system and block or protect self cells from attack. The inhibitory regulators ensure that the cascades are activated in a controlled manner such that effector activities occur at the right time and place and that host cells are protected from complement-mediated lysis (Zipfel and Skerka 2009).

SpC3, the Sea Urchin Homologue of the C3 Complement Component

The first evidence of a complement system in an echinoderm was suggested for the green sea urchin, *S. droebachiensis*, in which phagocytosis of RBCs by adult phagocytes could be augmented when opsonized by human C3 (Kaplan and Bertheussen 1977; Bertheussen 1981a, b, 1982; Bertheussen and Seljelid 1982), and could be decreased or blocked by inhibitors of mammalian complement (Bertheussen 1983). The first molecular evidence of an echinoderm complement component was based on transcript sequences from *S. purpuratus* phagocytes with homology to vertebrate C3 and called *SpC3* (see Smith et al. (1996), Al-Sharif et al. (1998), and Gross et al. (2000), reviewed in Smith et al. (2001)). *SpC3* transcripts are present in unfertilized eggs and persist throughout embryogenesis; however, *SpC3* gene expression increases in gastrulae when the embryos are cultured with *Vibrio diazotrophicus* (Shah et al. 2003). In adults, the preprocessed SpC3 form is stored in vesicles (Fig. 18a) as a single protein of 210 kDa, which is slightly larger than human C3 (190 kDa) (Al-Sharif et al. 1998; Gross et al. 2000). Upon secretion, SpC3 is cleaved at the βα junction during final processing to the α chain (130 kDa) and β chain (80 kDa), which are linked by disulfide bonds between cysteines in conserved positions (Al-Sharif et al. 1998). SpC3 protein levels are very low in the CF of IQ sea urchins but are readily induced upon injection with LPS (Clow et al. 2000, 2004). Homologues of C3 and complement receptor type 2 (CR2) are among the most abundant proteins in the CF of *S. purpuratus* and *H. erythrogramma*, which underscores the importance of these proteins in the echinoderm immune system (Smith 2002; Dheilly et al. 2013).

The SpC3 opsonization function in sea urchins is likely similar to that in vertebrates and is enhanced by challenge with LPS (Clow et al. 2004). Preincubation of

Fig. 18 (continued) an irrelevant antibody (Smith et al. 1992). [2]Yeast opsonized with LPS-activated CF was incubated with α-SpC3 antibody and subsequently washed with 1 mM glycine, pH 2, to remove the α-SpC3 but not the covalently bound complement protein. [3]Yeast opsonized with LPS-activated CF and incubated with α-SpC3. [4]Yeast opsonized with LPS-activated CF. [5]Yeast opsonized with ASW. [6]The phagocytic stimulation index (PSI) is the number of yeast cells phagocytosed per 100 coelomocytes. PSI are shown as means ±SEM from six phagocytosis experiments. *Significance ($p < 0.05$) is based on a Student's two-tailed *t* test. (Republished from Clow et al. (2004) in the Journal of Experimental Biology.) (**c**) SpC3 undergoes autolysis, indicating a functional thioester site. The western blot shows autolysis of SpC3 in CF under denaturing conditions plus heating at pH 10 to generate the 50 kDa fragment of the α chain that is recognized by the α-SpC3 antibody (lane 1). The full-length α chain in SpC3 is 130 kDa. The 80 kDa fragment of the α chain after autolysis is not recognized by the antibody and is not visible. Incubation of SpC3 with methylamine ($MeNH_2$) or yeast to engage the thioester site blocks autolysis (lanes 3–5). The autoradiograph demonstrates that the SpC3 α chain is labeled after incubation with ^{14}C-labeled methylamine (^{14}C-$MeNH_2$). M protein standard. (Modified from Smith (2002) and reprinted with permission from Elsevier)

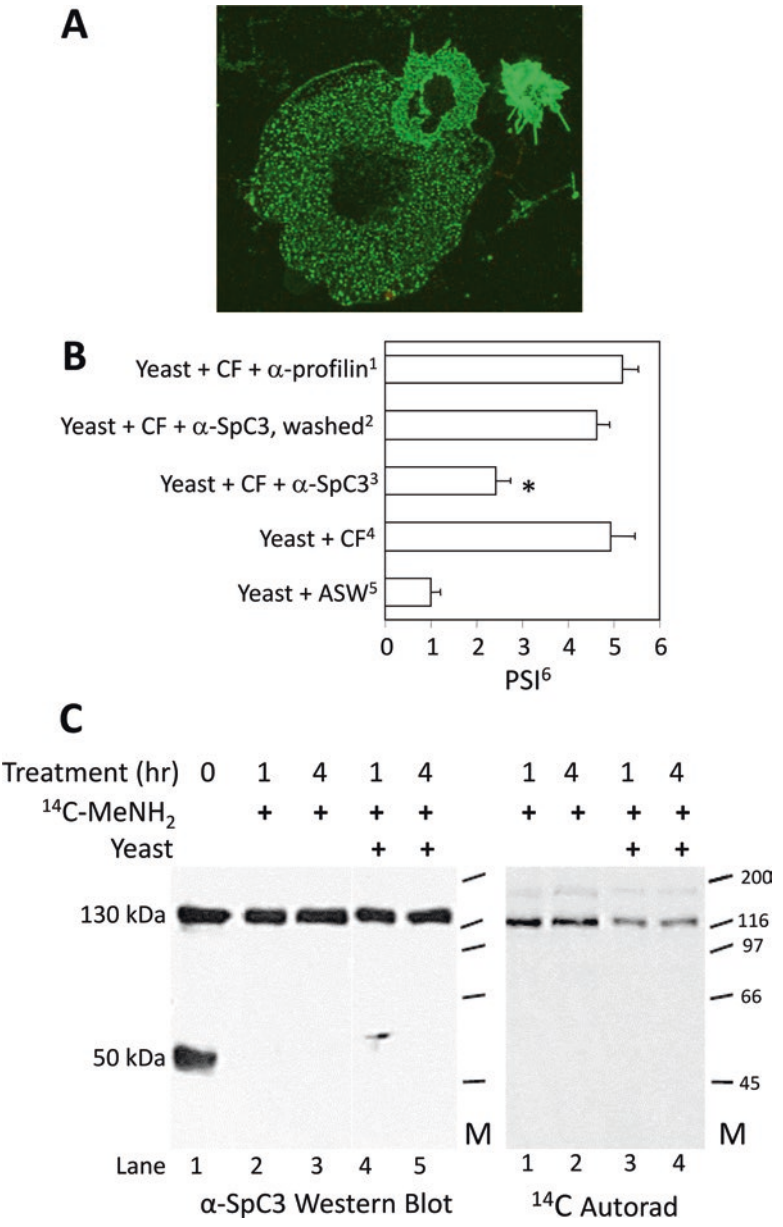

Fig. 18 *The complement system in echinoderms*. (**a**) Three coelomocytes from the purple sea urchin, *Strongylocentrotus purpuratus*, show SpC3 (green) within small cytoplasmic vesicles. The antibody to SpC3 (Al-Sharif et al. 1998) binds to the N-terminal end of the α chain. The central dark area within the cells is the unstained nucleus. The image was captured by confocal microscopy (republished from Gross et al. (2000), with permission from Springer Publishing). (**b**) SpC3 opsonizes yeast and increases phagocytosis by sea urchin coelomocytes. Cell-free coelomic fluid (CF) was obtained on day 3 from sea urchins after injections of LPS on days 1 and 2. Yeast (*S. cerevisiae*) was mixed with either CF or artificial seawater (ASW; Instant Ocean) and evaluated for phagocytosis by sea urchin coelomocytes. [1]Yeast opsonized with LPS-activated CF followed by incubation with α-profilin,

yeast with CF that includes SpC3 significantly increases phagocytosis by sea urchin phagocytes in comparison with nonopsonized controls, demonstrating the opsonization function of SpC3 (Fig. 18b) (Clow et al. 2004). Mammalian C3 and C4 undergo autolysis under heat and high pH, which requires an active thioester site and results in α chain cleavage into an N-terminal fragment of 50 kDa (Sim and Sim 1981). A similar autolytic reaction is observed for sea urchin SpC3, which also generates an α chain fragment and can be blocked by deactivation of the thioester (Fig. 18c) (Smith 2002). These data support homology between echinoderm SpC3 and vertebrate C3, which is based on both sequence and function.

SpBf, the Sea Urchin Factor B Homologue

In vertebrates, the second component in the alternative pathway is Factor B (Bf), which is a serine protease that interacts with cleaved and activated C3b. A transcript sequence with homology to Factor B (*SpBf*) is also expressed by coelomocytes (Smith et al. 1998). *SpBf* is constitutively expressed by phagocytes, and the deduced protein has a typical mosaic domain structure of five short consensus repeats (SCRs) at the N terminus, a conserved Factor D cleavage site, a von Willebrand Factor domain, and a serine protease domain (Smith et al. 1998). Although Factor D has not been identified in echinoderms, the positions of its cleavage sites are conserved in all Bf/C2 orthologues identified to date. Alternative splicing varies the number of SCRs in SpBf, which may result in different binding affinities for SpC3 (Terwilliger et al. 2004) or may interact with the several thioester-containing proteins encoded in the genome (see section "Additional Complement Homologues in the Sea Urchin Genome Sequence" and Table 1). If the sea urchin complement system functions as in other animals, SpBf may interact with activated SpC3, forming an SpC3–convertase complex similar to that in the alternative pathway of vertebrates, including a positive feedback loop and effective opsonization of foreign targets (Fig. 19). Variable expression of SpC3 may be a simple mechanism for regulating complement activity that is based on SpC3 protein concentration in the CF in response to immune challenge (Terwilliger et al. 2004).

Additional Complement Homologues in the Sea Urchin Genome Sequence

Annotation of genes in the *S. purpuratus* genome sequence indicated that initial reports of transcript sequences had identified only a subset of the genes homologous to vertebrate complement components. There are two *SpC3* genes, of which the second, *SpC3-2*, is expressed in embryos, and four additional genes that encode thioester-containing proteins—of which three are embryonically expressed—have not been investigated for function (Table 1) (see supplementary material in Hibino et al. (2006)). There are three paralogous *SpBf* genes that all encode SpBf proteins with five SCR domains (also called sushi domains or complement control protein (CCP) modules). It is noteworthy that a BAC clone insert identified a genomic linkage between *SpC3* and *SpBf*, which are also linked in the class III region of the major histocompatibility complex in vertebrates (Rast et al. 2000). A single mannose-binding lectin (*SpMBL*) homologue and four paralogues of *SpC1q* may

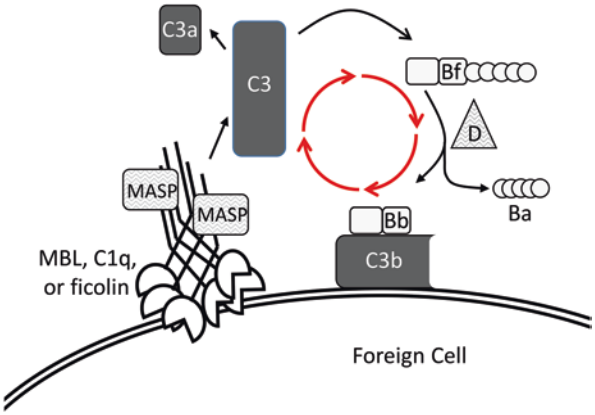

Fig. 19 *The echinoderm complement system* The echinoderm complement system may be activated by a lectin-like pathway and augmented by an alternative pathway feedback loop. Pathogens or other foreign surfaces may be detected by homologues of mannose-binding lectin (MBL), C1q, or ficolins that lead to cleavage and activation of the C3 homologue by mannose-binding-lectin-associated serine protease (MASP) homologues. Cleaved C3b binds to the foreign surface and associates with a Factor B (Bf) homologue, which is cleaved (Bb) and activated by a Factor D (D) homologue. This forms a C3-convertase-like complex (C3b and Bb) that cleaves and activates more C3 through an activation feedback loop (red arrows) to coat the pathogen with opsonizing C3b. The genes encoding Factor D and the MASPs have not been identified in the sea urchin genome sequence, although they have been identified in other echinoderms (see Table 5), and are indicated by patterned shapes. Properdin function is not shown. (Modified from Smith et al. (1999))

encode proteins that may activate a lectin-like complement pathway (Fig. 19) (Smith et al. 1999). Orthologues of members of the terminal or lytic pathway have not been identified in echinoderm genome sequences. Although there are 21 gene models that encode perforin-related proteins with MACPF domains (Table 1), the proteins are unlikely to function in the sea urchin terminal complement pathway, because they do not have the expected domain architecture. However, it is noteworthy that in the *S. purpuratus* genome there are two genes encoding CD59 homologues that, in vertebrates, block assembly of the membrane attack complex (MAC).

Because complement proteins with thioester sites can form covalent bonds with exposed amines or hydroxyls on any molecule, they can bind any cell surface, including self, and can result in inappropriate cell lysis, opsonization, and inflammatory reactions. Given that covalent binding between C3 or C4 and nonself is an important mechanism for identifying and clearing pathogens, self cells in vertebrates are protected from autologous complement attack by the complement regulatory system. Initial evidence for a complement regulatory system in the sea urchin is predicted from conserved cleavage sites in the SpC3 sequence (Al-Sharif et al. 1998). Two possible regulatory proteins identified from *S. purpuratus* coelomocyte transcripts encode the mosaic proteins: Sp Complement Related protein Long form (SpCRL) and Short form (SpCRS). The deduced protein sequences have 4–18 SCR domains with diverse sequences but which show sequence similarities to SCR

sequences in Factor I, Factor H, and a Factor I membrane attack complex (FIMAC) domain shared with members of the terminal or lytic pathway in vertebrates (Multerer and Smith 2004). Both genes are expressed in coelomocytes, gut, gonad, pharynx, esophagus, and axial organs, and are not induced by immune challenge with LPS, suggesting a constitutive protective function against complement attack for all self cells. Many complement regulatory proteins and several of the complement receptors are characterized by domain compositions that often include multiple tandem SCRs. Domain-based searches of the *S. purpuratus* genome (v4.2) indicate that 252 additional gene models contain SCRs. Although SCRs are present in a wide variety of complement proteins, many with SCRs are also likely to have nonimmune functions. Hence, functional analysis of these candidate complement regulatory proteins and receptors will be required to identify which of these proteins are additional components of the sea urchin complement system.

Complement Genes in Other Classes of Echinoderms

Since the original identification of complement homologues in the purple sea urchin, complement homologues have been identified in many other invertebrates (Table 5). This conservation underscores the importance of the antipathogen activities of a complement system that is likely based on opsonization. Contrary to initial reports (Ramírez-Gómez et al. 2008), complement homologues are present in the sea cucumber *Apostichopus japonicus*, including *MBL* (Vasilenko et al. 2012), two gene copies of *C3* (*AjC3* and *AjC3-2* (Zhou et al. 2011)) and one homologue of *Bf* (*AjBf* (Zhong et al. 2012)). AjC3-2 has all of the expected domains for a C3 protein; however, the sequence shows poor conservation of binding sites for CR1, CR2, C3aR, Bf, and Factor H, as well as cleavage sites for Factor I (Zhou et al. 2011). Maternal transcripts for both *AjC3* isoforms are present in eggs, and gene expression increases gradually during embryogenesis and peaks during larval stages. In adults, *AjC3* homologues are expressed in coelomocytes and upregulated in response to immune challenge. The *Bf* gene (*AjBf-2*) in *A. japonicus* is highly expressed in the tentacles, body wall, and coelomocytes (Zhong et al. 2012). Unlike *SpBf* in *S. purpuratus*, *AjBf-2* is expressed in the coelomocytes and body wall, and responds to LPS challenge. Only a few homologues of complement proteins have been identified in the Asteroidea. In the sea star *Asterias rubens*, the deduced peptide sequence from a partial sequence shows similarities to C3. Expression increases in both coelomocytes and the hepatopancreas in response to LPS (Mogilenko et al. 2010). Expression of complement homologues is also noted in the antiviral response in the sunflower star, *Pycnopodia helianthoides*, with a strong complement response to SSaDV (see section "Sea Star Wasting Disease") (Fuess et al. 2015). A complement system is likely present in all echinoderms that relies on lectin-based and C3 tick-over activation to opsonize and augment phagocytosis of foreign targets.

Complement System Activation in Echinoderms

The activities of the echinoderm complement system, based on the factors described above, is likely amplified by the positive feedback loop of the alternative pathway

Table 5 Complement components are present in a wide range of invertebrates

Phylum	Species	Common name	Component	References
Echinodermata	*Strongylocentrotus purpuratus*	California purple sea urchin	C3, Bf	Smith et al. (1996), Al-Sharif et al. (1998), Smith et al. (1998), Terwilliger et al. (2004)
	Apostichopus japonicus	Sea cucumber	MBL, C3, Bf	Vasilenko et al. (2012), Zhou et al. (2011), Zhong et al. (2012)
	Asterias rubens	Sea star	C3	Mogilenko et al. (2010), Leclerc et al. (2013)
	Pycnopodia helianthoides	Sunflower star	C3, Bf/C2, ficolin, properdin	Feuss et al. (2015)
Chordata	*Halocynthia roretzi*	Tunicate	C3	Nonaka and Azumi (1999)
	Botryllus schlosseri	Colonial tunicate	C3, Bf, MASP1, MBL, ficolin	Franchi and Ballarin (2014, 2017)
	Branchiostoma japonicum	Amphioxus	Properdin	Gao et al. (2017)
			C3, Bf	Suzuki et al. (2002), He et al. (2008)
			C1q	Gao et al. (2014)
			Ficolin	Huang et al. (2011)
	Ciona intestinalis	Tunicate	MASP, MBL	Skjoedt et al. (2010)
			C3	Marino et al. (2002)
Arthropoda	*Tachypleus tridentatus*	Chinese horseshoe crab	C3	Ariki et al. (2008)
	Hasarius adansoni	Adanson's house jumper	C3	Sekiguchi et al. (2012)
Mollusca	*Euprymna scolopes*	Hawaiian bobtail squid	C3	Castillo et al. (2009)
	Sinonovacula constricta	Razor clam	C3	Peng et al. (2017)
	Ruditapes decussatus	Carpet-shell clam	C3, Bf	Prado-Alvarez et al. (2009)
	Mytilus galloprovincialis	Mussel	C3	Gerdol and Venier (2015)
Cnidaria	*Swiftia exerta*	Gorgonian coral	C3	Dishaw et al. (2005)
	Nematostella vectensis	Starlet sea anemone	C3, Bf, MASP	Kimura et al. (2009)
	Diadumene lineata	Orange-striped green sea anemone	C3	Fujito et al. (2010)
	Acropora millepora	Branching stony coral	C3	Miller et al. (2007)

(Fig. 19). This activity would be mediated by the formation of a C3–convertase–like complex consisting of a C3b homologue bound to a target surface through the thioester bond and associated with a Bf homologue. Echinoderm genomes likely encode a Factor D homologue (currently unidentified) that would cleave Bf and activate the serine protease function of Bb. Bb plus C3b would associate to form the C3–convertase–like complex to cleave and activate additional C3 in the feedback loop of the alternative pathway. Although homologues of MASP genes have not been identified in the sea urchin genome sequence, they have been reported in other invertebrates (Table 5) and their protease activity associated with target binding by homologues of MBL, C1q, and ficolins in echinoderms is assumed. On the basis of the inability to identify members of the terminal or lytic pathway in echinoderms or the equivalent of the MAC through either biochemistry, transcriptomics, or genomic analysis, it is likely that the ancestral function of the complement system in deuterostomes is opsonization of targets with covalently bound tags to augment phagocytosis, which results in effective host protection.

Antimicrobial Peptides in Echinoderms

Antimicrobial Peptide Characteristics

The survival and fitness of echinoderms in a marine environment near coastlines or estuaries that are heavily populated by microorganisms suggest that these invertebrates have an effective innate immune system (Tincu and Taylor 2004). In many invertebrate lineages, a central component of immunity is mediated by the activities of humoral components such as antimicrobial peptides (AMPs). AMPs are typically low molecular weight proteins (2–50 kDa; 50–100 amino acids) (Ganz 2003; Maroti et al. 2011) with significantly different structures, conformations, and functions, with antimicrobial activity against a broad spectrum of bacteria, viruses, and fungi. Many are evolutionarily conserved and are widely distributed in a wide range of organisms (Leippe 1999; Pag and Sahl 2002; Garcia-Olmedo et al. 1998). Many AMPs have a net positive charge, many form amphipathic structures that are stable in aqueous and hydrophobic solutions, and some are posttranslationally modified, including proteolytic release from larger precursors (Zasloff 2002; Hancock and Sahl 2006; Li et al. 2015). AMPs are typically classified as α helical, β pleated sheet, or a mix of both, in addition to extended regions of disordered loops (Melo et al. 2009). Some AMPs have no stable structure in solution and are considered to be IDPs that fold to their final active conformation upon binding to a target (Zhang et al. 2014).

The recruitment and interaction of AMPs with bacterial membranes is initially based on an attraction between the cationic portion of the AMPs and the negatively charged microbial membrane containing phosphatidylglycerol, cardiolipin, or phosphatidylserine (Scott et al. 1999; Zhao et al. 2001; Yeaman and Yount 2003). Some AMPs act as multifunctional microbicides that interfere with bacterial membranes through electrostatic attraction (Scott et al. 1999; Zhao et al. 2001), followed by

attachment (Heller et al. 2000; Huang, 2000), insertion (Yang et al. 2001; Lee et al. 2004), and formation of pores with hydrophilic channels in the lipid bilayer (Lee et al. 2004; Wang et al. 2015), in which the pore lumen is partly lined by peptides and phospholipid head groups (Matsuzaki et al. 1996). Other AMPs interact with intercellular molecules (Wang et al. 2015; Yonezawa and Sugiura 1992; Park et al. 1998) and inhibit protein and/or DNA synthesis (Boman et al. 1993; Subbalakshmi and Sitaram 1998) or cell wall synthesis (Brotz et al. 1998; Le et al. 2016). AMPs may neutralize endotoxins and cellular chemotaxis, and may modulate immune responses by altering cytokine production, angiogenesis, and wound repair (Hancock and Sahl 2006; Li et al. 2000; Shi et al. 1996; Rosenfeld et al. 2006; Veldhuizen et al. 2014). These potential antipathogen activities of AMPs have focused attention on their development for novel drugs.

Echinoderm Antimicrobial Peptides

Strongylocins and Centrocins

Several AMP families have been characterized in echinoderms (Table 6). These include the echinoid AMPs strongylocins and centrocins, which were originally characterized in the green sea urchin, *Strongylocentrotus droebachiensis* (Li et al. 2008, 2010b), with orthologues in the other sea urchins *S. purpuratus* (Li et al. 2010a), *Echinus esculentus* (Solstad et al. 2016), and *Arbacia lixula* (Perez-Portela et al. 2016). Strongylocins are cysteine-rich peptides, with six cysteines that may form three disulfide bridges to stabilize the peptide structure. Strongylocins have a leader that targets the peptide for secretion (Coleman et al. 1985; von Heijne 1990; Reddy et al. 2004), which is followed by a prosequence and the mature peptide (Li et al. 2008; Solstad et al. 2016). Both native and recombinant AMPs from echinoderms show antibacterial activity against both Gram-positive and Gram-negative bacteria. Recombinant (r)SpStrongylocins do not alter bacterial membrane permeability, suggesting intracellular targets or other means of microbial killing (Li et al. 2010a). SdStrongylocin 1 is expressed in phagocytes, vibratile cells, and colorless spherule cells, whereas SdStrongylocin 2 is expressed in phagocytes and red spherule cells (Li et al. 2014a). The diversity of the signal sequences and the expression profiles suggest that strongylocins likely have unique effects during immune responses among different echinoids.

The centrocin AMP family is present in several sea urchin species (Table 6) (Li et al. 2010b; Perez-Portela et al. 2016; Solstad et al. 2016). Centrocins are heterodimeric peptides consisting of a heavy chain plus a light chain, which is likely involved in the formation and stabilization of the active heterodimeric structure and resistance to bacterial proteases (Li et al. 2010b). Comparison of the deduced peptide sequences with native centrocin sequences indicates that the precursor peptide is cleaved from the preprosequence, the interchain, and the C-terminal dipeptide (Gly-Arg) after the posttranslational modification (Li et al. 2010b). In other species, the dimeric structure of these peptides enhances antimicrobial activity, solubility, and

Table 6 Antimicrobial peptides and proteins in echinoderms

Class and genus	Origin	Peptides	References
Asteroidea			
Asterias rubens	Coelomocytes	Fragments of actin, histone H2A, and filamin A[a]	Maltseva et al. (2007)
		Peptides	Maltseva et al. (2007)
Echinoidea			
Paracentrotus lividus	Coelomocytes	Fragments of β-thymosin[a]	Schillaci et al. (2010)
		Paracentrin 1	Schillaci et al. (2010)
Strongylocentrotus droebachiensis	Coelomocytes	SdStrongylocin	Li et al. (2008)
		SdCentrocin	Li et al. (2010b)
Strongylocentrotus purpuratus	Coelomocyte cDNA	SpStrongylocin[b]	Li et al. (2010a)
Echinus esculentus	Coelomocytes	EeStrongylocin 2 EeCentrocins	Solstad et al. (2016)
Arbacia lixula	Coelomocyte cDNA	AlStrongylocin	Perez-Portela et al. (2016)
	Gut and ovary cDNA	AlStrongylocin 2b	Perez-Portela et al. (2016)
	Coelomocyte, gut, testis, and ovary cDNA	AlCentrocin 1b	Perez-Portela et al. (2016)
Holothuroidea			
Cucumaria echinata	Whole body	Fragments of CEL-III protein[a,c]	Hatakeyama et al. (2004)
Cucumaria frondosa	Coelomic fluid	Sequence unknown	Beauregard et al. (2001)
Holothuria tubulosa	Coelomocytes	Holothuroidins[a,c]	Schillaci et al. (2013)

[a]Putative AMPs, derived from larger precursor molecules
[b]Recombinantly produced peptides
[c]Synthetic peptides

resistance to bacterial proteases (Dempsey et al. 2003; Pini et al. 2005; Lee et al. 2008; Dewan et al. 2009; Liu et al. 2010a, b; Shin et al. 2010).

Although both the strongylocin and centrocin families of AMPs are expressed in sea urchin coelomocytes, transcripts are also detected in digestive and reproductive tissues (Perez-Portela et al. 2016). Strongylocins and centrocins have different gene expression profiles in the four coelomocyte subclasses and are also expressed during embryogenesis (Li et al. 2014a, b). In larvae, centrocin 1 is present in the vesicles of blastocoelar cells that surround the stomach and esophagus and also present in the larval arms in locations that correspond to larval immune cells, which are central mediators of responses to ingested bacteria (see section "Blastocoelar Cells") (Ho et al. 2016). The identification and analyses of echinoderm AMPs indicate that they may be involved in various immune activities and that they respond to invading bacteria in both larvae and adult sea urchins.

Antibiofilm Activities of Antimicrobial Peptides from the Sea Urchin *Paracentrotus lividus*

In aquatic environments, bacteria may be present as single, free-swimming cells, which can form sessile, surface-associated multicellular communities called biofilms (Costerton et al. 1995; Hall-Stoodley et al. 2004; de la Fuente-Nunez et al. 2013). Biofilms occupy diverse environments and are highly resistant to antimicrobial therapies (Fey 2010; Spizek et al. 2010). Furthermore, damage to host tissues or microbiota allows access for secondary infections by opportunistic pathogens that can also form biofilms (Kostakioti et al. 2013). Almost all Gram-negative and Gram-positive bacteria form biofilms, and many are highly pathogenic when in this form. Biofilm formation is coordinated by a quorum-sensing communication system through small molecules to facilitate optimal cell density (Spoering and Gilmore 2006; Horswill et al. 2007; Brogden and Brogden 2011). Investigations of AMPs from a wide range of animals that may be used as novel drugs with activity against biofilms are of significant importance.

Novel cationic peptides from the sea urchin *Paracentrotus lividus* inhibit biofilm activity (Schillaci et al. 2010), as well as the growth of the pathogens *Staphylococcus aureus*, *S. epidermidis*, and *Pseudomonas aeruginosa* (Schurr et al. 1994; Schillaci et al. 2010, 2013, 2014). The acid extract of coelomocyte lysates from *P. lividus* shows antimicrobial activity for peptides of less than 5 kDa (collectively called 5-CC). 5-CC has a range of minimal inhibitory concentrations (MICs), depending on the bacterial species, and blocks biofilm formation effectively for *S. epidermidis* and *S. aureus* at sub-MIC concentrations (Fig. 20a–d). The biological activity of 5-CC is due to the peptide Paracentrin 1 (SP1; 1.251 kDa), which shares a short region of sequence identity with β thymosin from *P. lividus*. The structural properties of SP1 and the thymosin fragment, $hT\beta_4$, based on molecular dynamics, show that both have a central hydrophobic core and peripheral charged amino acids (Fig. 21). β thymosin has numerous biological effects such as induction of chemotaxis, angiogenesis, expression of metalloproteinases, and inhibition of inflammation (Huff et al. 2001). Chemically synthesized SP1 is active against the aquatic form and biofilms of several staphylococcal strains, in addition to *P. aeruginosa* (Fig. 20e, f) (Schillaci et al. 2014).

Holothurian Antimicrobial Peptides

Holothurians also express proteins and peptides with antimicrobial activity (e.g., see Kuznetsova et al. (1982), Ridzwan et al. (1995), Mohammadizadeh et al. (2013), Kiani et al. (2014)). For example, a lectin with antimicrobial activity against Gram-positive and Gram-negative bacteria is induced in the sea cucumber *Holothuria scabra* by bacterial challenge (Gowda et al. 2008). Immune cell lysates from the congeneric sea cucumber *H. tubulosa* contain two novel peptides, Holothuroidin 1 (H1; 1.389 kDa) and Holothuroidin 2 (H2; 1.547 kDa), which have characteristics of cationic AMPS (Schillaci et al. 2013). Both H1 and H2 are active against human bacterial pathogens and inhibit biofilm formation (Schillaci et al. 2013). H1 and H2 are ~30% hydrophobic with amphipathic α helices arranged so that the peptides have a hydrophilic and a hydrophobic face. This amphipathic folding characteristic

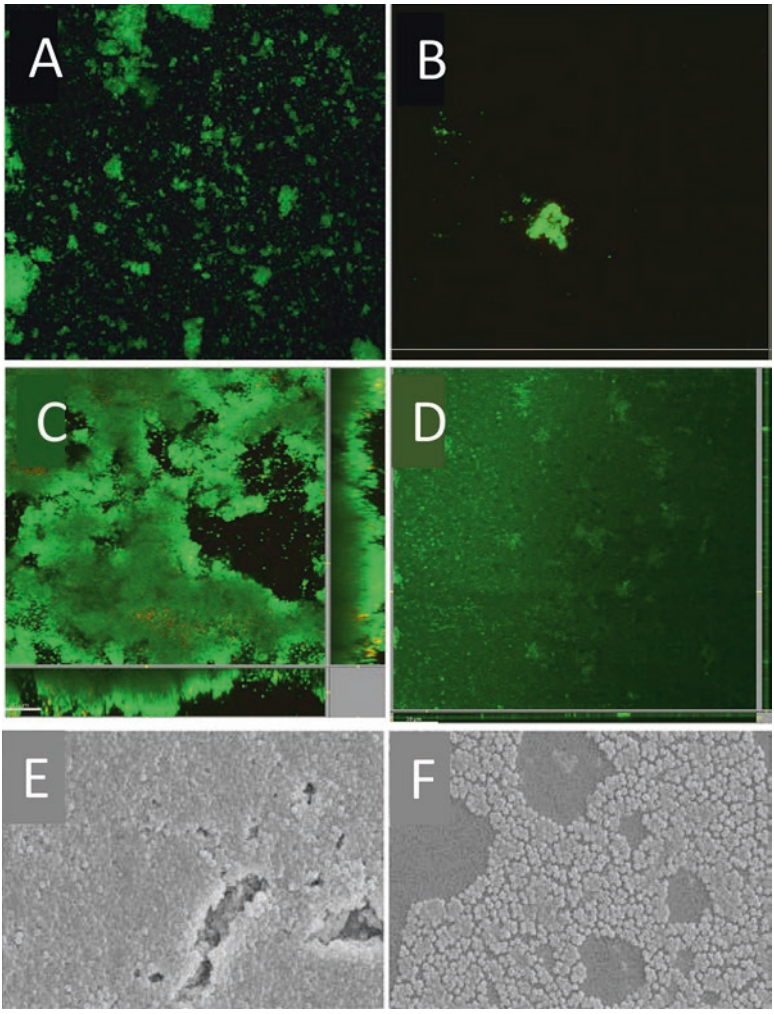

Fig. 20 *Peptides from the sea urchin Paracentrotus lividus have antibacterial activity*. (**a**–**d**) Biofilms of *Staphylococcus epidermidis* (strain 1457) were incubated for 6 or 24 h with the AMP mixture of Holothuroidin 1 (H1) and H2 (originally defined as 5-CC) (Schillaci et al. 2010). Dead cells were stained with propidium iodide (red) and live cells were stained with SYTO9 (green), followed by evaluation by laser scanning microscopy. The assays were repeated at least twice, with similar results. (**a**) After 6 h in the absence of 5-CC. (**b**) After 6 h with 5-CC. (**c**) After 24 h in the absence of 5-CC. (**d**) After 24 h with 5-CC. (**e**, **f**) Paracentrin 1 (SP1) inhibits growth and biofilm formation of *S. epidermidis* (strain RP62A) at subminimum inhibitory concentrations. (**e**) *S. epidermidis* grown without SP1. (**f**) *S. epidermidis* grown in the presence of SP1. Images (**e**) and (**f**) were captured by scanning electron microscopy. (Modified from Schillaci et al. (2014) in AMB Express)

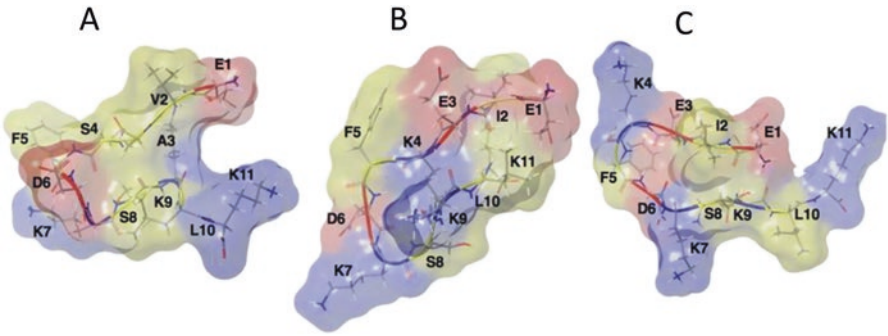

Fig. 21 *Molecular structure of the most stable conformations of SP1 and hTβ₄.* (**a**) SP1. (**b**) hTβ$_4$. (**c**) hTβ$_4$ rotated. The locations of some amino acids are labeled with colors to indicate acidic residues (red), basic residues (blue), and noncharged residues (yellow). (Reprinted from Schillaci et al. (2016), with permission from Springer Publishing)

facilitates interactions between the hydrophilic face of the peptides with the polar edges of bacterial membranes, and engages the nonpolar face with the hydrophobic core of membranes (Schillaci et al. 2013). Chemically synthesized H1 and H2 show broad-spectrum activity against most of the tested Gram-positive and Gram-negative strains, and they inhibit biofilm formation for a significant percentage of staphylococcal and *P. aeruginosa* strains (Fig. 22). Other AMPs from the Holothuroidea include one isolated from egg homogenates and CF from the sea cucumber *Cucumaria frondosa*, which has antibacterial activity against Gram-positive bacteria (Haug et al. 2002; Beauregard et al. 2001), and another that is a synthetic peptide of the α helical region of CEL-III from a congeneric sea cucumber, *C. echinata*, that also has antibacterial activity (Hatakeyama et al. 2004; Hisamatsu et al. 2008).

Although the results described here represent only a few AMPs from a few species, echinoderms are a potentially rich resource for the discovery of new AMPs. These natural compounds may offer novel and alternative strategies with applications in biotechnology and medicine to prevent and treat bacterial infections, including those associated with biofilm formation (Zilberman and Elsner 2008; Shukla et al. 2010; Glinel et al. 2012). The identification of novel AMPs from invertebrates is likely to provide innovative approaches for the design of new synthetic or recombinant derivatives with modified chemical–physical properties to improve antimicrobial activity against pathogens (Huang et al. 2010; Brogden and Brogden 2011).

Proteomics of Echinoderm Immune Responses

Cell processes are governed not only by gene expression or protein abundance but also by rapid variations in protein activity, localization, and interactions with other proteins, DNA, and RNA. Data generated through proteomics can be used to

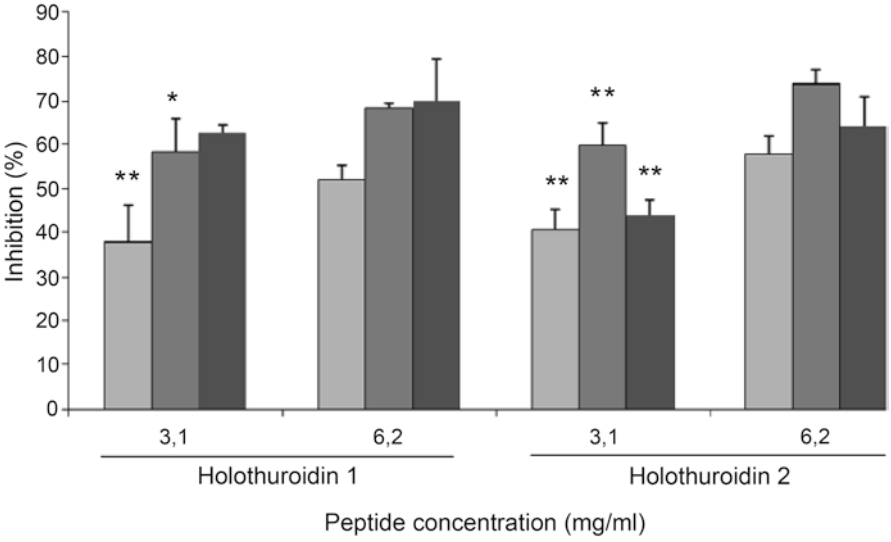

Fig. 22 *Holothuroidin 1 (H1) and Holothuroidin 2 (H2) from the sea cucumber Holothuria tubulosa have antibiofilm activity*. H1 and H2 have inhibitory activity against biofilms of *Staphylococcus aureus* (ATCC 25923) (light bars), *S. epidermidis* (ATCC 35984) (medium bars), and *Pseudomonas aeruginosa* (ATCC 15442) (dark bars). The data are expressed as the mean (± SD) of three experiments. $*p < 0.05$; $**p < 0.01$. (Reprinted from Schillaci et al. (2013) in AMB Express)

evaluate simultaneously all proteins involved in host defense mechanisms in a slice of time, which can be used to predict biological functions. Most proteomic approaches rely on fractionation of protein mixtures and the subsequent identification of separated proteins by mass spectrometry (MS). The process includes an enzymatic digestion of the proteins to produce peptides that are separated using liquid chromatography (LC) followed by ionization for MS analysis. Peptide mass fingerprinting is employed to search databases of protein sequences for predicted masses that would arise from peptide digestion. Tandem MS (MS/MS) evaluates the outcomes of additional fragmentation and also gives rise to a list of masses for each peptide fragment. The masses for each peptide and its fragments are also used to search databases of predicted masses that would arise from fragmentation of all peptides. The outcome from this analytic approach is the identification of the proteins in a sample.

Proteomic analyses rely heavily on the availability of reference genomes and transcriptomes to generate reference protein databases, and improved sequencing technologies have been a powerful engine for generating these databases. Proteomic strategies are now theoretically accessible for any species of interest (Dheilly et al. 2014) but have not been used extensively to investigate the molecular mechanisms underlying echinoderm immune responses. In addition to the genome resources described above, de novo transcriptomes are available for 23 species from all five echinoderm classes (Reich et al. 2015). Proteomic evaluation of coelomocytes shows changes in protein synthesis before and during an infection by various pathogenic microorganisms in the sea urchins *S. purpuratus* and *H. erythrogramma*, the

sea star *Marthasterias glacialis*, the sea cucumber *Apostichopus japonicus*, and the sunflower star, *Pycnopodia helianthoides* (see section "Sea Star Wasting Disease"). The functions of many potential immune response proteins in echinoderms remain unknown, but the currently available echinoderm genomes and transcriptomes can be used to generate reference databases for functional proteomics.

Proteomics of Immune Responses in Sea Stars and Sea Cucumbers

The proteome of the sea star *Marthasterias glacialis* was first characterized using a combination of one-dimensional (1D) SDS-PAGE followed by in-gel trypsin digestion and nanoliquid chromatography (LC) separation of peptides, plus two-dimensional (2D) SDS-PAGE followed by in-gel trypsin digestion before MS analysis (Franco et al. 2011). At the time, next-generation sequencing approaches were not developed sufficiently and protein characterization was based on the echinoderm database at GenBank (www.ncbi.nlm.nih.gov). The absence of a reference database for *M. glacialis* limited the efficiency and accuracy of protein identification and was likely the basis for why no sea star complement homologues were identified despite their known presence in members of other classes (Al-Sharif et al. 1998; Smith et al. 1996, 1998, 1999, 2001; Xue et al. 2015). However, this approach identified an abundance of homologous proteins involved in cytoskeleton regulation, cell adhesion, signaling, regulation, proliferation, and regeneration. Similarly, comparative proteomics for coelomocytes from the sea cucumber *Apostichopus japonicus* before and after response to a challenge from the Gram-negative bacteria *Vibrio splendidus* identified 40 proteins, of which 32 are upregulated (e.g., calreticulin, calumenin, ficolin, and NIPSNAP1) and eight are downregulated (Zhang et al. 2014). Of these differentially expressed proteins, approximately a third are immune response proteins. Despite the shortcoming of this study, based on the lack of an appropriate database, several proteins with predictions of crucial functions in sea cucumber immune responses were first identified by this proteomic approach (reviewed in Xue et al. (2015)).

Proteomics of Immune Responses in Sea Urchins

Similar proteomes are observed from the CF of the sea urchins *S. purpuratus* and *H. erythrogramma,* using an approach based solely on proteins separated by 1D SDS-PAGE, followed by in-gel trypsin digestion, nano-LC peptide separation, and MS analysis (Dheilly et al. 2012, 2013). A total of 323 unique proteins could be identified from *S. purpuratus*, with matches to 236 homologues in *H. erythrogramma*. Although the *S. purpuratus* genome was used as a reference, it was probable that some proteins in *H. erythrogramma* could not be identified because of sequence divergence between the species rather than absence. Proteins associated with cytoskeletal regulation and cell adhesion are the most abundant in both proteomes, which is consistent with their prevalence in highly mobile amoeboid

coelomocytes. These cells have previously been implicated in clotting reactions and phagocytosis, and express the complement component SpC3 (Al-Sharif et al. 1998; Gross et al. 1999, 2000). It is noteworthy that the complement component C3 and the complement receptor type 2 (CR2) are among the most abundant proteins in sea urchin CF, suggesting their involvement in the echinoderm immune system (Dheilly et al. 2013). Major yolk protein (MYP) is another abundant protein in the CF of sea urchins (Giga and Ikai 1985a; Dheilly et al. 2013). An isoform of MYP, a major constituent of sea urchin egg yolk, functions as an ironless calcium-binding transferrin with activities in cell–cell adhesion and positional information during embryonic development (Noll et al. 1985, 2007). The MYP isoform in the CF is larger and has greater zinc-binding capacity and greater glycosylation, and requires higher concentrations of Ca^{2+} for successful binding to liposomes (Giga and Ikai 1985a, b; Unuma et al. 2007; Dev and Robinson 2014). However, despite its extreme abundance, the functions of MYP in the CF in vivo are not known. Like the proteome of sea star CF (see section "Proteomics of Immune Responses in Sea Stars and Sea Cucumbers"), other proteins in the CF of sea urchins are predicted to have functions related to cellular regulation and proliferation, lysosomes, proteases, peptidases, stress responses, and detoxification (Dheilly et al. 2013).

Comparative proteomics can be used to predict the functions of proteins in the CF of echinoderms that are involved in antipathogen responses. Predictions are based, in part, on the assumption that variations in protein abundance in response to immune challenge indicate involvement in immune activities. For example, there is a substantial change in the proteome of the CF from the sea urchin *H. erythrogramma* after challenge with bacteria or LPS in comparison with the proteome after injection of buffer, which suggests differences in proteins involved with immune responsiveness versus injury repair (Dheilly et al. 2011b, 2012). The results imply significant changes in energy metabolism and cell signaling, which could ultimately regulate the recruitment of coelomocytes to the site of injury for wound repair (Dheilly et al. 2011b). Furthermore, a discrete modification of the coelomocyte proteome occurs in response to the injection of bacteria with increased expression of apextrin and calreticulin. Apextrin is found in the secretory vesicles of sea urchin eggs, is involved in cell adhesion during embryonic development (Haag et al. 1999), and has an MACPF domain that may be associated with cell lysis (Miller et al. 2007; Rosado et al. 2008). Similarly, expression of apextrin in the protochordate *Branchiostoma belcheri* is induced during acute immune responses (Huang et al. 2007), acts as an extracellular effector for bacterial agglutination and intracellular bacterial recognition, and activates signaling mediated by NFκB (Huang et al. 2014). Calreticulin is a multifunctional Ca^{2+}-binding protein, which regulates intracellular Ca^{2+} homeostasis and storage in the endoplasmic reticulum (Gelebart et al. 2005). Its increase in abundance following bacterial challenge demonstrates the importance of calcium signaling in immune cells of echinoderms.

Proteomics are also used to evaluate temporal responses of sea urchins to immune challenge. When the CF is collected over time from the sea urchin *H. erythrogramma* after injection with LPS or buffer, and is evaluated by 1D SDS-PAGE, in-gel trypsin digestion, and LC-MS, 345 proteins are identified and their quantification provides a picture of the successive steps in sea urchin responses to

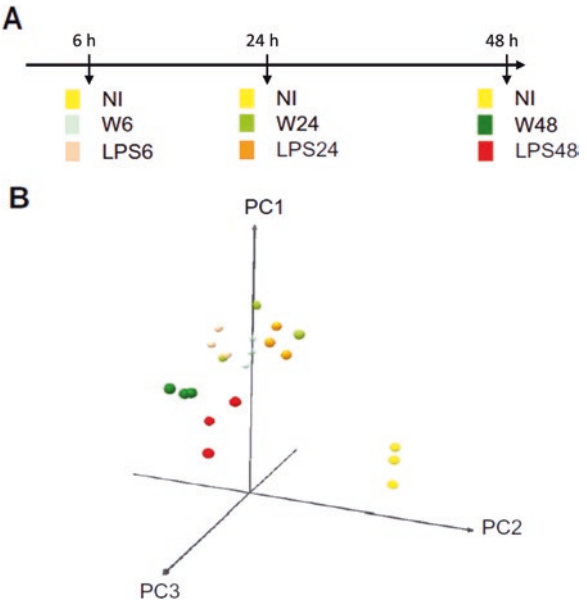

Fig. 23 *Temporal changes in the coelomic fluid proteome of the sea urchin Heliocidaris erythrogramma, responding to LPS*. (**a**) The experimenal design is illustrated showing the timeline over which samples were collected. Coelomic fluid samples from 21 sea urchins were collected for 48 h after treatment. Samples (n = 9) were collected from three sea urchins that were not injected (NI), including three samples after 6 h (NI6), 24 h (NI24), and 48 h (NI48). Samples (n = 9) were also collected after wounding (W), including three samples after 6 h (W6), 24 h (W24), and 48 h (W48). Additional samples (n = 9) were collected after the injection of LPS, including three samples after 6 h (LPS6), 24 h (LPS24), and 48 h (LPS48). (**b**) The three-dimensional score plot of the principal component (PC) analysis shows the cumulative data for all proteins identified in the CF of *H. erythrogramma*. (Reprinted from Dheilly et al. (2012) in Developmental and Comparative Immunology, with permission from Elsevier)

immunological challenge compared with injury (Dheilly et al. 2012). The injury response consists of an initial upregulation of cytoskeletal proteins consistent with an increase in cell motility, followed by an increase in MYP plus members of the large lipid transfer protein (LLTP) family, which are iron-binding proteins. The late-stage cellular response shows an induction of von Willebrand Factor, cyclophilin B, and selectins, suggesting cell migration to the site of injury and adhesion to collagen. The principal component analysis of the LPS response shows that most changes in protein abundances occur after 48 h, and that responses to injury are quite different (Fig. 23) (Dheilly et al. 2012). After LPS injection, there are relative increases in vesicular transport proteins such as coatomer proteins, receptor activated C kinase (RACK), and cellular signaling molecules such as G protein (q) and mitogen-activated protein kinase (MAPK). Many immune response proteins such as the sea urchin complement homologue SpC3, dual oxidase maturation factor, dual oxidase 1, α2-macroglobulin, and SRCRs are also more abundant 48 h after injection of LPS compared with injury, suggesting that these proteins are important in inducible immune responses in sea urchins.

Fibrocystin L and aminopeptidase N are identified only 48 h after LPS injection, indicating that these proteins are expressed only during late stages of PAMP-induced immune responses. In vertebrates, fibrocystin L is a lectin receptor expressed on immune responsive cells and participates in regulating phagocytosis (Hogan et al. 2003). Aminopeptidase N (also called CD13) is involved in immunomodulatory peptide degradation, antigen trimming, and antigen processing (Riemann et al. 1999). This approach for analyzing immune/injury response proteins in the sea urchin *H. erythrogramma* is sufficiently sensitive to detect the over expression of highly variable proteins in response to LPS, such as those of the diverse SRCR family. For example, 14 different SRCRs are present in the CF of *S. purpuratus* according to results from shotgun proteomics (Dheilly et al. 2013) in agreement with cDNA sequences and specific patterns of SRCR expression with respect to individual sea urchins (Pancer 2000). Similarly, shotgun proteomics of *H. erythrogramma* CF show that three SRCRs are expressed specifically in response to LPS challenge (Dheilly et al. 2012). The inducible expression of these cell surface proteins on sea urchin coelomocytes and those that are secreted into the CF suggests their involvement in the recognition of, or response to, pathogens.

Proteomic studies of echinoderm immune systems have yielded important results, and the lower cost and higher accessibility of next-generation sequencing approaches have provided genomes and transcriptomes of diverse sea urchins, brittle stars, sea stars, and sea cucumbers (see www.echinobase.org). This expansion to any echinoderm species is expected to revolutionize the proteomics of the immune system of echinoderms and to allow in-depth analyses of this complex system. In addition, reanalysis of previously published MS results, using newly sequenced genomes and transcriptomes as references, will increase significantly the amount of information extracted from previous studies (Franco et al. 2011; Xue et al. 2015). Applications of proteomics have also been used to evaluate the diversity of highly variable proteins that function in the immune response, and investigations of immune biomarkers (Fulton and Twine 2013) will be used to characterize the protein antigens targeted by the diverse families of PRRs that function in the sea urchin immune system. Such studies may enable an understanding of the extraordinary expansion of immune response gene families from a range of echinoderms, including TLRs, NLRs, SRCRs, lectins, and Trf proteins (see section "Immunogenomics: Immune Genes Encoded in Echinoderm Genomes") (Rast et al. 2006; Buckley and Rast 2012).

Negligible Senescence and the Immune System of Sea Urchins

The term "negligible senescence" is used to describe animals that do not show an association between increased age and an increased mortality rate or decreased fertility, physiological function, or disease resistance (Finch 1990; Finch and Austad 2001). Sea urchins appear to exhibit negligible senescence with indeterminate

Table 7 Estimated maximum life-spans for selected sea urchins

Species	Maximum life span (years)	References
Tripneustes ventricosus	3	Pena et al. (2010)
Lytechinus variegatus	3–10	Moore et al. (1963), Beddingfield and McClintock (2000), Hill et al. (2004), Russell et al. (2012)
Echinometra lucunter	≥40	Ebert et al. (2008)
Strongylocentrotus purpuratus	≥50	Ebert (2007, 2010)
Mesocentrotus franciscanus	>100	Ebert and Southon (2003), Ebert (2007)

growth, sustained ability to regenerate external appendages, lifelong reproduction, few reported cases of neoplasm, and no increase in the mortality rate at advanced ages (Jangoux 1987; Robert 2010; Ebert 2008; Bodnar and Coffman 2016). Despite these properties, different sea urchin species have very different life-spans that range over nearly two orders of magnitude (Table 7). Characterization of immune cell functions in sea urchins may help us to understand the contribution of the immune system to the negligible senescence and increased life-span that have been observed for many members of the echinoid lineage.

The accumulation of damage in somatic tissues with age is thought to contribute to a reduction in cell function, cell death, or cancer progression (Martin and Grotewiel 2006). Sea urchin species with life-spans ranging from ~3 to >100 years (Table 7) show no age-related increase in indicators of oxidative damage in coelomocytes and other somatic tissues such as protein carbonyls and 4-hydroxynonenal, indicating effective preservation with age. A marker for oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, measured in cell-free CF from sea urchins, also does not increase with age (Du et al. 2013). Antioxidant activities (e.g., superoxide dismutase and total antioxidant capacity) are largely maintained with age in coelomocytes and other somatic tissues, which may contribute to the lack of accumulated damage. Interestingly, coelomocytes from longer-lived species (*S. purpuratus* and *M. franciscanus*) have greater antioxidant properties than shorter-lived *L. variegatus*, suggesting that the immune cells of the long-lived animals are better equipped to mitigate oxidative damage.

Although the sea urchin *L. variegatus* is short-lived, coelomocytes from this species are highly resistant to a variety of DNA-damaging agents including ultraviolet radiation (UV), hydrogen peroxide (H_2O_2), methyl methanesulfonate (MMS), benzo[a]pyrene, and bleomycin (Loram et al. 2012; Reinardy and Bodnar 2015). The LD_{50} values calculated for coelomocytes 24 h after ex vivo exposure to these agents are many times higher than those for mesenchyme cells of other marine invertebrates (including larvae from *L. variegatus*) and values reported for cultured mammalian cells (Loram et al. 2012; El-Bibany et al. 2014; Reinardy and Bodnar 2015). Apoptosis is not detected in *L. variegatus* coelomocytes even at high doses

of these damaging agents (Loram et al. 2012), which is consistent with reports of low levels of apoptosis in coelomocytes of the sea urchin *Paracentrotus lividus* after exposure to UV-B (Matranga et al. 2006). Effective DNA repair is evident in *L. variegatus* coelomocytes following acute ex vivo exposures and is accompanied by an upregulation in the expression of genes encoding components of DNA repair pathways (Reinardy and Bodnar 2015). The DNA repair capacity of coelomocytes is maintained with age in the sea urchin *L. variegatus* and in the rock-boring sea urchin, *Echinometra lucunter*, following H_2O_2-induced DNA damage; however, the sample sizes were small in this study (El-Bibany et al. 2014). There is a general correlation between the DNA repair capacity of coelomocytes and the life-span of different sea urchin species (*Echinometra lucunter* > *Lytechinus variegatus* > *Tripneustes ventricosus*), with longer-lived species showing higher levels of repair 24 h after exposure to DNA damage (El-Bibany et al. 2014). This observation supports the notion that longer-lived species invest greater resources in cellular maintenance and repair (Kirkwood 2005).

The ability to protect the genome from harmful DNA damage is critical for maintaining genome stability and protection against neoplastic disease (Lombard et al. 2005). Sea urchins are noted for the lack of reported cases of neoplasm (Jangoux 1987; Robert 2010), although further study is needed to determine whether the high resistance to DNA damage correlates with effective DNA repair in sea urchins and if this contributes to a low incidence of neoplastic disease. Interestingly, DNA damage induced in somatic tissues following intracoelomic injection of the DNA-alkylating agent MMS is accompanied by increased immune gene expression in coelomocytes (Reinardy et al. 2016). This suggests a link between the DNA damage response and activation of the echinoid immune system and possible activities in the surveillance and removal of damaged cells.

Systematic studies that compare immune function with age across sea urchin species with different life-spans will be important for discerning the contribution of the immune system to the long-term maintenance of tissue homeostasis and resistance to disease that defines negligible senescence. Interspecies genomic comparisons indicate that the immune gene repertoire is more complex in long-lived sea urchin species (*S. purpuratus*, *M. franciscanus*) than in short-lived species (*L. variegatus*) (Buckley and Rast 2012). Aging is typically accompanied by a decline in immune function and increased vulnerability to infectious, inflammatory, and neoplastic disease. Understanding the extent and mechanisms by which sea urchins avoid age-related decline in immune function will provide valuable insight into the underpinnings of negligible senescence.

Toxicology and Effects of Pollution on Echinoderm Immunity

Marine ecosystems are vulnerable to anthropogenic stress because of their interface with terrestrial environments and the impacts of coastal city development related to urban runoff, industrial effluents, antifouling paints on boats, mining operations,

and atmospheric particulate pollutants (Islam and Tanaka 2004; Li et al. 2014b). Pollutants include heavy metals, insecticides and herbicides, waste from food processing, pollutants from livestock operations, volatile organic compounds, and chemical waste. Despite the continuous use and release of such substances, surprisingly little is known about the toxicological risk faced by marine organisms for many of these compounds. An increasing number of studies are combining approaches for monitoring chemical contaminant levels with measurements of biological responses to assess the environmental status across marine regions (Lyons et al. 2010; Connon et al. 2012; Andersen et al. 2016).

Pollutants affect survival, growth, reproduction, metabolism, and immunity in marine invertebrates (Ellis et al. 2011; Gallo and Tosti 2013; Ray et al. 2015). In echinoderms, heavy metals in contaminated sites alter the immune responses of the sea star *Asterias rubens* (Coteur et al. 2003a, b). In response to manganese, *A. rubens* induces the proliferation of hematopoietic cells and shows an increase in the number of coelomocytes (Oweson et al. 2008, 2010). In the sea star *Marthasterias glacialis*, zinc inhibits the lysozyme-like activity of the mucus (Stabili and Pagliara 2009), which may lead to increased disease susceptibility. The use of immunological parameters such as lysozyme activity has been proposed for risk assessment in echinoderms exposed to chemical contamination. In the sea urchin *Paracentrotus lividus*, zinc treatment causes phagocytes to change shape from petaloid to filopodial and increases the number of the red spherule cells (or red amoebocytes) (Fig. 24) (Pagliara and Stabili 2012). Coelomocytes respond to stress conditions such as temperature shock and pollution in both laboratory experiments and field studies (Matranga et al. 2000), and their use as sentinels of environmental stress was first proposed by Matranga et al. (2005). Short-term treatment with zinc also affects humoral parameters, causing decreases in both lysozyme-like activity and antibacterial activity against *Vibrio alginolyticus* (Fig. 25a, b) (Pagliara and Stabili 2012). The impacts of eight divalent heavy metal ions alter the activities of immune-related enzymes, including superoxide dismutase, phenoloxidase, acid phosphatase, alkaline phosphatase, and myeloperoxidase in CF from the sea cucumber *Apostichopus japonicus* (Jiang et al. 2016). Lead inhibits the activities of most immune-related enzymes in *A. japonicus*, whereas cadmium strongly inhibits myeloperoxidase. The results suggest that heavy metals have significant impacts on *A. japonicus* immunity and that metals are likely important stressors that modulate responses in echinoderm immune systems.

The sea urchin *Paracentrotus lividus* is a dominant predator in Mediterranean rocky reef ecosystems and serves as an important model organism for monitoring of the state of marine environmental health (Pinsino and Matranga 2015). Sea urchins are capable of adjusting to environmental changes, which enables investigations to uncover the conserved molecular signaling pathways involved in the protection, robustness, resistance, and plasticity of this invertebrate innate immune system (Rast et al. 2006). Immunological and eco-toxicological analyses of coelomocytes from *P. lividus* have been used to assess pollution in a marine coastal area of the

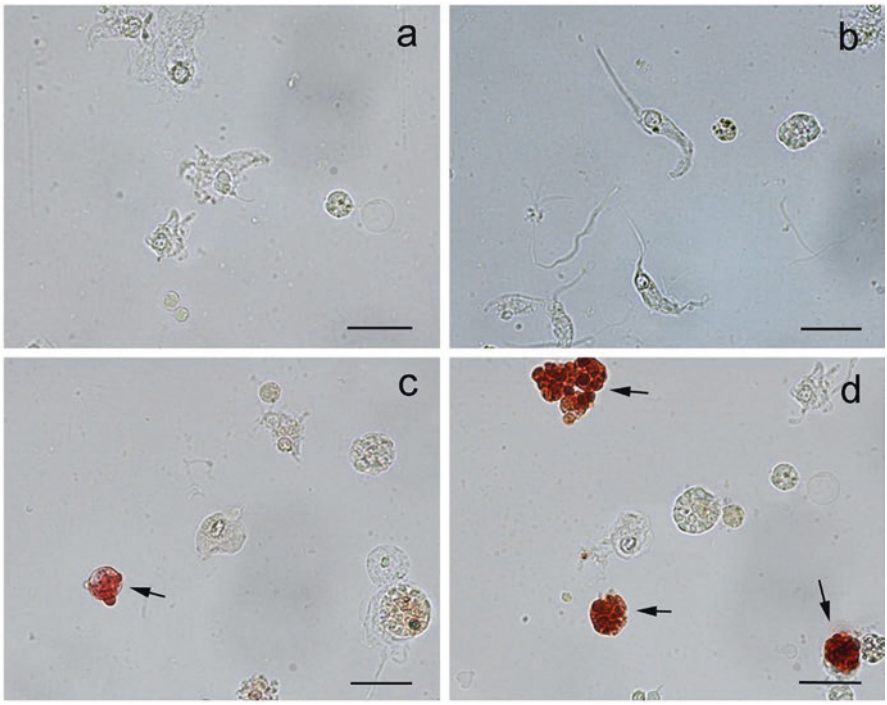

Fig. 24 *Coelomocytes from the sea urchin Paracentrotus lividus respond to zinc*. Sea urchins were housed at 20°C in filtered seawater at 37‰ salinity. Experimental animals were exposed to 18.4 μM $ZnCl_2$ in the seawater. Coelomocytes were collected from treated and untreated animals after 24 h. (**a**) Phagocytes from untreated sea urchins. (**b**) Phagocytes from sea urchins exposed to zinc. (**c**) Red spherule cells (arrow) from untreated sea urchins. (**d**) Red spherule cells (arrows) from sea urchins exposed to zinc. The scale bar is 5 μm. (Reprinted from Pagliara and Stabili (2012) in *Chemosphere*, with permission from Elsevier)

northern Adriatic Sea (Matranga et al. 2000). Coelomocytes isolated from animals collected from urban and industrially contaminated sites (in Rovinj, Croatia) exhibited increased numbers of the red spherule cells (or red amoebocytes) in comparison with animals collected from unpolluted sites or subjected to accidental injuries (Matranga et al. 2000; Pinsino et al. 2008). Under normal, nonpolluted physiological conditions, the relative percentage of red spherule cells from *P. lividus* constitutes only 4.70 ± 1.48% (mean ± SE) of the total cell population (Matranga et al. 2006). However, under stressful conditions, the percentage increases to 11.7 ± 0.99% (≥50% increase) although the total number of coelomocytes in the CF remains constant (Matranga et al. 2006; Pinsino et al. 2008). Immune cells harvested from animals from polluted seawater exhibit increased levels of the constitutive form of the 70kDa heat shock cognate (Hsc70) protein (Matranga et al. 2000; Pinsino et al. 2008). Similar increases in the Hsc70 concentration are observed in in vitro studies

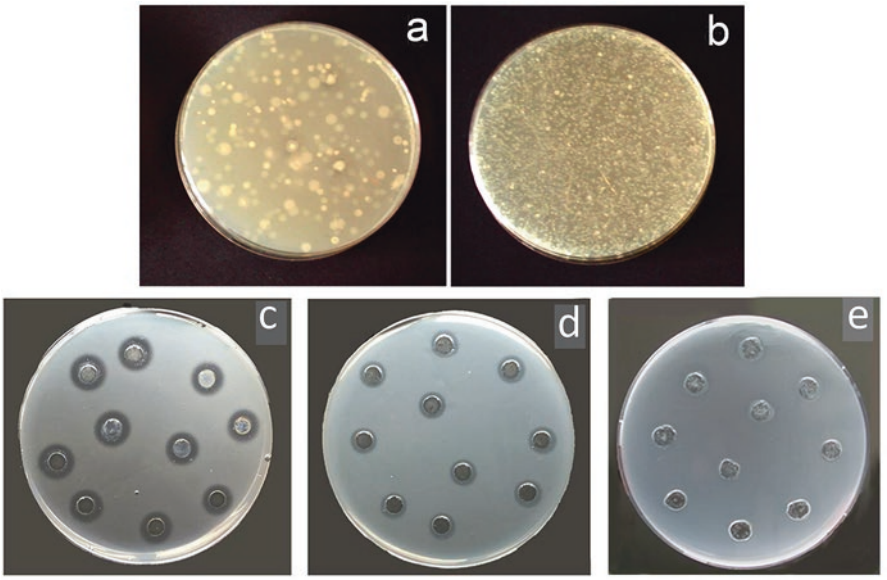

Fig. 25 *Antibacterial activity in coelomocyte lysates from Paracentrotus lividus inhibits growth of Vibrio alginolyticus*. Sea urchins were housed in filtered seawater or seawater with 18.4 μM $ZnCl_2$ as described in the legend for Fig. 24. *Vibrio alginolyticus* bacteria were incubated for 30 min with coelomocyte lysate from untreated sea urchins (**a**) or zinc-treated sea urchins (**b**) and evaluated for bacterial proliferation. (**c**–**e**) Coelomocytes from the sea urchin *Paracentrotus lividus* have lysozyme-like activity. Sea urchins were housed at 20 °C in filtered seawater of 37‰ salinity, and experimental animals were exposed to 0.5 mg/L lindane. Coelomocytes were collected after 24 or 48 h of treatment, and lysates were evaluated for lysozyme activity in a standard growth assay of *Micrococcus lysodeikticus*. The diameter of the cleared zone around each well filled with 30 μL of coelomocyte lysate was due to the lysis of bacterial cell walls and was recorded after overnight incubation at 37 °C. The diameter of the cleared zone was compared with a reference sample of hen egg white lysozyme. (**c**) *M. lysodeikticus* plus coelomocyte lysate from untreated sea urchins after 24 h. (**d**) *M. lysodeikticus* plus coelomocytes lysate from sea urchins treated with lindane for 24 h. (**e**) *M. lysodeikticus* plus coelomocyte lysate from sea urchins treated with lindane for 48 h

in which *P. lividus* coelomocytes are exposed to temperature shock, decreased pH, UV-B radiation, or heavy metals (Matranga et al. 2002, 2005, 2006). Hsc70 serves as an indicator of changes in the steady-state homeostasis with key activities in mediating stress resistance, immune resistance, and apoptosis (Mosser et al. 2000). Accordingly, the results demonstrate the utility of Hsc70 as a general stress response marker for reliable monitoring of both acute and chronic stresses in *P. lividus* immune cells.

Sea urchin coelomocytes are useful for evaluating the toxicity of nanoparticles (NPs) through changes in expression patterns of genes and proteins and alterations to cellular morphology. Coelomocyte exposure to a range of NPs has variable effects

on Hsc70 protein levels, and when taken up by the cells, NPs cause modifications to the trans face of the Golgi apparatus and to the endoplasmic reticulum (Falugi et al. 2012). Coelomocytes exposed to tin dioxide (SnO_2) NPs do not show altered Hsc70 levels relative to controls, whereas immune cells exposed to cerium dioxide (CeO_2) or iron oxide (Fe_3O_4) NPs show decreased Hsc70 protein levels. Furthermore, the activity of acetylcholinesterase (AChE), butirylcholinsterase (BChE), and propionylcholinesterase (PrChE), as well as the basal protein levels of glucose-regulated protein 78 (GRP78), are significantly inhibited in immune cells exposed to all of these NPs. Thus, as in human immune cells, coelomocytes from *P. lividus* exposed to NPs show selective changes to specific pathways and biomarkers (Boraschi et al. 2012). Accordingly, phagocytes from *P. lividus* that interact with titanium dioxide (TiO_2) NPs elicit a receptor-mediated phagocytic mechanism that involves the TLR/p38 MAPK signaling pathway but does not activate an inflammatory response or the Hsc70-dependent stress response (Pinsino et al. 2015). In general, these findings demonstrate that sea urchin immune cells can be employed as a tool for analysis of toxicity and safety of NPs, and can also be used to provide useful data on the pollution status of marine ecosystems.

Unlike many other chemicals, phosphate pollutants and some pesticides are deliberately added to the environment to kill selective organisms (Vijgen et al. 2011; Szabo and Loccisano 2012). Highly toxic, persistent, and bioaccumulating pollutants (e.g., polychorobiphenyls (PCBs)) may have major ecological consequences. For the sea star *Asterias rubens*, PCB exposure results in increased production of reactive oxygen species (ROS) (Coteur et al. 2002b, 2003a; Danis et al. 2004a, b, 2006). Similarly, chronic exposure of the sea urchin *L. variegatus* to phosphate decreases bactericidal activity against pathogenic *Vibrio* sp. (Böttger and McClintock 2009). Exposure to subchronic concentrations of the pesticide lindane alters both cellular and humoral immune responses in *P. lividus*, decreasing total coelomocyte numbers in the CF and increasing the proportion of red spherule cells (Stabili and Pagliara 2015). Hemolytic and lysozyme-like activities, as well as antibacterial activity toward *Vibrio alginolyticus*, decrease in sea urchins treated with lindane (Fig. 25c–e). These immunological changes in response to lindane exposure highlight the use of coelomocytes from *P. lividus* and other echinoderms as novel biosensors to assess the impact of pollutants on invertebrate health and to provide invaluable information on marine ecology (Stabili and Pagliara 2009; Luna-Acosta et al. 2010, Pinsino and Matranga 2015).

Conclusion

Elia Metchnikoff opened the field of immunology through his investigations of blastocoelar cell chemotaxis and encapsulation of the tip of a rose prickle with which he had impaled a sea star larva (Metchnikoff 1893). This first report and subsequent work on larvae, plus tissue rejection and clearance of pathogens from the adult body cavity, demonstrated the presence of an echinoderm immune system. Clear echinoderm larvae and observations of larval immune cells, particularly

after manipulating the expression levels of transcription factors that function in hematopoiesis, demonstrate the conservation and ancient ancestry of the transcriptional circuitry required to differentiate an immune system in deuterostomes. Although the echinoderm phylum is ancient, which is consistent with species diversity, phagocytic coelomocytes are pervasive within the classes and indicate that phagocytosis and encapsulation are essential and basal attributes of immunity. The genome of the purple sea urchin was the first basal deuterostome to be analyzed and provided information to identify and characterize the astounding numbers of immune system genes that function in the echinoderm immune system (Sodergren et al. 2006; Rast et al. 2006; Hibino et al., 2006). It was the first assessment of the entire system, which documented it as surprisingly complex, robust, and likely highly sophisticated.

Many humoral factors that are secreted into the CF—including antipathogen proteins, complement components, and some AMPs—augment phagocytosis through opsonization, whereas other humoral factors, including other AMPs, are microbicidal. The involvement of large protein families that function in immune detection and response, plus the possibility of regulated mechanisms for diversifying immune genes, suggest that many or perhaps all multicellular organisms have molecular mechanisms for immune gene sequence diversification. Although the echinoderm mechanisms for immune diversification are unknown and may be unlike mechanisms in other groups, gene sequence diversity illustrates the robustness of the echinoderm immune response. Echinoderms inhabit a wide range of ecological niches from the abyssal depths of the oceans to the intertidal zone, are often key, long-lived species that shape and maintain the status of many marine ecosystems, and can be employed as sentinels of change in ecological health. The marine habitats in which echinoderms live are shared with multitudes of microbes, many of which are opportunist pathogens that can complicate and exacerbate infections initiated by true pathogens. Understanding the flexibility of the echinoderm immune response in its arms race for survival against ever changing pathogens will be the challenge for the future.

Dedication This work is dedicated to Valeria Matranga who passed away too young in April 2016 after a long and courageous battle against cancer. Valeria contributed immensely to our understanding of cellular and molecular immune processes in the sea urchin, *Paracentrotus lividus*. Her dedicated research on echinoderms led to an understanding of how they interact with their environment and how coelomocytes can be employed to evaluate environmental toxins and pollutants. She and her insight for creative approaches in eco-immuno-toxicology will be missed because her approach to thinking about how to answer difficult scientific questions would have been more and more valuable in the future.

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Urochordata: *Botryllus* – Natural Chimerism and Tolerance Induction in a Colonial Chordate

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Natural Chimerism and Tolerance Induction During Pregnancy

Chimerism is the presence of two or more genetically distinct and separately derived populations of cells in the same organ or individual. Natural chimerism has been detected in a wide variety of multicellular organisms, including humans (review in Buss 1982; Grosberg 1988; Bianchi 2007, 2010; Lakkis et al. 2008; Voskoboynik et al. 2009; Rinkevich 2011; Eikmans et al. 2014). In placental mammals, natural chimerism occurs during pregnancy between a mother and fetus or between fetuses (Owen 1945; Billingham et al. 1952; Gengozian et al. 1964; Herzenberg et al. 1979; Tippett 1983; Bianchi et al. 1996; van Dijk et al. 1996; O'Donoghue et al. 2004; Loubiere et al. 2006; Ross et al. 2007; Mold et al. 2008, 2010; Betz 2010; Kallenbach et al. 2011; Johnson et al. 2012; Eikmans et al. 2014). Progenitor cell trafficking over the placenta often leads to a stable engraftment of hematopoietic stem cells

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(HSCs) and non-HSCs in mother and child (Van Rood et al. 1958; Bianchi 2007; Betz 2010; Kallenbach et al. 2011; Johnson et al. 2012; Eikmans et al. 2014). From an immunological perspective, pregnancy represents a state akin to organ transplantation, in which fetal antigens are foreign to the mother's immune system. The fetus expresses a combination of maternal and paternal antigens; this combination is referred to, in immunological terms, as semi-allogeneic. The immune system of placental mammals has therefore evolved mechanisms to prevent immune-mediated rejection of semi-allogeneic fetuses.

In the middle of the twentieth century, Owen (1945, 1954) and Billingham et al. (1953) suggested that fetal exposure to foreign antigens during pregnancy induced immunological tolerance in the fetus. Years later, Mold et al. (2008, 2010) further found that a substantial number of maternal cells cross the placenta to reside in fetal lymph nodes and induce the development of regulatory T cells (Tregs) that suppress fetal antimaternal immunity. Tregs persist at least until early adulthood and are now recognized as key mediators of immunological tolerance (Mold et al. 2010; Betz 2010). Mold et al. (2008, 2010) discovered a form of antigen-specific tolerance in humans induced in utero via chimerism, highlighting the important role of cell chimerism in the induction of fetal tolerance to maternal tissues. Furthermore, research in transplantation and stem cell biology over the last 70 years has revealed that chimerism plays a role in successful, long-term engraftment (review in Mold et al. 2008, 2010; Weissman and Shizuru 2008; Betz 2010; Kallenbach et al. 2011; Eikmans et al. 2014; Weissman 2014). Successful HSC transplantation regenerates the entire blood and immune system and induces long-term immune tolerance to donor-specific histocompatibility antigens, allowing engraftment of any organ or cells from the same donor without a need for immunosuppression (Weissman and Shizuru 2008; Weissman 2014; Chhabra et al. 2016). Colonial ascidians may offer a unique evolutionary perspective on stem-cell-mediated chimerism and immunological tolerance. In these organisms, a genetically controlled allorecognition system, similar to the natural killer cell "missing self" hypothesis in vertebrates (Ljunggren and Karre 1990), enables the creation of a natural chimeric state (through vasculature fusion) with kin (allograft acceptance) and prevents chimerism with nonrelated individuals (allograft rejection) (Oka and Watanabe 1957; Sabbadin 1962; Scofield et al. 1982; Voskoboynik et al. 2013a). Ascidians are considered the closest living sister group of vertebrates (Delsuc et al. 2006; Voskoboynik et al. 2013b). Studying the immunobiology of the tolerance to partial allogeneic allograft in these organisms may reveal the evolutionary precursors to the maternal tolerance of the fetus and the outcomes of blood or tissue transplants.

A Single Gene Determines Allograft Acceptance or Rejection in Colonial Chordates

In colonial chordates, like *Botryllus schlosseri,* homeostasis is defined by the generation of all organ systems every 2 weeks. Colonies are initially formed by asexual reproduction of founder individuals, a product of sexual reproduction (review in Manni et al. 2014; Gassparini et al. 2014). The colony individuals are united under

a single gelatinous tunic by a network of anastomosed extracorporeal blood vessels. Throughout adult life, *B. schlosseri* generates its entire body every 2 weeks (20 °C). This cycle of development includes the formation of all body organs including the heart, respiratory system, digestive system, and neural complex. Ovary and testis are formed within each individual when sexual reproduction commences (Manni et al. 2014). In addition to their extraordinarily high developmental activity, *B. schlosseri* colonies engage in a natural transplantation reaction, whereby colonies undergo self–nonself recognition, which leads to either the formation of parabionts with a fused vasculature (i.e., fusion) or an inflammatory rejection response (i.e., rejection) (Fig. 1) (Bancroft 1903; Sabbadin 1962, 1982; Scofield et al. 1982; Scofield and Nagashima 1983; Boyd et al. 1990; Sabbadin et al. 1992; Rinkevich and Weissman 1992; Rinkevich et al. 1994; Saito et al. 1994; Chadwick-Furman and Weissman 1995a, b; Ballarin et al. 1995, 1998, 2002; Cima et al. 2004, 2006; Rinkevich 2005; Oren et al. 2007, 2008, 2010; Voskoboynik et al. 2013a).

In seminal work, Oka and Watanabe showed that a single polymorphic gene locus with multiple, codominantly expressed alleles underlies fusion/rejection outcomes in *Botryllus primigenus* (Oka and Watanabe 1957, 1960, 1967; Oka 1970; Mukai and Watanabe 1975; Watanabe and Taneda 1982). Oka and Watanabe performed classical genetic experiments to investigate the inheritance pattern of fusibility among *B. primigenus* colonies (Fig. 2). They found that fusibility in *B. primigenus* is controlled by a single polymorphic gene with multiple, codominantly expressed alleles; sharing one of these alleles is required for fusion (Oka and Watanabe 1957, 1960, 1967; Mukai and Watanabe 1975; Watanabe and Taneda 1982; Oka 1970). The progeny from crosses between *B. primigenus* colonies that shared one allele in the fusibility locus resulted in colonies heterozygous for the fusibility gene. However, these crosses did not produce homozygous colonies for the fusibility gene or heterozygous colonies identical to the maternal colony (Fig. 2) (Oka and Watanabe 1957, 1960, 1967; Mukai and Watanabe 1975; Oka 1970; Watanabe and Taneda 1982). Furthermore, homologous heterozygous colonies

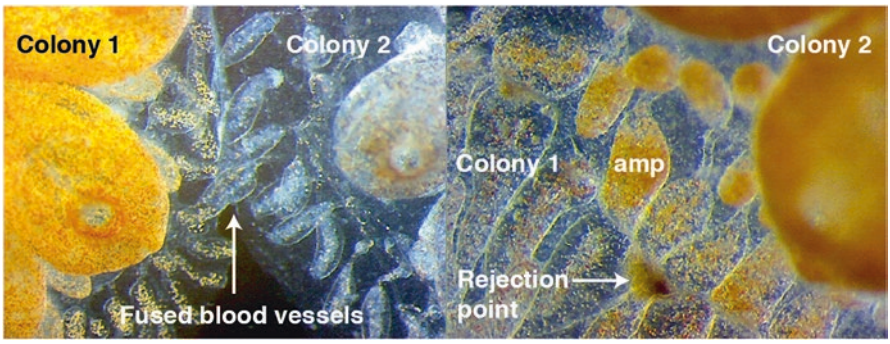

Fig. 1 Fusion/rejection between *B. schlosseri* colonies is governed by the BHF gene. Colonies that share an allele will fuse (left panel), whereas colonies that do not will reject (right panel). Major anatomical features are indicated (amp = ampullae, the colony vasculature blind ended tissue). (Adopted from Voskoboynik et al. 2013a)

	Botryllus primigenus	*Botryllus schlosseri*
Cross between rejecting wild type	P1 ⟷ P2	P1 ⟷ P2
Hypothesized genotypes	AB ⟷ CD	EF ⟷ GH
Fusibility and genotypes among F1	AC ⟷ BD BC ⟷ AD	EG ⟷ FH FG ⟷ EH
Cross between F1 fusing pairs	AC (maternal) X BC (paternal)	EG (maternal) X FG (parental)
Fusibility among F2	AB CB	EF ⟷ GG EG FG
Missing genotypes F2	AC CC	None
Cross between homologous heterozygote colonies	AB X AB	EG X EG
Fusibility among F2	Sterile	EE ⟷ GG EG

Fig. 2 Inheritance of histocompatibility trait in *Botryllus*. Classical genetic studies performed by Oka and Watanabe (1957, 1960, 1967) and Sabbadin (1962) demonstrated that fusibility in *Botryllus* is controlled by a single polymorphic gene locus with multiple, codominant expressed alleles. ◀▶ Nonfusion, otherwise fusion

were sterile if both alleles (e.g., AB X AB) were shared (Fig. 2) (Oka and Watanabe 1957, 1960, 1967; Oka 1970). Based on these observations, Oka and Watanabe hypothesized that the fusibility gene is also involved in self-sterility, that is, fertilization occurs only when the alleles controlling spermatozoa/ova fusions are different (Oka and Watanabe 1957, 1960, 1967; Oka 1970). Self-sterility in *B. primigenus* prevented Oka and Watanabe from directly testing and proving their fusibility genetic control hypothesis. Turning to *B. schlosseri,* which does not exhibit self-sterility, Sabbadin (1962) studied the inheritance pattern of fusibility among *B. schlosseri* colonies. He found that progeny from crosses between histocompatible *B. schlosseri* colonies segregate in a manner consistent with a monogenic trait and further confirmed Oka and Watanabe's genetic control hypothesis on fusibility (Fig. 2) (Sabbadin 1962). Scofield et al. (1982) further supported the conclusion that fusibility in *B. schlosseri* is controlled by a single locus and segregates as expected in a monogenic trait. The rules governing fusibility reactions are as follows: AB = AB leads to fusion, AB = CD to rejection, and AB = BC to fusion (Fig. 2) (Sabbadin 1962; Scofield et al. 1982).

To map the fusibility histocompatibility (termed Fu/HC; Weissman et al. 1990) genomic region in *B. schlosseri*, classical breeding experiments were performed, and defined homozygous and heterozygous lines for distinct Fu/HC alleles were developed (De Tomaso et al. 1998). These lines, established by Yasunori Saito, were crossed and maintained in our mariculture for three decades and added compelling evidence that histocompatibility in *Botryllus* is controlled by a single gene (it would otherwise be impossible to maintain these lines via crosses). Using these lines and a bulk segregant analysis, allelic fragment length polymorphism markers that cosegregate with the Fu/HC phenotype were identified (De Tomaso et al. 1998). A genetic map of the Fu/HC region was built, and a highly polymorphic gene (*cFuHC*) was identified (De Tomaso et al. 1998, 2005). At that time, preliminary data indicated that the *cFuHC* appeared to correlate with known fusion/rejection outcomes

(De Tomaso et al. 2005). However, incomplete sequence across the histocompatibility region compromised these data, and new evidence (Rinkevich et al. 2012; Nydam et al. 2013; Voskoboynik et al. 2013a) forced us to reevaluate its role in histocompatibility.

The original *cFuHC* gene model consists of two dominant isoforms, a short secreted form and a membrane-bound form encompassing the entire predicted gene (De Tomaso et al. 2005). By integrating the *B. schlosseri* genome assembly (Voskoboynik et al. 2013b) with a wide variety of sequencing data and polymerase chain reaction validation experiments, we and others have now concluded that *cFuHC* is composed of two genes separated by 250 bp (Rinkevich et al. 2012; Voskoboynik et al. 2013a; Nydam et al. 2013). One of the two genes at this locus is predicted to be targeted to the secretory pathway (called *FuHC*^{sec}) and is identical in sequence to the original secreted isoform (De Tomaso et al. 2005). The other gene encodes a putative transmembrane domain (called *FuHC*™) and has a newly identified N-terminal exon encoding a putative signal peptide (Nydam et al. 2013; Voskoboynik et al. 2013a). Notably, BLAST analysis revealed a homolog of *FuHC*™ in *Ciona intestinalis* (gi|198,429,243; e-value = 4e-37), with identical predicted functional domains.

BHF, the *Botryllus* Histocompatibility Factor

Given the simple inheritance of the histocompatibility trait observed for defined crosses (Sabbadin 1962, Scofield et al. 1982; De Tomaso et al. 1998), we tested whether any single gene identified in the *B. schlosseri* genome assembly (Voskoboynik et al. 2013b), including the *FuHC*^{sec} and *FuHC*™ genes, could stratify colony pairs by known fusibility outcomes. Homozygous or heterozygous lines for the fusibility histocompatibility phenotype raised in our mariculture facility, identical to the lines used by De Tomaso et al. (1998, 2005) to find the Fu/HC region (Figs. 3 and 4), as well as pairs of related rejecting colonies and unrelated wild-type fusing colonies, were sequenced and analyzed for the histocompatibility phenotype. After sequencing transcriptomes from 21 defined and wild-type colonies and using new genomics tools, we discovered a gene unique to ascidians, whose protein sequence in many alleles is absolutely concordant with fusion/rejection outcomes. This concordance was demonstrated by analyzing high-throughput, deep-coverage RNA sequencing data from 21 colonies encompassing 32 known fusion/rejection outcomes. Because this newly discovered gene is also (1) highly polymorphic, (2) concordant in sequence with our defined fusibility histocompatibility lines and with all known fusibility outcomes, pedigree relationships, and zygosity of defined lines (Figs. 3 and 4), (3) expressed in the vasculature, the tissues that participate in allorecognition, (4) 100% predictive of new fusibility outcomes, and (5) significantly upregulated in colonies poised to undergo fusion or rejection, and (6) the histocompatibility reaction can be inhibited by translation-blocking morpholino based on this gene, it is the leading candidate for a "*Botryllus* histocompatibility factor" (termed BHF). No other protein-encoding gene in the current *B. schlosseri*

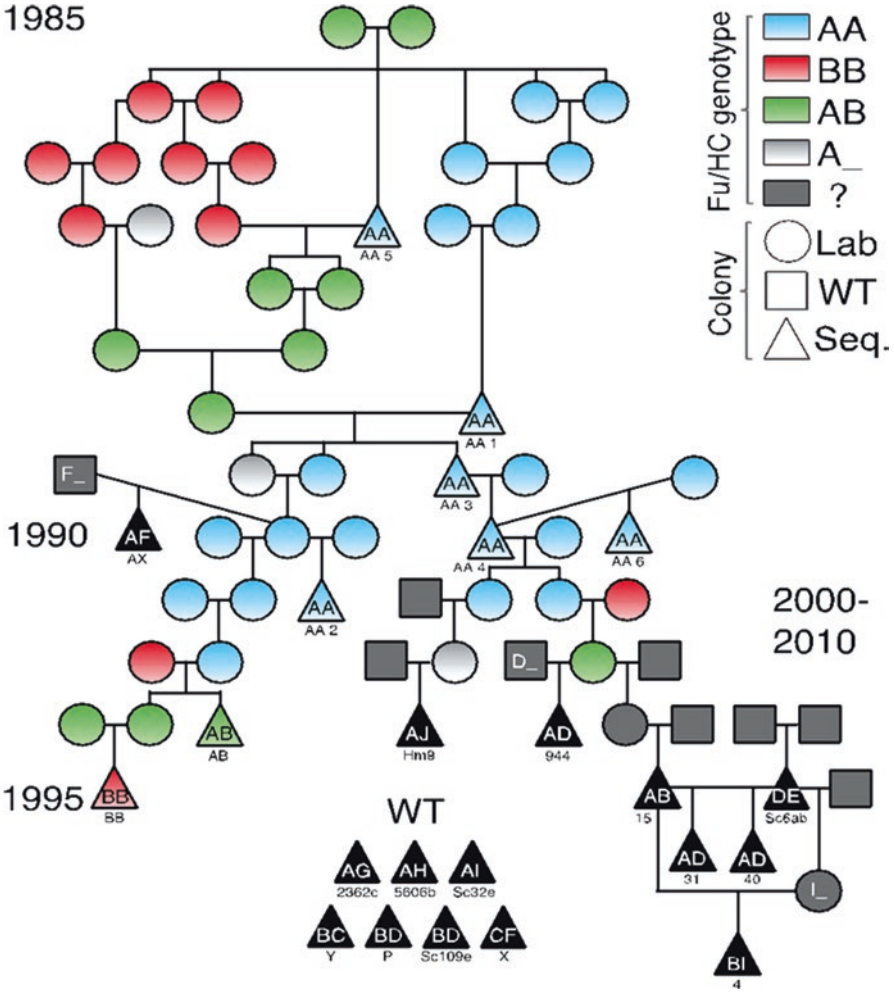

Fig. 3 Pedigree of defined and wild-type fusibility histocompatibility lines, and *BHF* genotypes derived for 23 colonies sequenced. Crosses made by our lab over the last three decades to precisely identify the fusibility histocompatibility (Fu/HC) locus (approximate years are indicated). The pedigree includes homozygous (AA or BB) and heterozygous (AB or AX) breeding lines that were developed in our mariculture facility, as were the wild-type (WT) colonies. *BHF* genotypes derived for all 23 colonies sequenced, including exploratory and validation cohorts were analyzed by high-throughput, deep-coverage RNA-Seq and two Sanger-sequenced colonies with the AA genotype (AA 5 and AA 6). Abbreviated colony names are indicated beneath the sequenced genotypes. Colonies sequenced in this work are depicted as triangles. (Taken from Voskoboynik et al. 2013a)

genome assembly satisfies all of these critical criteria (Voskoboynik et al. 2013a) (Figs. 3 and 4). Strikingly, analysis of the 1 cM genomic region governing *B. schlosseri* histocompatibility that was narrowed down by a bulk segregant analysis (the fosmid sequence used to identify *cFuHC*) revealed that *BHF* is located

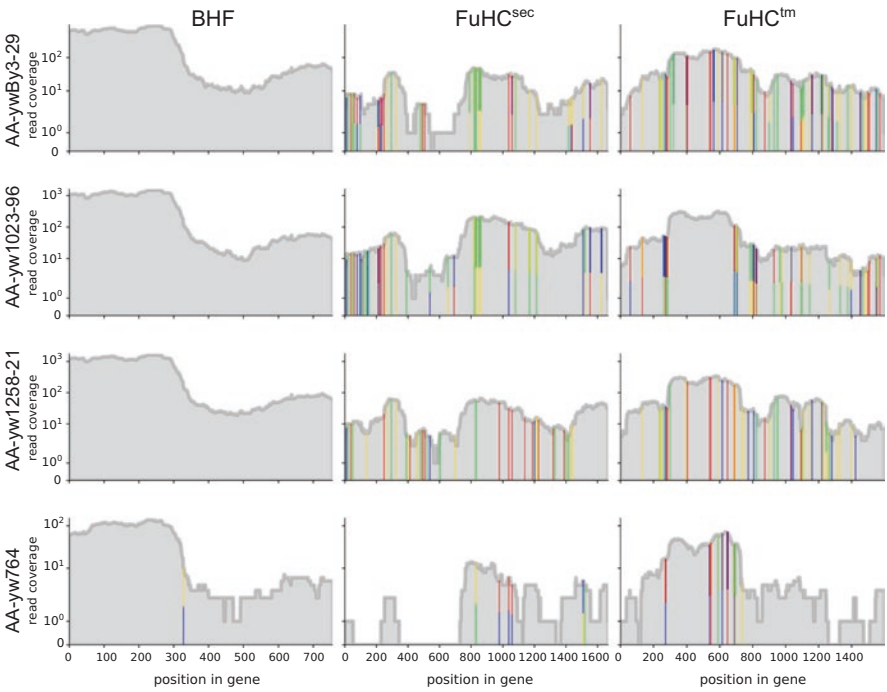

Fig. 4 Concordance of *BHF* alleles with genetically homozygous defined lines for distinct fusibility histocompatibility alleles (AA line). Coverage and single-nucleotide variants of RNA-seq reads (2 × 100 bp) from four different genetically defined homozygous *B. schlosseri* lines for distinct fusibility histocompatibility alleles (AA line) demonstrate concordance of *BHF* alleles but not *FuHC*^{sec} or *FuHC*^{tm} alleles with genetically homozygous defined lines. The gray area is the coverage; vertical lines indicate homozygous (single color) or heterozygous (two colors each spanning half the coverage) variants. Colors indicate nucleotide variants: A – green, C – blue, G – yellow, T – red. Alignments were performed with BWA ("mem" algorithm), duplicates were removed using picard (MarkDuplicates tool), and variants were called using varscan (*p*-value cutoff of 0.01, default coverage filtering)

approximately 62 kb away from *FuHC*^{sec} and *FuHC*™. Analysis of the *B. schlosseri* genome confirmed physical linkage for these three genes (chromosome 9) (Voskoboynik et al. 2013b). BHF is a highly charged and partially unstructured protein with no detectable domains or signal peptide (Voskoboynik et al. 2013a) (Fig. 5a).The *BHF* gene produces two transcripts; isoform 1 is composed of three exons, encoding a 252–amino acid protein, and isoform 2 is composed of two exons, exon 1 and extended exon 2, encoding 219-amino acid protein (Taketa et al. 2015; Fig. 5b). Although lacking transmembrane domain, BHF expression in HEK293T cells suggests localization of BHF to the membrane (Taketa et al. 2015). *BHF* has three remote homologs in the National Center for Biotechnology Information (NCBI) database, all of which encode uncharacterized proteins from solitary tunicates. However, since solitary tunicates do not participate in fusibility reactions, we attempted to amplify BHF from two other colonial tunicate species (*Botrylloides*

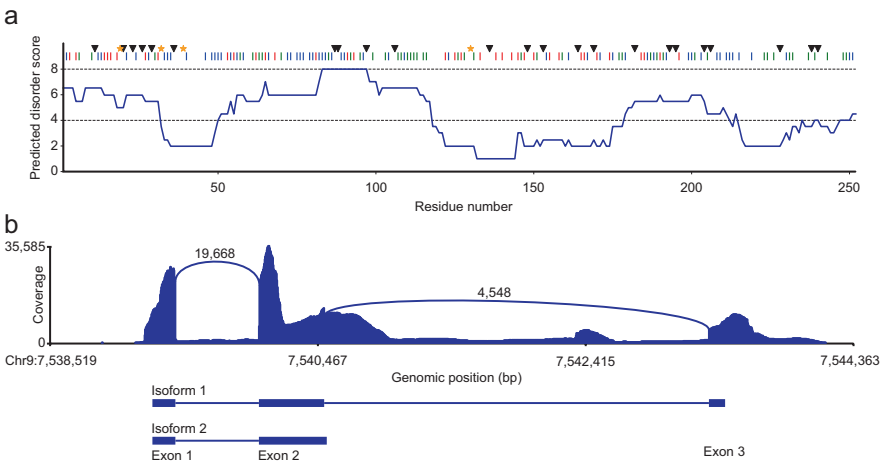

Fig. 5 BHF protein sequence analysis. (**a**) The lack of any structurally or functionally characterized homologs of BHF allows only a qualitative analysis of its sequence. The upper panel highlights major characteristics of the sequence, while the lower panel summarizes the outcomes of several independent servers for disorder prediction. Blue, red, and green bars indicate positive (lysine, arginine, and histidine), negative (aspartic and glutamic acids), and polar (asparagine, glutamine, and serine) residues, respectively. Black triangles indicate glycine or proline residues, and the orange stars indicate cysteine residues. Overall the protein has a + 21 net positive charge. The relative scarcity of core-forming hydrophobic residues as well as the structurally destabilizing effect of proline, glycine, and high net charge suggest that the protein is at least partially unstructured. To quantify this hypothesis, we submitted a BHF sequence to the DisMeta metaserver, which provides a consensus disorder prediction based on several independent methods. The lower panel summarizes these predictions, indicating that more than half of the protein (residues score above four) is disordered (taken from Voskoboynik et al. 2013a). (**b**) Genomic characterization of *BHF* locus in *B. schlosseri* reveals two isoforms. Paired end RNA sequences (2 × 100 bp) aligned to *B. schlosseri* chromosome 9 revealed two isoforms. Isoform 1 includes three predicted exons, encoding a 252-amino acid protein; isoform 2 includes two exons, exon 1 and extended exon 2, encoding a 219-amino acid protein. Values above the arcs in the upper panel are the number of reads spanning that splice junction. Alignments were performed using STAR 2.5.1b

spp. and *Diplosoma* spp.). We succeeded in recovering highly similar sequences from both species, which indicated that *BHF* may represent a general colonial tunicate allorecognition factor (Voskoboynik et al. 2013a).

As described previously, when two genetically distinct *B. schlosseri* colonies meet, they either anastomose extracorporeal blood vessels to form a chimera with a common vasculature, or they reject one another. In some chimeras, one of the chimeric partners undergoes partial or complete reabsorption (i.e., morphologically shows death and disappearance of tissue) (Corey et al. 2016). We found that *BHF* and genes that promote immune response highly expressed during resorption in the resorbing chimeric partner (the resorption loser) (Fig. 6) (Corey et al. 2016). A comparison of differential expression of genes (based on RNAseq) in the resorption chimera loser and the resorption chimera winner during resorption revealed that the *BHF* gene is highly expressed in the resorbing part (Fig. 6). Resorption can also be

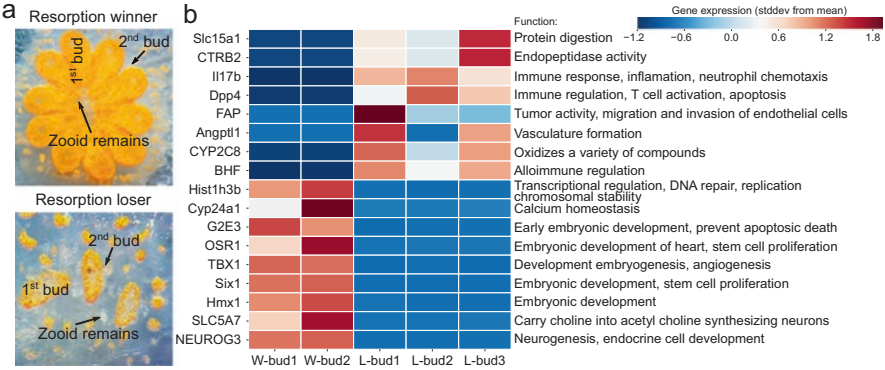

Fig. 6 *BHF* and genes that promote immune response highly expressed during resorption in resorbing chimeric buds. We sequenced distinct RNA-seq libraries made from chimeric tissues taken during resorption (resorption winner vs resorption loser). When allogeneic stem cells home to primary and secondary buds in chimeras to form the new generation zooids, we sampled chimeric primary and secondary buds from the chimera resorption winner and the chimera resorption loser (**a**) and sequenced the RNA-seq of each sample individually. Genes with significant differential expression between samples (resorption winner vs. resorption loser) were identified using edgeR. A heat map (**b**) shows the difference in expression of genes identified as having the highest fold change between the secondary bud resorption winner and loser. Values plotted are normalized gene expression, calculated by standardizing the library size and then adjusting each gene to have zero mean expression and unit standard deviation across the samples of interest. Genes with the highest log fold change (LFc) that expressed in the resorbing loser secondary buds (promoting immune response and death) and the resorption winner secondary buds (involved in embryogenesis stem cell differentiation and development) are presented in the table. Scale bar: 500 um; Differentially expressed FDR < 0.05, *p*-values adjusted using Benjamini–Hochberg method. Each experimental group sequenced included two to three biological repeats [e.g., three different samples of the second bud, resorption loser (L-bud)]. W-bud: resorption winner secondary bud; L-bud: resorption loser secondary bud

defined by distinguishing molecular characteristics: the buds of the resorption loser, unlike the winner, has cells with (a) high expression levels of genes associated with immune response, apoptosis, and death (i.e., *ILI-17B, FAP, DPP4*) (Fig. 6); (b) lower expression of genes associated with stem cell proliferation, embryogenesis, and development across species (i.e., *Six1, HMX1, OSR1, TBX1*) (Fig. 6) (Corey et al. 2016). These molecular characteristics of resorption suggest, first, that it is similar to rejection (because *BHF* is the gene that determines fusion vs. rejection) and, second, that it is related to stem cell fates because the stem-cell-associated genes are downregulated in the loser and upregulated in the winner, and stem cells are a crucial and large component of the secondary buds (that will generate an individual body within the colony). We believe resorption not only is mediated by stem cells and the immune response of the resorption winner to loser but may also be an early indicator of the outcome of somatic stem cell competition, since in many cases the resorption winner is also the somatic stem cell winner (Fig. 6) (Stoner and Weissman 1996; Stoner et al. 1999). These expression patterns suggest that BHF has an important role during resorption and led us to hypothesize that the resorption

event in chimeras following fusion represents a second wave of rejection and might demonstrate an ancient form of smoldering rejection to nonshared histocompatibility alleles (Corey et al. 2016).

Our genome-wide comprehensive study revealed a single gene, *BHF*, that not only encodes self/nonself recognition but is the primary determinant of allogeneic stem cell activity by restricting fusion partners to kin (Voskoboynik et al. 2013a). *BHF* is embedded in a haplotype of several genes with high polymorphism (Voskoboynik et al. 2013a, b). Its structure does not follow biological precedence by either sequence or motifs to be either a membrane or secreted protein by classical criteria, and therefore study of this gene will likely reveal new mechanisms of recognition.

Historically, the major histocompatibility complex (MHC), a set of cell surface molecules encoded by a large gene family that controls a major part of the immune system in all vertebrates and determines self from nonself, was discovered because of its role in the rejection of transplants (Little 1941; Snell and Higgins 1951; Hirano et al. 2011). In *B. schlosseri* the Fu/HC region must encode colony specificity in fusion and rejection reactions (Sabbadin 1962; Scofield et al. 1982; De Tomaso et al. 1998) and also regulates the cosettlement of histocompatible kin during the larval tadpole stage (Grosberg and Quinn 1986). Within the Fu/HC haplotype, there are at least three distinct loci that have a structure that could be involved in fusion/rejection, cosettlement, and as yet unknown other functions. The secreted form (FuHC^{sec}) could be the candidate for recognition leading to cosettlement, as the ocean rather than air is the medium for signal spread and recognition, making this more like a chemokine than a volatile odorant/pheromone for organismal movements. While the membrane form (FuHC™) was the obvious candidate for the fusion/rejection gene, our comprehensive genomics and transcriptome analysis showed that allelic differences do not give a coherent pattern of rejection and fusion. Instead, the nearby BHF locus has a strict concordance of protein sequence and fusion/rejection. As discussed earlier, the lack of any known functional domains in BHF, including a canonical signal peptide or transmembrane region, leaves open the question of how BHF functions to initiate allorecognition, both spatially and mechanistically. This novel protein may have novel intracellular trafficking signals to initiate the fusion/rejection allorecognition process, or it may use other mechanisms. The existence of a linkage group containing the *cFuHC* genes, both of which have putative signal peptides, suggests that BHF may heterodimerize with either protein. Analogously to peptide presentation by MHC, BHF may undergo cytoplasmic degradation followed by surface display on FuHC™ or other surface proteins. Alternatively, BHF may be transmitted in soluble form by intracellular vesicles, such as neurotransmitters, or via specialized membrane channels. We produced the BHF protein and developed BHF monoclonal antibodies and are currently conducting experiments that test these hypotheses. Future investigations of BHF function will add important information about the underlying commonality of integrated networks and signaling processes involved in self–nonself recognition as well as what most certainly represents an important mechanism for preserving population diversity (Litman and Dishaw 2013).

Competing Stem Cells

Inspired by the genetic control for allograft acceptance and creation of chimerism within kin in *Botryllus*, Burnet (1971) hypothesized the emergence of intraspecific parasitism during the evolution of the immune system. Indeed, his hypothesis was confirmed in *B. schlosseri* chimeras, where the replacement of the host germline by a donor genotype was demonstrated (Sabbadin and Zaniolo 1979).

Pancer et al. (1995), Stoner and Weissman (1996), and Stoner et al. (1999) used genetic markers to confirm these results and further showed that in a chimera, the blood, soma, and germ cells demonstrated the combined genotypes of both chimeric partners. Moreover, in many cases the circulating pluripotent cells of one partner parasitized either the soma or the germline of the other partner and replaced the gonads or the soma (bud/zooid) of several individuals in the host colony (termed germline or somatic cell parasitism) (G/SCP). In a few cases, a complete takeover of donor genotype occurred, and the gonads in the chimeric colony solely expressed the donor's genotype (Sabbadin and Zaniolo 1979; Pancer et al. 1995; Stoner and Weissman 1996; Stoner et al. 1999). Under invariant environmental conditions, both GCP and SCP followed repeatable hierarchies of "winner strains" and "loser strains" (Stoner et al. 1999). However, breeding experiments proved that only the hierarchical position of GCP is sexually inherited (Stoner et al. 1999). The hierarchy of somatic parasitism in *B. schlosseri* chimeras is a plastic trait, as variations in the environmental conditions (such as seawater temperature) can be reversed, that is, the winner–loser hierarchy at the somatic parasitism level (Rinkevich and Yankelevich 2004). Germline parasitism is not limited to invertebrates and was detected in chimeric marmosets (primates), which often transmit sibling alleles acquired in utero to their own offspring, so an individual that contributes gametes to an offspring is not necessarily the genetic parent of that offspring (Ross et al. 2007). *B. schlosseri* colonies propagate asexually through budding, so somatic stem cell parasitism in host colonies can induce the development of partial allogeneic entities (buds) within the host colony. As a result, chimerism in protochordates could serve as a state that enables the development of a "virtual embryo" within the host colony (Voskoboynik et al. 2009).

In addition to cell parasitism, chimerism may alter the tolerance and intolerance state in the colonies. Chimeras might fuse with colonies that they used to reject (on their nonchimeric phase) or reject colonies rather than fuse (Mukai 1967; Sabbadin and Astorri 1988). Moreover, in some cases, chimeric colonies will simultaneously fuse and reject another colony (Taneda 1985; Sabbadin and Astorri 1988). The presence of a simultaneous fusion and rejection in a genotype where one of the chimeric partners previously fused and the other partner previously rejected proves the persistence of both genotypes and suggests an uneven distribution of each genotype within the chimera. Different fusibility patterns observed in chimeric entities at different time points suggest genome fluctuation and competitive interaction of the different genomes within chimeras (Sabbadin and Astorri 1988). Sabbadin and Astorri observed changes in the tolerance state of chimeric colonies and linked them to changes in the dynamic of chimeric cells within a chimera. Genetic analysis of the dynamic of chimeric cells revealed that chimeras exhibit either a sectorial

pattern in which both genotypes are detected within some systems but not others or a uniform pattern in which tissues throughout the entire chimera exhibit both genotypes (Stoner and Weissman 1996). Colonies that showed rejection and fusion at the same time probably expressed a sectorial pattern and the others expressed a uniform pattern. Different expression patterns are observed during different time points, indicating a change in the dynamics of chimeric cells within the host (Pancer et al. 1995; Stoner and Weissman 1996; Stoner et al. 1999). These studies show that chimerism can alter the genetically controlled ability of *B. schlosseri* colonies to tolerate or reject other colonies. The temporal and spatial dynamic of chimeric cells, patterns of host/donor cell competition, niche occupation and immunoregulatory mechanisms for routing, and the timing and frequency of chimeric cells probably play an important role in the induction of tolerance or intolerance.

Chimerism and the long-term ability of cells from one genotype to replace the germline and somatic cells of the host led to the hypothesis that chimerism, cell parasitism, and budding in *B. schlosseri* are mediated by stem cells (Sabbadin and Zaniolo 1979; Rinkevich and Weissman 1987; Pancer et al. 1995; Stoner and Weissman 1996; Stoner et al. 1999). By transplanting a single cell, which expresses a high enzymatic activity of aldehyde dehydrogenase and a set of serial engraftment assays, Laird et al. (2005) revealed that multipotent stem cells are responsible for stable long-term chimerism in adult *B. schlosseri* colonies. We further identified a major adult somatic stem cell niche in the anterior ventral side of the endostyle (Voskoboynik et al. 2008) and a germline potential in cells that harbor the cell islands (Rinkevich et al. 2013). These first ascidian stem cells and stem cell niches were identified by direct visualization of cells that exhibit fundamental and important aspects of mammalian stem cell biology, including self-renewal capacity, homing to developing buds, expansion, differentiation and multilineage potential, and takeover of the genotype of new developing buds (Laird et al. 2005; Voskoboynik et al. 2008; Rinkevich et al. 2013). Furthermore, cells in these niches express markers associated with stem cells and gene products that implicate signaling centers for stem cells (Voskoboynik et al. 2008; Rinkevich et al. 2013).

The discovery that stem cells could compete in both the germline and soma of *B. schlosseri* chimeras led us to study clones of competing stem cells in mammals. We found that such competitions existed, and almost certainly were responsible for the emergence of rare myeloid biased HSC clones in aging, arising at the expense of the lymphomyeloid balanced clones that dominate in young mice (Rossi et al. 2005, 2007, 2008; Beerman et al. 2010). Over the past decade, we have elucidated a basic HSC competition not only in aging but also in the clonal progression of some clones toward the production of leukemia stem cells (LSCs) (Miyamoto et al. 2000; Jamieson et al. 2004; Weissman 2005a, b; Majeti et al. 2009) and among itinerant germline stem cells (Ueno et al. 2009). This hypothesis is now accepted as an important paradigm in stem cell aging and cancer (Weissman 2015). The biology of tolerance induction and stem cell engraftment in *B. schlosseri* is regulated on four different levels. First there is fusion or rejection; second, if fusion occurs, the body of one chimeric partner is resorbed; third is competition between somatic stem cells that circulate from one chimeric partner to another for asexual whole body

development; and fourth is stem cell competition among germline stem cells. We have found the gene (*BHF)* that controls the first level of interaction and discovered that the other levels, of resorption and stem cell competition, are governed by heritable genetic factors. Thus, genetically distinct strains of *B. schlosseri* have stem cells that, in a chimera, vary in their vulnerability to being resorbed and in their ability to "win" or "lose" in a competition to replace differentiated tissue. It is important to note that each level of stem cell competition has biomedical implications: resorption is a model for graft rejection, somatic stem cell competitions relate to stem cell transplant engraftability, and germline stem cell competition determines which genotypes are inherited, a phenomenon that occurs naturally in mammalian testis development, and that may be important in fertility.

The *BHF* is a single gene that controls histocompatibility and allows or prevents stem-cell-mediated chimerism—a mechanism that can determine the germline and or soma of the host in *B. schlosseri*. The discovery of this gene establishes a link between stem cell parasitism and allorecognition and promotes *B. schlosseri* as an evolutionary model for studying the molecular regulation of stem cell competition and tolerance induction.

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The Inflammatory Response of Urochordata: The Basic Process of the Ascidians' Innate Immunity

Nicolò Parrinello, Matteo Cammarata, and Daniela Parrinello

Introduction

The Ascidians—New Insights into an Old Problem

Tunicata (phylum Chordata) are filter-feeding marine invertebrate protochordates that occupy a key phylogenetic position in chordate evolution, representing modern-day descendants of the chordate progenitor. At the larval stage, most of them present temporary chordate characters including a notochord and dorsal nerve cord. In addition, the adults are provided with a wide respiratory pharynx, equipped with a ventral ciliated channel, structurally distinguishable (endostyle), for collecting food particles. The endostyle is also provided with a glandular thyroid-like structure secreting iodoproteins (Burighel and Cloney 1997). According to genome-wide sequence information, Tunicata are considered the sister group of Vertebrata (Delsuc et al. 2006, 2008; Swalla and Smith 2008), thus assuming a deep meaning in the study of the evolutionary biology (Fig. 1).

Ascidiacea (sea squirts) are a representative class of the Tunicata subphylum. They are sessile and include both solitary and colonial organisms widespread all over the seas (about 3000 species). This class includes the most common and favored model species studied for developmental biology as well as for immune-related gene annotation; comparative analysis of conserved protein sequences such as domains, modules, or motifs; and the upregulation of gene transcription challenged by harmful agents. Genome-wide surveying and evolutionary history disclose—besides conservation of genes across metazoans—genes shared with vertebrates and ascidian/tunicate–specific genes that diverge between orders, while polymorphism can characterize distinct populations. In tunicate species, the rates

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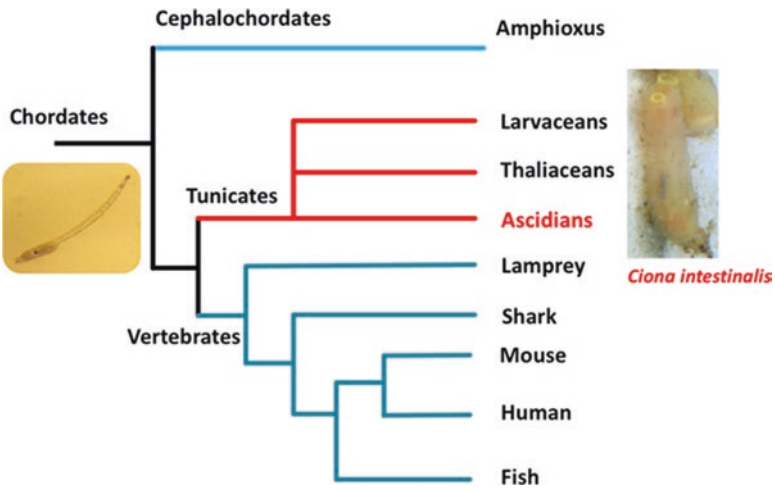

Fig. 1 Chordata phylogenetic tree

and patterns of molecular evolution are peculiar and they appear to be fast evolving (Berná and Alvarez-Valin 2014). The debate on the phylogenesis of the ascidian orders and families remains open.

The findings reported here mainly result from species belonging to different orders, different families of a same order, and species within the same family, as well as from ascidians with opposite lifestyles (solitary/colonial). Therefore, differences between evolutionary lineages can be expected.

The different species here that are mainly mentioned include *Ciona intestinalis*, *Ciona savigny*, and *Phallusia mammillata*, which belong to two distinct families of the order Phlebobranchiata; *Styela plicata*, *Styela clava*, *Molgula manhattensis*, the colonial *Botryllus schlosseri*, *Botrylloides leachi*, and the budding *Polyandrocarpa misakiensis*, which belong to the same family (styelids) of stolidobranchs, whereas another family of this order includes *Halocynthia roretzi*, *Halocynthia papillosa*, and *Pyura stolonifera*. The colonial lifestyle has been independently acquired; many colonial species are Aplousobranchiata, while solitary forms prevail in Phlebobranchiata and Stolidobranchiata. They differ in the type of budding and colony structure. A molecular study of styelids indicates several independent acquisitions of coloniality (e.g., *Botryllus*, *Botrylloides*) (Pérez-Portela et al. 2009).

C. intestinalis and *B. schlosseri* have been preferably chosen as model species to study immunoevolution.

In spite of the established classification, care must be taken with ascidian specific status, which could affect the homogeneity of the results from geographically distinct populations. On the basis of genetic divergence and the geographic distribution, the *C. intestinalis* populations have been temporarily named as type A (Mediterranean, Pacific, and Southern Atlantic coast of Europe) or type B (North Atlantic) (Suzuki et al. 2005; Caputi et al. 2007; Nydam and Harrison 2007; Sato et al. 2012). These types have also been regarded as taxonomically different species

(Pennati et al. 2015). Nonetheless, evidence of incomplete reproductive isolation in the wild populations, as well as laboratory hybridization experiments (Nydam and Harrison 2011), have raised the taxonomic issue.

Similar phylogenetic and population genetic data have been reported for the colonial *B. schlosseri*. Mitochondrial and nuclear genes, as well as polymorphic microsatellites for colonies sampled from the southern and northern coasts of Europe and the eastern–western coasts of North America, have shown that this well-known model organism comprises three highly divergent and probably reproductively isolated cryptic species. Among these, the "type A" recovered in all of the surveyed regions is by far the most common and widespread (Bock et al. 2012).

Anyway, on the basis of the collection sites, most published reports on innate immunity mainly refer to *C. intestinalis* populations designed as type A, as well as to *B. schlosseri* designated as type A. Therefore, while waiting for the taxonomic *status* to be precisely defined and taking into account the sampling geographic area, in the present work both ascidians are referred as belonging to type A.

Genome sequencing analyses (Dehal et al. 2002; Voskoboynik et al. 2013a, b) revealed that ascidians have a basic, nonduplicated set of a chordate-type genome. In several species (mainly *C. intestinalis* and *B. schlosseri*), gene sequencing and transcriptional profiling significantly contribute in disclosing gene upregulation; meanwhile cell labeling with riboprobes and immunohistochemistry performed with specific or cross-reactive antibodies identify cell types and indicate their functions. A comprehensive picture of immune-related genes and their phylogenetic lineages helps to clarify the evolution of a system pivotal for survival, also supporting the evolutionary meaning of multifunctional genes.

Some Topics Relevant to the Subject

Inflammation is the first nonspecific response for innate self-protection and tissue repair, triggered when tissues are injured by harmful stimuli including mechanical stress and intrusion of invasive agents (or their products) (Janeway et al. 2001; Medzhitov 2008; Ashley et al. 2012). It is a vital basic part of the immune system; the initial cause is cleared out and tissue repair initiated. The response, largely based on the extent and size of the injuring and/or invading agents, underlies a wide variety of physiological and pathological processes. Among vertebrates, the inflammatory cascade is a complex network of immunological, physiological, and behavioral events which, starting from self or nonself recognition, are coordinated by signaling and production of bioactive molecules. Mediators act as autocrine and paracrine, and interact with various cell types to amplify the inflammatory response.

In mammals, the mononuclear phagocyte system (monocytes, tissue macrophages, and dendritic cells), and the polymorphonuclear cell family (neutrophils, eosinophils, and basophils) are the main cells involved in the nonspecific innate immunity. The inflammatory response proceeds with the recruitment of leukocytes and degranulation (delivering of secretory vesicles/granules) of neutrophils, mast cells, and eosinophils, and with an orchestrated reciprocal functional regulation

with macrophages (Guilliams et al. 2014). The permeability of the involved vasculature increases, and neutrophils and monocytes detecting gradients of chemokines (chemotaxis) migrate (transendothelial and transepithelial migration) to the site of inflammation. Concurrently, proinflammatory and anti-inflammatory cytokines and effector molecules are produced. Some stimuli evoke a fast (occurring within minutes or hours), acute, and short-lived inflammation that may switch to a long-term chronic phase.

Upon exposure to proinflammatory cytokines, LPS or other microbial products, heat shock proteins, and molecular fragments of the extracellular matrix, macrophages acquire a proinflammatory "classically activated phenotype," act as phagocytes, mediate cytotoxic activity, and produce a large number of mediators including complement components and several other factors. Macrophages that are "alternatively activated" or with a "reparative phenotype" function in resolution of inflammation and wound tissue repair (Koh and Di Pietro 2011; Mantovani et al. 2013; Wynn and Vannella 2016). In chronic inflammation, macrophages can collect in layers surrounding the foreign material and form a compact structure (granuloma) with a significant protective function such as efficient intracellular bactericidal activity and prevention of microbe dissemination.

Neutrophils and macrophages (originating from monocytes) are professional phagocytes that recognize and engulf pathogens and have a role in the removal of apoptotic corpses. Also dendritic cells are phagocytes, and sets of them act as peripheral sentinels. They detect signals displayed by foreign agents and, after intake and processing, they present antigenic determinants (antigen-presenting cells (APCs)) to T lymphocytes through a process that is MHC dependent. After phagocytosis, macrophages can also be APCs. Thereby a linkage between innate and adaptive immune systems occurs; the innate immune response traced back to invertebrates has evolved into a more complex system interacting with the adaptive immunity that in jawed vertebrates responds to different and various environmental stimuli in their habitats.

Ascidian Tissues Involved in Inflammatory Responses

The Tunic

The tunic, of epidermal origin, is the physical barrier against intruders. The tissue matrix is made up of an amorphous ground substance containing fibrous components ("tunicin": cellulose-like polysaccharide filaments associated with collagen, elastin, and mucopolysaccharides) (Endean 1961; Deck et al. 1966). The tunic external margin is bordered by a thin layer (cuticle, containing keratin), and the inner border is lined by a monolayered epidermis, in turn enveloped in a connective tissue that forms a lacunar network. Tunic cells, scattered in the matrix, and the epidermis produce the tunic matrix (Burighel and Cloney 1997; Di Bella et al. 1998, 2009). In vascularized tunics, the cells can directly derive from tunic vessels, otherwise they—crossing the epidermis—come from the connective tissue and the

circulating hemolymph. Cells also derive from the proliferating activity of the epidermis (Di Bella et al. 2005; Hirose 2009). The body contractions are due to longitudinal and circular muscles. In *C. intestinalis*, tunic cells express a type IX collagen α-chain (cloned and sequenced), with structural features of fibril-associated collagens with interrupted triple helices (FACIT) (Vizzini et al. 2008). In addition, antibodies specific for mammalian collagen have identified a type I–like collagen (Vizzini et al. 2001) that, with the type IX, may stabilize the matrix (Shaw and Olsen 1991).

The Circulatory System

The circulatory system consists of a tubular heart, enclosed in a pericardium, that pumps the hemolymph by means of peristaltic contractions regulated by two pacemakers, one at each end of the heart. The peristalsis originates at one end of the heart and the direction reverses periodically. The hemolymph flows from each end of the heart, through a single vessel lined by monolayered epithelium. Sinuses or lacunae in the connective tissue are the terminal of the system.

In the tunic of many solitary ascidians (e.g., *C. intestinalis*), vessels are absent, whereas in other species (e.g., *Phallusia mammillata* (Endean 1961) and *B. schlosseri* (Burighel and Cloney 1997)), vessels delimited by epithelium ramify through the tunic and terminate in knob-like bulbils.

In colonial ascidians, the individuals (zooids) are embedded in a common tunic and each of them has a complete body plan (heart, gastrointestinal tract, nervous system). In the tunic matrix, an extracorporeal common vascular system is interconnected by a network of vessels joined to the unique marginal vessel that runs along the contour of the colony. The vessels give rise to many finger-like blind endings (ampullae) bordered by columnar epithelial cells.

According to Konrad (2016) the ascidian circulatory system shows structural characteristics that allow to define it as "closed." The epithelial wall of vessels or ducts, as well as the lacunar network of the connective tissue, prevent the hemolymph from percolating around the cells of the body tissue. Here, the term "hemolymph," instead of "blood," is used to distinguish vertebrate blood from the ascidian circulatory tissue.

The Pharynx

The pharynx, which usually is anterior to the visceral organs, extends in the greatest part of the body. It consists of two epithelial monolayers perforated by rows of ciliated stigmata. The hemolymph flows inside a mesh of vessels called transversal and longitudinal bars, delimited by monolayered epithelium. In the lumen, abundant mature and immature hemocyte types are contained (Konrad 2016). In the bars, clusters of stationary cells called hemopoietic lymph nodules consist of dividing hemoblasts collected in groups surrounded by maturing hemocytes (Ermak 1976,

1982). Besides respiration and food particle collection, this organ is retained as the main organ of immunity, in which stem cells proliferate (Giacomelli et al. 2012).

The inflammatory components mainly originate from the pharynx. When soluble harmful agents (including LPS preparation) are locally inoculated into the tunic, they permeate the underlying tissues, reach the pharynx, and stimulate the response.

The findings here reported support the concept that the pharynx is directly involved in immune responses.

Hemocytes

Internal defense of ascidians mainly relies on hemocytes circulating in the hemolymph and therefore in the pharynx, which reach the lacunar connective network and infiltrate the tissues including the tunic. Several hemocyte populations can be inflammatory cells synthesizing and releasing bioactive proteins, carrying out phagocytosis, cytotoxicity, and encapsulation (De Leo 1992; Arizza and Parrinello 2009; Cima et al. 2016). In a cDNA/EST study to identify the genes expressed in hemocytes from *C. intestinalis*, 62 out of 530 of the obtained clusters had significant homology with vertebrate innate defense mechanisms (Shida et al. 2003).

The hemocytes show distinct morphological and functional features. In different species, light and electron microscopy observations distinguish various cell types that cannot not take into account hemocyte differentiation stages. In addition, seasons and/or mutable environmental conditions can affect the frequency of cell types in wild specimens. Nevertheless, basic hemocyte types can be distinguished as follows (Arizza and Parrinello 2009; Wright and Cooper 1983): (i) undifferentiated stem cells (hemoblasts/lymphocyte-like); (ii) agranular (hyaline/vacuolated) amoebocytes; and (iii) granular amoebocytes. Here, pigmented cells are disregarded. Agranular and granular hemocyte populations can be inflammatory cells (Fig. 2): (1) hyaline amoebocytes with fine granules and small vacuoles; (2) vacuolated cells including "signet ring cells" (SRCs) with a single very large vacuole, containing electron-transparent material, and compartment cells (CCs) in which vacuoles of medium size fill the cytoplasm and contain electron-transparent material and small granules; (3) granulocytes with small granules; and (4) granulocytes with large granules, including "morula cells" (MCs) in which large granules in the cytoplasm give them a raspberry-like shape. A possible characterization of MCs concerns their phenoloxidase (PO) content. In several botryllids, differences in frequency, morphology, PO level, and amoeboid behavior, have been reported (Shirae and Saito 2000). In *B. scalaris* and *C. intestinalis*, amoebocytes with granules varying in size also show weak PO activity (Shirae and Saito 2000; Parrinello et al. 2001). When activated, granulocytes can degranulate hyaline and granular amoebocytes (small granules) can be phagocytes. In *C. intestinalis* a particular granulocyte (URG) contains a single and large electron-dense granule that fills the whole cytoplasm.

Activated vacuolated cells display various features originated by a vacuolization process leading to vacuoles varied in size and content. Similarly, granules of granulocytes undergo processing phases in their content before being released, and their

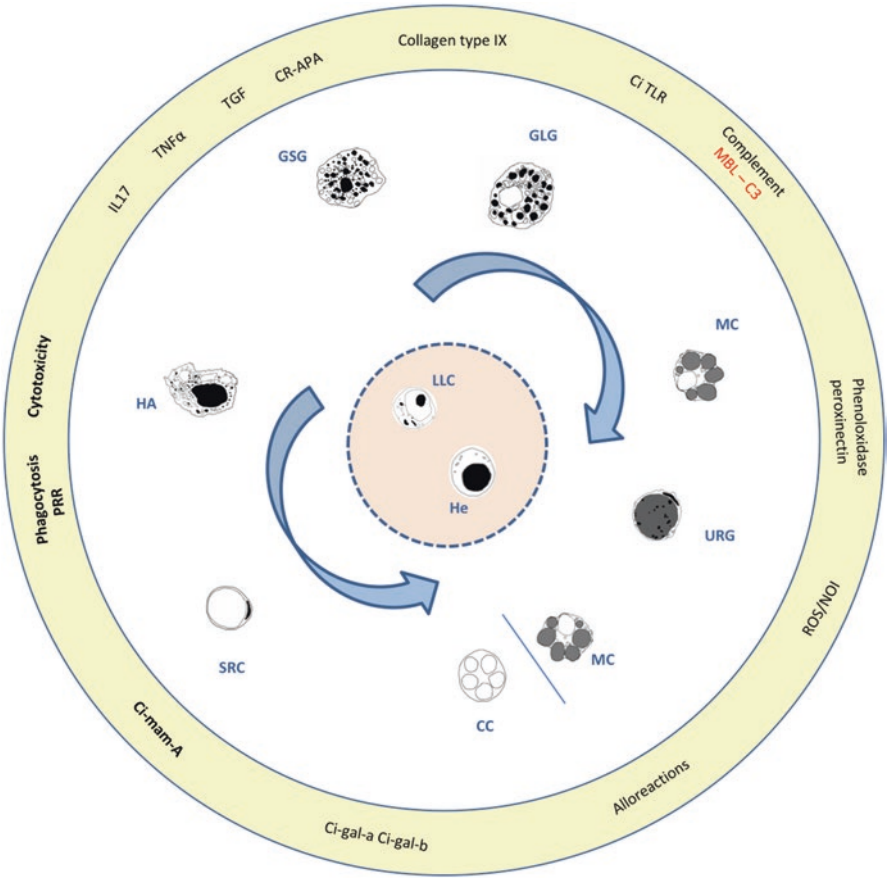

Fig. 2 The *Ciona intestinalis* pharynx inflammatory response: hemocytes involved, products, and activities. CC: compartment cell, GLG: granulocyte with large granule, GSG: granulocyte with small granules, HA: hyaline amoebocytes, hemoblast, LLC: lymphocyte-like cell, SRC: signet ring cell, URG: granulocyte with a sole large granule

features (as seen by TEM) change, assuming the appearance of vacuolated cells (De Leo 1992). MCs and CCs with granules or vacuoles containing small electron-dense granules could be interchangeable with each other in terminology (here called MCs/CCs) (Fig. 2). In a *B. schlosseri* nonfusion reaction, a macrophage-like cell type (MLC) originating from granulocytes has also been described (Ballarin et al. 2013).

Various models, often confusing, have been proposed for hemocyte differentiation lineages and functional maturation. The hemocyte renewal mainly occurs in the pharynx and connective lymph nodules, as well as in nodules associated with the postbranchial digestive tract (Ermak 1975a). Hemoblasts (mainly in nodules) and circulating lymphocyte-like cells (LLCs; bigger in size than hemoblasts, with a nucleolus) are retained stem cells that give rise to the hemocyte types (Fig. 2). Normally, in the hemolymph, LLCs occur at low frequency.

It is presumable that each hemocyte type may include distinct populations with morphological and functional peculiarity. In a recent paper, hemocyte types from *H. roretzi* were examined using flow cytometry and morpho-functional parameters (Donaghy et al. 2017). The following hemocyte populations were identified: (i) one of the three granulocyte populations is deeply involved in phagocytosis; (ii) one of the two main hyaline amoebocyte populations, provided with lysosomal content, inducible oxidative activity, and no proteases, does not show phagocytic activity; (iii) the second hyalinocyte population mainly contains proteases; LLCs and a population of hyalinocytes present with different sizes and complexity but similar profiles, suggesting that they may be intermediate/maturation stages. These findings suggest that several morpho-functional characters of ascidian hemocyte populations remain to be clarified.

As an effect of the inflammatory stimulus and cytokine network, stem cells proliferate and differentiate, enhancing the frequency of mature cells. Granulocytes degranulate and release signaling molecules. Complement cascades (alternative and/or lectin-dependent), phagocytosis, cytotoxicity, and encapsulation are activated, and finally the possible wound in the tissues is repaired.

In the hemolymph plasma, bioactive substances could contribute to the inflammatory process. In *S. plicata*, heparin, sulfated heteropolysaccharides (glucose and galactose), and sulfated disaccharides have been found in the hemolymph. Heparin and histamine colocalize in the intracellular granules of granulocytes. In mammals, histamine is associated with heparin in the granules of mast cells and basophils; therefore, this hemocyte type (or a granulocyte population) appears to be circulating basophil-like cells. Finally, histamine-containing cells have been also detected in the pharynx. The possibility exists that heparin- and histamine-containing granulocytes may be presumptive counterparts of mammalian basophils. They could perform immunological functions and tissue regeneration (de Barros et al. 2007).

A search of the *C. intestinalis* genome identified no reliable orthologs of vertebrate blood coagulation factors, although paralogs and/or constituent domains were evident (Jiang and Doolittle 2003). The findings concern plasminogen-like carboxyl-terminal domains of fibrinogen, a scaffold conceivably related to factors V and VIII, a number of serpins that do not match with antithrombin, and a carboxypeptidase paralogous to thrombin-activated fibrinolysis inhibitor, as well as numerous domains that are similar to those identified in tissue factor, tissue factor inhibitor, and thrombomodulin.

Phagocytes

Phagocytosis is the most phylogenetically ancient process. First observed by Elie Metchnikoff (1887) in amoeboid cells from marine invertebrates, phagocytosis has a pivotal role in internal defense of invertebrates and vertebrates.

In vertebrates, antimicrobial proteins (e.g., lysozyme), peptides (e.g., defensins), binding proteins (e.g., lactoferrin), reactive oxygen species (ROS) (respiratory burst), and reactive nitrogen species (RNS) are the main phagolysosome effectors.

These toxic molecules can also damage host tissues when inflammatory cells are inappropriately activated. Germ line–encoded receptors discriminate potential pathogens, enabling phagocytes to internalize and kill an array of pathogens (phagosomes mature into phagolysosomes) without the need for opsonization (Di Meo et al. 2016; Robinson 2008). In general, these receptors are called "pattern recognition receptors" (PRRs) and their ligands are "pathogen-associated molecular patterns" (PAMPs) on the surface of Gram-negative and Gram-positive bacteria (e.g., mannans, peptides, lipopolysaccharides, and lipoteichoic acids). PAMPs bind to PRRs and initiate signaling cascades leading to cell activation. The key elements of this framework can be found in ascidians. Hyaline amoebocytes, granulocyte populations, and their transition types can be retained functional analogs of neutrophils and macrophages. They are recruited, cross the vessel epithelium to reach the injured tissue, represent the dominant cells in the earliest inflammatory stages, and can exert phagocytic activity.

In *B. schlosseri*, circulating professional phagocytes are represented by hyaline amoebocytes and macrophage-like cells, which may be transition stages; the former is the active phagocyte that upon ingestion takes the globular form of a macrophage-like cell (Voskoboynik et al. 2004; Ballarin 2008; Cima et al. 2016). Both cells have similar cytochemical properties and common content of lysosomal enzymes (such as phosphatases, 5′-nucleotidase, β-glucuronidase, and esterases), share the same surface glycans, and cross-react with anti-CD39 antibody (a tool used in mammals for monitoring immune activation) (Ballarin and Cima 2005). During the phagosome formation, reactive oxygen metabolite production, nitrite ion release, and acid phosphatase secretion increase. A comparison of the major hemocyte types reported in several botryllid species showed that SRCs can also be equipped with the phagocyte enzymatic apparatus, and they have been retained to belong to the same cell lineage.

In *B. schlosseri*, phagocytosis is modulated by cross talk with MCs that, when activated, release IL-1α-like and TNFα-like factors that enhance the phagocytic activity (Menin et al. 2005; Menin and Ballarin 2010). In the colonial *Aplidium yamazii*, phagocytic activity of tunic cells containing phagosomes has also been shown. Presumably these phagocytic cells engulf extraneous substances (including bacteria) and also function as scavengers to keep the tunic free of discarded tunic cells and other debris (Hirose et al. 1994).

Phagocytosis can be facilitated by opsonins such as lectins and complement pathway products, which bind to the target and enhance the phagocyte activity. In *H. roretzi*, products of C3 complement cascade are opsonins (Nonaka and Azumi 1999).

LLCs as Stem Cells

In general, stem cells have been defined as clonogenic cells capable of self-renewal and multilineage differentiation. These cells, provided with physical and cell surface characteristics, give rise to renewal of lineage progenitors, from which progeny

more restricted in their differentiating potential originate, and finally mature cells are formed (Weissman 2000). The role of ascidian LLCs/hemoblasts is intriguing (Fig. 2); they are a retained primordial form of vertebrate lymphocyte/stem cells (Peddie and Smith 1995; Cooper and Parrinello 2001; Cooper 2009).

The topic has been mainly examined in colonial ascidians, and the question as to whether hemoblasts are stem cells or tissue-restricted progenitor cells has been posed (Kawamura and Sunanaga 2010).

In *B. schlosseri*, somatic stem cell populations exist in "niches" in the anterior ventral region of the endostyle and in the vasculature, where they proliferate in developing buds and migrate to regenerate organs (Voskoboynik et al. 2008). X-ray treatments of primary hemocyte cultures from *B. primigenius* and *B. schlosseri* colonies decrease the LLC proliferative response to mitogeneic factors (Rinkevich and Rabinowitz 1993).

Homologous genes predominantly expressed in human hematopoietic stem cells, myeloid populations, and early lymphoid populations have been identified in the *B. schlosseri* genome (Voskoboynik et al. 2008, 2013a, b). The findings indicate that at least some genetic circuitry relevant for vertebrate immunity appeared to be already in place in the protochordates' and vertebrates' common ancestor. However, the meaning of CD34 epitopes identified by immunocytochemical assay in LLCs remains to be established. CD34, first identified in mammalian hematopoietic stem and progenitor cells, is expressed by a multitude of other nonhematopoietic cell types and identifies progenitor cells from many tissue types (Sidney et al. 2014). In Botrylloides at least two LLC differentiation pathways have been proposed, and phagocytes (hyaline amoebocytes) also show the CD34 marker (Cima et al. 2001; Ballarin and Cima 2005).

In solitary ascidians, electron micrographs of lymph nodules indicate hemocyte differentiation from hemoblasts (Ermak 1975a, b, 1976, 1982). In circulating hemolymph, hemoblasts are rarely distinguished, and LLCs can have the potential for differentiation into hemocyte lineages (Donaghy et al. 2017).

Recombinant human IL-2 and phytohemoagglutinin (PHA) stimulation increases the LLC proliferative activity in *S. clava* pharynx explants (Raftos et al. 1991a, b). PHA binds glycan components of the cell surface glycome; human IL-2 interacts with specific receptors and exhibits a variety of affinity states depending on the subunit composition (Wang et al. 2000). Therefore, a cross-linking with hemocyte receptor–like can be expected, whereas notable differences, including a low level of stimulation in pharyngeal cultures, have been reported.

Histological observations do not show LLCs directly involved in *B. schlosseri* nonfusion reaction, and only a few LLCs have been observed in *C. intestinalis* inflammatory response. The immunocompetence potential is indicated by significantly greater proliferative activity among individuals immunized with allogeneic tissues (Raftos and Cooper 1991; Cooper 1992, 2009). The enhanced proliferation was restricted to discrete crypts of dividing cells within the body wall of the recipients, and in *S. plicata* allograft rejection, adoptive transfer of alloimmune memory has also been reported (Raftos et al. 1988; Raftos 1996a, b).

The Ascidian Inflammatory Response Is Orchestrated

Ascidians have evolved complex inflammatory reactions characterized by molecular and functional homologies with mammals (Azumi et al. 2003; Cha et al. 2001; Voskoboynik et al. 2013a, b). The basic value of the inflammation in ascidian innate immunity is emphasized by the absence of vertebrate-type adaptive immunity (Cooper 2016). *C. intestinalis* and *B. schlosseri* genome-wide sequence analyses have provided a comprehensive picture of immunity-related genes (Azumi et al. 2003; Satoh et al. 2003; Voskoboynik et al. 2013a,b).

The genomes lack significant homologies to genes known to play a pivotal role in the vertebrate adaptive immune system, including assembled MHC genes; dimeric immunoglobulin molecules; genes with homology to *RAG1*/*RAG2*, which are involved in Ig and TCR rearrangements; terminal deoxynucleotidyl transferase, which adds nucleotides to the rearrangement; VDJ elements to create receptor diversity, V-region subgenic elements encoding T cell and Ig antigen receptor domains; or VLR-like immune receptor elements. Nevertheless, outside the jawed vertebrate lineage, a RAG1/2–like gene pair in the purple sea urchin has been identified. An evolutionary scenario of significant gene loss from the highly compacted genome of the ascidian lineage, or horizontal gene transfer, may be suggested (Fugmann et al. 2006; Fugmann 2010). In this respect, it is intriguing that proto-MHC regions, Ig-like domains and transcripts, and activating and inhibitory receptors with MHC-independent functions have been reliably traced throughout ascidian genomes (Du Pasquier 2004; Satake et al. 2003; Azumi et al. 2003; Voskoboynik et al. 2013a, b). A number of genes predict integral membrane proteins with extracellular C-type lectin or Ig-like domains, intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs), and immunoreceptor tyrosine-based activation motifs (ITAMs) (plus their associated signal transduction molecules). *C. intestinalis* expresses immunoglobulin variable region–containing chitin-binding proteins (VCBPs), which are not found in vertebrates (Cannon et al. 2002, 2004; Dishaw et al. 2016). Unlike V-region-containing antibodies and T cell antigen receptors, the VCBPs do not undergo somatic rearrangement, but some exhibit regionalized hyperpolymorphism due to haplotypically variable alleles. The variable region consists of two variable (V) Ig domains and a single chitin-binding domain. These domains bind and promote the opsonization of bacteria, and the distinctive C-terminal chitin-binding domain (CBD) likely is also integral to overall function. The expression of CiVCBP genes is confined largely to the gut epithelium (stomach and intestine); the protein is secreted into the lumen where they bind bacteria. The VCBPs, through association with an extensive network of chitin fibrils, an integral component of the gut-specific mucus, may also influence settlement of bacterial communities by modulating adherent biofilms on epithelial surfaces. In addition, hemocytes (granular amoebocyte population) scattered within the lamina propria and in the circulatory system express the VCBPs. These localizations are significant because the gut is an entry portal for pathogens and a site of complex microbial communities, including commensals (Dishaw et al. 2011). Thus, before the evolutionary emergence of adaptive immunity, soluble immune mediators encoding

V-type Ig domains likely served a role in the establishment and maintenance of gut homeostasis.

The *B. schlosseri* genome encodes homologs of Foxn1, the thymus epithelial gene marker of the thymopoietic microenvironment in vertebrates, and a polymorphic Hsp is involved in allorecognition (see the section: Inflammatory Events Characterize Colonial Ascidian Take Over and Allorecognition). In addition, genes homologous for complement components, Toll-like receptors (TLRs), and genes involved in intracellular signal transduction of immune responses have been identified.

These data indicate that genetic circuitries relevant for vertebrate immunity were already in place in the common ancestor of the protochordates and vertebrates.

PRRs

Response and effector mechanisms start from direct hemocyte/tissue receptor–ligand interaction. Hemocytes (phagocytes, cytotoxic cells) are recruited crossing the epithelium, innate immunity genes are upregulated, and a network of inflammatory factors is produced.

In all invertebrates, a key facet of defense responses lies in the ability to recognize and respond to invading microbes and cell disturbance through a set of germ line–encoded pattern recognition receptors (PRRs), which detect invariant pathogen motifs (PAMPs) and put in place a variety of cellular and molecular inflammatory responses, including phagocytosis, pathogen killing, nodule formation, and encapsulation. PRRs comprise an array of sensors whose basic characteristics include a protein domain for detection coupled to a protein domain that interacts with downstream signaling molecules. The ligand-bound PRR delivers a signal that activates specific transcription factors and creates a network of cross talk by which they regulate multiple host proinflammatory genes and coordinate an appropriate immune response toward the detected pathogen (Hansen et al. 2011; Mogensen 2009; Amparyup et al. 2012). PRRs are expressed by the first responder cells (in mammals: monocytes, macrophages, dendritic cells, and neutrophils), as well as by tissue-specific epithelial and endothelial cells. They include complement receptors, C-type lectin family members, and members of galectin and TLR families. Scavenger receptors, which are structurally heterogeneous, recognize several ligands and structures (including glycans) and function directly as phagocytic receptors. Soluble PRRs (e.g., C-type lectins, pentraxins, and galectins) mediate the binding signaling for cellular responses and can opsonize pathogens, facilitating recognition and ingestion. Structural properties allow a single PRR to recognize a wide range of microbial agents (Silva and Correia-Neves 2012).

Endogenous nonmicrobial signals, termed “damage-associated molecular patterns” (DAMPs), could be involved in stimulating inflammatory responses (Matzinger 1994, 2002). In this respect, Matzinger’s DAMPs cannot be retained as an alternative to self/nonself recognition, but they may be additional signals from

distressed or damaged cells that could share the same receptors with PAMPs (Pradeu and Cooper 2012).

TLRs

Toll-like receptor (TLR) genes that initiate defensive responses against a wide variety of pathogens have been identified throughout the animal kingdom (Voogdt and van Putten 2016). At first, the *Toll* gene was discovered in *Drosophila* to control dorsal–ventral patterning during embryonic development. The protein product was then identified as a transmembrane receptor important for antifungal immunity in the adult fly. The genome sequencing of *Drosophila* showed that there are eight Toll-like receptors and these may also function in innate immunity (Parker et al. 2001; Valanne et al. 2011).

In mammals, TLR genes encode 10–12 membrane molecules with diverse specificities for extracellular and endosomal ligands. They are expressed by lymphocyte populations, macrophages, and dendritic cells (Schmitz et al. 2004; Takeda and Akira 2005). Acting as transmembrane receptors, they recognize PAMPs and express signaling pathways leading to cell activation for appropriate responses to various classes of pathogens. Different TLRs activate distinct patterns of gene expression and instruct the development of antigen-specific acquired immunity participating in activation of antigen-presenting cells (APCs). TLRs recruit adapters to initiate a proinflammatory signaling cascade culminating in the activation of several transcription factor families, also promoting T helper–dependent inflammation. At the cellular level, TLR signals affect many aspects of the cellular response, including cell survival, proliferation, and regulation of the proinflammatory response (Akira and Takeda 2004; Billack 2006; Reynold and Dong 2013). In macrophages and neutrophils, TLR activation enhances phagocytosis and increases the oxidative burst, while resident macrophages secrete proinflammatory cytokines.

They are type I integral membrane receptors, each with an N-terminal ligand recognition domain, a single transmembrane helix, and a C-terminal cytoplasmic signaling (TIR) domain. The solenoid-like ectodomain, made up of leucine-rich repeat (LRR) motifs, shows variations in structure and organization, and mediates recognition and signaling to activate transcription factors. On the basis of sequence homologies, vertebrate TLRs have been grouped into six subfamilies, and not all vertebrate species express all TLR paralogs (Botos et al. 2011).

LPS is a potent activator that involves both TLR4 and the CD14 protein required for LPS-induced TLR4 endocytosis, LPS transport to the receptor, and delivery of the TLR to the endosomal signaling machinery (Zanoni et al. 2011; Liu et al. 2001). TLR4 and TLR2 can also respond to endogenous molecules from traumatic tissue injury. The stress-induced heat shock proteins Hsp60 and Hsp70 released from dying cells are recognized by both TLR4 and TLR2, and a form of fibronectin, expressed in situations of tissue injury, binds to TLR4. Engagement of the TLRs leads to NF-κB activation and production of the proinflammatory cytokines IL-1, IL-6, IL-8, IL-12, and TNF, as well as stimulating inducible nitric oxide synthase (iNOS) and the

production of reactive nitrogen intermediates by macrophages (Mak and Saunders 2006).

Genome-wide analyses have shown that TLRs or related genes, essentially conserved in the genome of nonmammalian organisms, diverge in number, structural organization, and biological roles (Satake and Sasaki 2010; Satake and Sekiguchi 2012). In invertebrate deuterostomes, TLR-like genes are paralogous, and the expansion of TLR-related genes may occur in a species-specific manner, whereas in vertebrates, the number of TLRs does not significantly differ among species (Coscia et al. 2011).

In ascidians, TLR-like receptors have been identified. *C. intestinalis* possesses only two authentic TLR-like genes (CiTLR1 and CiTLR2), expressed in the hemocytes and gut (Nonaka and Satake 2010; Sasaki et al. 2009). This finding contrasts with the large number of TLR genes found in the echinoderm *Strongylocentrotus purpuratus*, in which more than 200 gene models have been classified into a number of distinct subgroups (Rast et al. 2006; Tu et al. 2012; Hibino et al. 2006). Most sea urchin TLR genes display greater similarity to each other than to TLRs of other species, and they are encoded in tandem arrays, suggesting an enormous gene expansion. The amphioxus genome has numerous predicted TLR complete gene models; 72 TLR or TLR-related genes have been detected in the genome of *Branchiostoma floridae* (Satake and Sekiguchi 2012; Huang et al. 2008). Most of these genes seem to have been generated via species-specific gene duplication. The presumptive evolutionary scenario indicates that only a few TLRs or their related genes might have existed in a common deuterostome ancestor. In this case, *C. intestinalis* conserves the ancestral characteristics, whereas sea urchins and amphioxus have expanded their gene paralogs during their divergence in concert with variations in their lifetimes, life cycles, and environments.

In *C. intestinalis* the putative amino acid sequence has a unique structural organization with similarity to mammalian TLRs. However, the CiTLRs are localized in both the cell plasma membrane and endosomes, providing evidence that the CiTLRs are functionally "hybrids" of the vertebrate TLRs that are located on either the cell surface or endosomes (Sasaki et al. 2009; Satake and Sekiguchi 2012). CiTLRs have more extensive binding affinity for PAMPs (CiTLR1 and CiTLR2 bind multiple ligands triggering signal transduction), whereas in mammals different TLRs are necessary. Genes expressed by hemocytes in the hemolymph and pharynx, or associated with the gut, respond to the pathogenic ligands, and this supports the view that TLR-mediated innate immune functions are conserved in ascidian tissues.

CiTLR–ligand interaction elicits a dose-dependent induction of NF-χB transcription factor that upregulates cytokine-like genes. In the anterior and middle intestine, where both CiTLRs are abundantly expressed, ligands differentially upregulate CiTNFα gene expression. In this ascidian, LPS activates pharynx inflammatory responses including CiTNFα production and lectin complement activation. However, neither CiTLR1 nor CiTLR2 recognizes LPS (Sasaki et al. 2009; Satake and Sekiguchi 2012); therefore, the possibility exists that other receptors could be involved in the induction of CiTNFα or, as in mammals, *Ciona* TLRs could utilize accessory molecule(s). Interestingly, according to Sasaki et al. (2009), the expression profiles for CiTLRs may be implicated in recognition of endogenous ligands.

Lectins

Glycans are components of the outer surface of all cells and form large parts of the extracellular matrices. They have extraordinary structural diversity, biochemical specificity, and regulatory flexibility. The diversity of the glycome, including considerable intra- and interspecies variations, reflects the central role played by oligosaccharides, glycoproteins, and glycolipids in numerous biological systems and evolutionary machinery (Springer and Gagneux 2013; Cummings et al. 2017; Bianchet et al. 2008). The enormous combinatorial possibility of glycan presentation is manifested during immune cell activation, differentiation, and signaling, as well as in their aberrant expression in inflamed or neoplastic tissue. Glycans have a prominent role as PAMPs and DAMPs and are crucial for self/nonself discrimination (Varki et al. 2009; Rabinovich and Croci 2012).

Lectins are proteins or glycoproteins that mainly bind glycans (including glycoproteins and glycolipids) with weak bonds forming three-dimensional arrangements of multivalent lectins and glycans. Most of them are oligomers of subunits covalently or noncovalently bound, thus determining the avidity of lectin–glycan interactions and amplifying both recognition and effector capabilities.

They are soluble or integral membrane components and act as PRRs characterized by the carbohydrate recognition domain (CRD). Integral membrane lectins are mostly type II transmembrane proteins with a short hydrophobic domain and an extracellular C-terminal region that carries the CRD. Soluble lectins agglutinate a wide variety of erythrocytes and, at first, they were identified by hemagglutination assays. Aside from the CRD, the lectins exhibit domains with variable structures. The presence of conserved or variant residues within the CRD, the structure of the other domains, the Ca^{2+}-dependence/independence, and glycan specificity or protein binding distinguish several lectin families with intrafamily variations, representing a very heterogeneous group of proteins.

The lectin domains are functionally connected with inflammatory reactions, as supported by gene upregulation and tissue localization compatible with internal defense roles. In ascidian hemolymph, several humoral and cellular lectins have been reported; they can display opsonic activity and mediate inflammatory responses (Parrinello 1995; Vasta et al. 2004; Quenseberry et al. 2003).

Galectins

The galectin molecular family, formerly named S-lectins, is defined by the evolutionarily conserved CRD and Ca^{+2}-independent binding to β-galactoside-containing glycans (such as lactose and N-acetyllactosamine). They are nonglycosylated proteins with a wide taxonomic distribution and structural conservation in vertebrates, invertebrates, protists, and fungi (Houzelstein et al. 2004; Yu et al. 2007; Vasta et al. 2012). The conserved β-sandwich structure is formed by six strands with the CRD and five distinct strand sheets.

In the cytoplasm, they bind endogenous ligands performing several intracellular functions, and can be translocated into the nucleus. Missing a secretion signal peptide, they are released into the extracellular matrix by direct translocation across the

plasma membrane. Once released, galectins bind glycoproteins or other glycoconjugate ligands on target cell surfaces or in the extracellular environment, recognizing exogenous ligands such as glycans and LPS (Rabinovich and Gruppi 2005; Rabinovich et al. 2002; Vasta 2012; Vasta et al. 2012).

Their binding capacity, functional multivalence, and cellular effects are improved by oligomerization. Some galectins have diverged to bind ligands in a carbohydrate-independent manner (Nesmelova et al. 2008). Galectins are involved in acute and chronic inflammation (Liu et al. 2008, 2012).

In mammals, more than 15 galectins have been identified and structurally classified into three groups: (i) prototype galectin monomers with a single CRD, which are noncovalently linked in dimers for effective binding and signaling; (ii) tandem galectins, with two distinct but homologous CRDs per monomer in which the flexible linker domain allows formation of dimers that increase their potency; and (iii) chimera-type galactins, in which the oligomerization results in multivalent carbohydrate ligand binding. In mammals, the chimera-type Gal-3 is a multifunctional lectin with proinflammatory activity, inducing migration of monocytes and macrophages involved in endocytosis and antigen presentation (Norling et al. 2009; Sano et al. 2003).

In *C. intestinalis*, two galectins—CiLgals-a and CiLgals-b—form distinct oligomers (Vizzini et al. 2012; Ballarin et al. 2013). The galectin genes recorded in the genome (Dehal et al. 2002) are organized into three exons with two subtypes: N-terminal F4 subtype CRD and C-terminal F3 subtype CRD (F4-CRDs and F3-CRDs). A similar exon/intron organization has been found in echinoderm orthologs (Houzelstein et al. 2004). CiLgals-a exhibits the F4-CRD-liker-F3-CRD gene organization; CiLgals-b shows an F4-CRD-linker-F4-CRD structure not known in vertebrate genes.

Comparative analysis of the CiCRD deduced amino acid sequences showed that the N-CRD and C-CRD, like vertebrate CRDs, are included in two distinct clusters, suggesting a domain duplication model and an early domain divergence. The divergence between the vertebrate N-CRD and C-CRD was greater than that between invertebrate deuterostomes (Shida et al. 2003; Azumi et al. 2007; Terajima et al. 2003; Vasta et al. 2004). The vertebrate galectin signature sequence, directly involved in galactoside binding, is conserved in the N-CRD and C-CRD of CiLgals-a and in the N-CRD of CiLgals-b. CiLgals-a is considered orthologous in the deuterostome galectin lineages. On the contrary, the CiLgals-b C-CRD is so divergent that the signature sequence could not be suitable as a sugar-binding motif and has been related to a distinct functional role.

The homology molecular modeling (human Gal-3-C-CRD, Gal-9 N-CRD, Gal-4-C-CRD superimposition) shows a CiLgals-a common structural model that includes two antiparallel β-sheets composed of five and six β-strands, respectively, with a CRD suitable for binding to β-galactosides. The divergent sequence of the CiLgals-b C-CRD lacks superimposition. Both galectins are constitutively expressed by hemocytes as well as by the stomach epithelium, where they can interact with environmental microorganisms (Parrinello et al. 2017). According to Houzelstein et al. (2004), although CiLgals-b is outside the CiLgals-a group, it is orthologous to

the *S. clava* mono-CRD galectin supporting tandem duplication events from a mono-CRD galectin to bi-CRD galectins. A prototype galectin was also found in the colonial ascidian *D. candidum*, in which multiple members of the galectin family have been identified (Vasta et al. 1986).

Galectins Participate in the Inflammatory Response

In mammals, pathogens upregulate the expression of galectin genes and participate in the inflammatory response (Rubinstein et al. 2004; Klyosov 2008). In *C. intestinalis* pharynx hemocytes, the LPS stimulus significantly upregulates the transcription of the CiLgals-a and -b genes (Vizzini et al. 2012). In this respect, since the two CiTLRs do not bind to LPS, the possibility exists that galectins are involved directly or as TLR-associated molecules (Sasaki et al. 2009). The Gal-3 discriminates *Saccharomyces cerevisiae* and *Candida albicans* in association with TLR2 for signaling (Jouault et al. 2006; Martchenko et al. 2007). More generally, the triggering via the galectin-mediated signal transduction pathway depends on cross-linking with β-galactoside glycojugate or glycoprotein receptors. The amphioxus *Branchiostoma belcheri tsingtauense* galectins (BbtGals, F4-CRD-linker-F3-CRD-type bi-CRD) may function like their vertebrate homologs, directly binding to bacteria, and so the transcription of BbtGal-L mRNA is increased (Yu et al. 2007). In mammals, galectins upregulated in infections are required for the specific recognition of fungi.

In comparison with the mRNA expression profiles of the other inflammatory components (see below), the perceptible beginning of the transcription is delayed and the maximum level was reached at 24 h post-inoculation (p.i.). An increased number of riboprobe-labeled hemocytes are engaged inside the vessels, and CCs and SRCs express both CiLgals. The riboprobes are localized in the nucleus and in the surrounding cytoplasm, and specific antibodies label the proteins mainly associated with granules and the nuclear envelope. Galectins expressing cells migrate into the tunic, while both galectins outline the endothelium basal membrane. Functions can be deduced from domain organization and amino acid sequence homologies. Structural differences and the highest CiLgal-a transcription level suggest that CiLgals-a has a more major role than CiLgals-b in the LPS-challenged pharynx response. Findings on galectin-like molecules released by cultured *C. intestinalis* and *B. schlosseri* hemocytes also suggest an opsonic role (Parrinello et al. 2007; Ballarin 2008).

Galectins can also sense damage signals by transmission of the information to effector cells (Sato and Nieminen 2004).

RBLs

Rhamnose-binding lectins (RBLs) are Ca^{2+}-independent lectins, specific for rhamnose and galactosides, which have been found in marine invertebrates and fish (Jimbo et al. 2007; Terada et al. 2007; Ogawa et al. 2011; Cammarata et al. 2014; Ballarin et al. 2013). RBLs share one or multiple CRDs with a unique α/β fold, eight highly conserved Cys residues engaged in four disulfide bridges, and conserved

motifs (YGR, DPC, and KYL). They are involved in glycan metabolism regulation, cell proliferation, phagocytosis, and cytotoxicity.

The hemolymph of *B. schlosseri* contains soluble RBLs, and sequences of five isoforms have been identified. The predicted proteins contain a single CRD, Cys and characteristic motifs, a signal peptide, and no glycosylation sites. A phylogenetic tree, built with the RBL sequences in databases, clearly shows that BsRBLs are located within the protochordate cluster (Gasparini et al. 2008). Specific antibodies and riboprobes label BsRBLs expressed by professional phagocytes, whereas MCs do not express them. BsRBLs exert multiple roles in immunosurveillance and immunomodulation, acting as opsonins, stimulating the respiratory burst and ROI production, exerting chemotactic activity, and challenging MCs to release cytokine-like molecules. During the allogeneic immune response, activated MCs release BsIL1α and BsTNFα (Menin and Ballarin 2010). The BsTNFα further induces the synthesis of BsRBL by a limited number of phagocytes, thus additional phagocytes become activated and migrate toward the inflamed tissue. The released BsRBLs are involved in MC degranulation and act as opsonins favoring clearance and encapsulation, and potentiate positive feedback with a progressive increase in the local concentration (Ballarin et al. 2013).

C-Type Lectins

These lectins form a large protein superfamily sharing a CRD basic structure in which a fold shows highly variable amino acid sequences. They are Ca^{2+} dependent or independent and can bind ligands other than glycans, thereby the typical CRD has been designed as CTLD (Zelensky and Gready 2005; Cummings and McEver 2009; Drickamer and Taylor 2015). The CTLD structure is characterized by a double loop (loop in a loop) stabilized by two highly conserved disulfide bridges at the base of the loops, and a set of conserved hydrophobic and polar interactions. The second long loop is structurally and evolutionarily flexible, and it is involved in glycan binding and interactions with diverse ligands. Generally, the structural diversity between the different C-type lectins is higher in the loop regions, mainly because of amino acid insertions or deletions. The diversity within families is amplified by subunit oligomerization that affects the avidity for multivalent ligands. Multiple gene copies, allelic variation, posttranscriptional and posttranslational modifications produce multiple isoforms that further expand the lectin recognition capabilities, providing wider recognition and effector capacity and functions (Kerrigan and Brown 2009; Gijtenbeel and Inghuis 2009; Drickamer and Fadden 2002).

Serving as PRRs, they are transmembrane or soluble proteins (glycoproteins). As signaling receptors they have diverse functions depending on the motifs in their cytoplasmic domain, and are crucial in shaping immune responses. They induce endocytic, phagocytic, antimicrobial, proinflammatory, or anti-inflammatory responses (Hoving et al. 2014). On the basis of molecular phylogeny and domain organization, various families have been distinguished, including collectins, selectins, and pentraxins.

C-type lectin genes have radiated independently in each animal lineage (mammals, ascidians, flies, nematodes), and they have diverged in chordate lectin families.

In ascidians, despite the literature description of "bona fide" mammalian homologs, the multifunctional roles of C-type-like lectins—including immune responses and regulation of cell growth, adhesion, and differentiation—have been widely recognized (Matsumoto et al. 2001). The structure of a C-type lectin (TC14) isolated from the budding ascidian *P. misakiensis*, specific for D-galactose and related monosaccharides, has been resolved in detail (Poget et al. 1999). This lectin is a dimer that adopts a typical CTLD fold with differences in the loop regions and in the second α-helix involved in the formation of a dimeric interface. The binding site, coordinated by a calcium ion per monomer, is quite exposed and located on the surface of the loop region. The TC14 lectin plays a role in generalized defense mechanisms, such as strong antibacterial activity.

In *C. intestinalis*, C-type lectin genes have been recorded in the genome, and soluble lectins are contained in the hemolymph. In *S. plicata*, they are components of the acute-phase response (Green et al. 2003; Raftos et al. 2001).

A putative C-type lectin with CTDL and an Ig domain (BsCLT) has been cloned from the *B. schlosseri* genome; the deduced amino acid sequence features three building blocks: (i) a Greek-key motif signature (a class of β-sheet) at the N terminus; (ii) a CTDL domain signature; and (iii) an immunoglobulin (Ig) domain at the C terminus. The nonpolymorphic Ig domain has been classified as an intermediate-type Ig domain. Antibodies raised against recombinant BsCLT cross-reacted with a polypeptide in tunicate crude extract, suggesting that they may play a systemic defense role (Pancer et al. 1997).

CTLD Lectins that Bind Protein Targets

In mammals, Natural Killer (NK) cells are lymphocytes classified as "innate" lymphocytes that respond quickly to a variety of pathological challenges through a distinct repertoire generated by the combinatorial assortment of germ line–encoded activating and inhibitory receptors expressed on their surface (Kelly et al. 2015; Bartel et al. 2013). One of two classes of NK receptors is the C-type lectin–like superfamily encoded in the natural killer gene complex (NKC). In this respect, the divergent evolution of ancient C-type lectins, acting on the CTDL fold that loses the Ca-dependent sugar binding capacity and binds proteins or lipids, and the components of the NKC are expressed by natural killer (NK) cells. Most NK cell–associated CTLRs are known to bind glycoproteins with an MHC class I–like fold: these include classical and nonclassical MHC class I molecules and MHC class I–like molecules. A prominent member of this group is NKG2D, an activating receptor that binds to several MHC class I–like molecules induced by various forms of cellular stress such as viral infection, tumor formation, tissue damage, and heat shock protein expression. In distinct mammal orders, the NKCs diverged in their binding affinity; thereby, in the mouse, Ly49 receptors detect allelic variants of MHC I molecules and CD94/NKG2x receptors interact with a nonclassical MHC class I molecule presenting signal peptides of MHC class I molecules.

The receptors are type II transmembrane glycoproteins with an N-terminal cytoplasmic domain and a single transmembrane domain, followed by a stalk region and a single extracellular C-type lectin–like domain (CTLD) at the C terminal. The receptors are basically built up by two α-helices and two antiparallel β-sheets forming a compact homoheterodimer structure stabilized by two or (mostly) three conserved intramolecular disulfide bonds (Bartel et al. 2013; Wada et al. 2004; López-Botet et al. 1997). The mammalian NKC encodes for several dozen CTLRs. These sensors allow the release of NK cell cytotoxicity toward self-MHC-deficient cells (viral infections or tumor cell lines) and hence represent the molecular substrates of the "missing-self" recognition mode.

The human invariant CD94 glycoprotein covalently assembles with different C-type lectins of the NKG2 family and forms disulfide-linked heterodimers. Five different molecular species of NKG2 (NKG2A, B, C, E and H) have been reported. NKG2A and B, produced by alternative splicing, have two receptor tyrosine-based inhibitory motifs in their cytoplasmic domains and form inhibitory receptors complexed with CD94. CD94 forms heterodimers with NKG2 family molecules and, with CD94/NKG2A binding to the specific ligand, suppresses activation signaling processes; thus, the NK cytotoxic activity toward "self" is inhibited, whereas it is displayed when a missing-self target is met (Borrego et al. 2005).

In both *B. schlosseri* and *C. intestinalis* a CD94-like gene (CD94/NKR–like) provided with a CTLD that recognizes proteins and a homolog of the vertebrate NK receptor have been reported (Zucchetti et al. 2008; Khalturin et al. 2003; Boyington et al. 1999). Both deduced amino acid sequences share structural features that recognize proteins, connecting them to human CD94 functionality.

The comparative analysis of CiCD94 displays 50/66% identity/similarity with BsCD94 and 30/49% with *H. sapiens* CD94 (Zucchetti et al. 2008). The deduced amino acid sequence discloses that the receptor is provided with a single CTDL, a transmembrane sequence, and a short cytoplasmic tail at the N terminus that is a typical feature of type II C-type lectin, and contains three possible sites for glycosylation. Four cysteines form two of the four intrachain disulfide bonds, and hydrophobic residues are involved in the dimerization. The CTLD of the CiCD94-1 lacks Ca^{2+}-binding sites. The CiCD94-1 receptor shares structural features with the CTLDs that recognize proteins; the amino acids that in human CD94 are involved in the interaction with peptides presented by the MHC class I molecules are conserved (Cambi and Figdor 2003; Brown and Gordon 2001).

Unlike mammalian CD94, the identified BsCD94-1 presents a short cytoplasmic domain; therefore it is presumable that it requires a partner chain to become functional NKR (Khalturin et al. 2003). However, the extent of structural conservation between the Botryllus BsCD94-1 molecule and the vertebrate orthologs strongly implies functional conservation. Specific antibodies raised toward the recombinant BsCD94-1 protein label three groups of hemocyte types: granulocytes with a relatively small nucleus and small cytoplasmic granules, granulocytes with a large nucleus and a small cytoplasm to nucleus ratio, and SRCs. The label was limited to

the cell surface, confirming the transmembrane localization of the BsCD94-1. MCs do not show epitopes of the protein.

In *C. intestinalis*, the gene is upregulated by LPS stimulus, and both mRNA and protein are expressed in the majority of granular amoebocytes that populate the tunic and the hemolymph following the LPS stimulation. These hemocytes are phagocytes and their activity is inhibited by specific anti-CiCD94 protein, suggesting that the receptor is involved in phagocytosis (Zucchetti et al. 2008).

The deduced molecular characters of BsCD94/NKR and CiCD94/NKR receptors forecast a cell lineage with the NK receptors functioning in the missing-self model.

Collectins

In mammals, Ca^{2+}-dependent collectins recognize PAMPs; activate the lectin complement cascade counteracting bacteria, parasites, and transformed cells; and are relevant in triggering effector responses. The structure is characterized by a mannose- or N-actylglucosamine (GlcNAc)-specific CTDL, a coiled neck region joining the CTDL to a large collagen domain, and a short N-terminal tail region. Subunits assemble in large oligomeric complexes via interactions by the collagenous tail. As a type I transmembrane protein, collectins are expressed by macrophages and several tissues, and their expression is upregulated by several cytokines (Marshall and Gordon 2004; East and Isacke 2002; Turner 2003). In soluble form, collectins facilitate in vitro phagocytosis, promote chemotaxis, stimulate the production of cytokines and ROIs by inflammatory cells, and are implicated in the phagocytic uptake of apoptotic corpses. Collectins, such as MBL and ficolins with associated serine proteases (MASPs), have a pivotal role in activating the lectin complement pathway. MBL recognizes mannose and mannans, and appears to be a spatially coordinated TLR coreceptor increasing the microbial uptake. It is also localized in the phagosome (Ip et al. 2009). The ficolins are a group of GlcNAc-specific proteins containing collagen-like and fibrinogen-like (FBG) sequences; they can be secreted or function as PRR cellular receptors. They have overall collectin structure and activity similar to those of MBL, but in contrast to MBL, it is the FBG domain that binds GlcNAc (Matsushita et al. 2000; Gupta and Surolia 2007; Sim and Laich 2000).

In ascidians, collectin-like lectins show the typical CTDL and variations in the remaining structure and in glycan specificity. The ascidian MBL-like and ficolin-like lectins, complexed with MASPs, activate C3, leading to the complement cascade (Sekine et al. 2001; Nair et al. 2005; Fujita et al. 2004a, b; Raftos et al. 2001; Skjoedt et al. 2010).

In *H. roretzi*, the complement activating Hr-collectin binds to glucose (thereby designated GBL) but not to mannose or GlcNAc (Ji et al. 1997; Kenjo et al. 2001; Nonaka and Azumi 1999). The GBL C-terminal half contains the CTDL, but the collagen domain is replaced by another sequence with an α-helix structure similar to the configuration of Gly-X-Y repeats.

Phylogenetic analysis showed that CiMBL clusters with vertebrate MBL, indicating a common ancestor, while CiMBL and HrMBL form separate clusters, supporting a common ancestor before the divergence of the two taxonomic orders.

In *S. plicata*, the subunit of the dimeric collectin-like (TC14) includes a collagenous domain and a short, cysteine-bearing N-terminal domain; it is specific for D-galactose and related monosaccharides. TC14 is expressed by circulating hemocytes, and the expression increases following challenges by an inflammatory agent (LPS, carrageenans) (Green et al. 2003). In activated circulating hemocytes (presumably hyaline amoebocytes) a C3 homolog is promptly upregulated and exocytosed (Raftos et al. 2001, 2002, 2003, 2004).

Transcripts for MBL-like proteins have been identified in several ascidian species (Franchi and Ballarin 2017; Vasta et al. 1999). Transcripts for ficolins are present in *H. roretzi* (Kenjo et al. 2001), *B. leachii* (Rinkevich et al. 2007), and *B. schlosseri* (Franchi and Ballarin 2017). The transcription of the *H. roretzi* ficolin-3 gene is significantly impaired in organisms with soft tunic disease (Cha et al. 2011), and the collectin-like (GBL) involved in the recognition of microbes interacts with MASP and leads to HrC3 activation (Sekine et al. 2001). In *S. plicata*, increased collectin secretion has been related to the inflammatory response (Nair et al. 2000; Green et al. 2003). In colonial ascidians the MBL-like pathway has been identified; genes for MASPs and ficolins are upregulated in MCs during the *B. schlosseri* nonfusion reaction (Oren et al. 2007; Franchi and Ballarin 2017).

In *C. intestinalis*, which expresses the complete lectin-triggered complement activation pathway, a CiMBL has been cloned and sequenced (see below). The CiMBL initiates the lectin pathway of the complement and promotes phagocytosis, killing of pathogens, and induction of other cellular responses. In addition, two CiMBLs and a CiMBL-associated serine protease are expressed in the gut epithelia (Skjoedt et al. 2009). The deduced CiMBL amino acid sequence shows a protein structure that includes a Cys-rich N-terminal domain, presumably engaged in disulfide bridges between monomers, two collagen-like domains, one α-helix domain, and one CTLD that binds mannose/glucose residues. The CiMBL mRNA transcription profile, after LPS stimulation, shows the rapid expression and the enhanced level of the transcript: at 1 h p.i. the CiMBL level is sixfold increased, then it decreases (2–4 h p.i.) and after a further increase it reaches its maximum peak at 24 h p.i. The CiMBL is mainly expressed by amoeboid granulocytes in the tunic matrix and CCs in the pharynx bars and connective tissue (Bonura et al. 2009).

Complement

Complement pathways and their products are pivotal in inflammation and largely detectable in invertebrates.

The complement system is a complex innate immune surveillance system. Complement components are activated in a cascade fashion after a triggering event; each step of the pathway results in conformational changes or cleavage of the downstream components, which become activated and gain the capacity to activate the subsequent cascade components (Merle et al. 2015a; Nesargikar et al. 2012). Proteolytic cleavage is, in part, performed by enzymatic complexes originating

from association of products from the same complement cascade. The complement core components are named with a simple number designation in the order of their discovery, while the sequence of the cascade reactions is C1, C4, C2, C3, C5, C6, C7, C8 and C9. In ongoing inflammatory reaction, the level of the complement components increases—contributing to acute and chronic inflammation, immunostimulation, lysis of bacteria and foreign cells, and opsonization and chemotaxis—and moreover it participates in B cell activation.

C3 is the central component shared by three routes: antibody-dependent, lectin-dependent (detailed below), and alternative.

Alternative and lectin-dependent pathways can be traced back in invertebrates; the lectin pathway has been described in deuterostome invertebrates, including ascidians (Nonaka 2014; Smith et al. 1999; Fujita et al. 2004a, b; Nonaka and Kimura 2006; Nonaka and Yoshizaki 2004; Nair et al. 2005). In this pathway, MBL or ficolin lectins form a complex with proenzymes, i.e., MBL-associated serine proteases (MASPs) that are provided with a modular structure including a serine protease C-terminal domain (Matsushita and Fujita 2001; Dinasarapu et al. 2013). Upon binding of the MBL– or Ficolin–MASP complex to pathogens, the MASP zymogen is converted into the active form that cleaves C3, C2 and C4, leading to fragments, some of which are necessary for continuing the pathway. Several MASPs have been identified and each of them has a defined role (Bobó et al. 2016). At the end, the triggered lectin-dependent downstream cascade (as well as the alternative pathway) merges into the classical pathway involving terminal complement components (C6, C7, C8, and C9) that form the C7–C9 membrane attack complex perforin domain (MAC). This complex causes pores to appear on the plasma membrane of the target cells, leading to their lysis (Kondos et al. 2010). Proteins containing the MACF domain, but lacking the other terminal complement components, have been found in organisms in a broad range of phyla (Nonaka 2014).

One of the major consequences of complement activation is the generation of three small cationic peptides (C3a, C4a and C5a) usually referred to as complement anaphylatoxins. They are mainly involved in several activities: (1) attraction of phagocytes by chemotaxis (mainly C3a and C5a) and promotion of extravasion of leukocytes (that bear the specific receptors C3aR and C5aR) into the injured site; (2) upregulation of adhesion molecule expression by neutrophils and endothelium, increasing (mainly C5a) vascular permeability and “leukocyte rolling”; (3) opsonization of potential pathogens for facilitating phagocytes in recognition and ingestion of targets; (4) induction of the oxidative burst by macrophages and neutrophils; (5) induction of C3 receptor expression; and (6) C5a stimulation of the secretion of proinflammatory cytokines such as IL-1 and IL-6, which can also stimulate the proliferation of T cells, and modulation of dendritic cells (APCs) influencing the adaptive immune response. The functional profile of C4a, usually included among the anaphylatoxins, is questionable (Barnum 2015; Merle et al. 2015a, b). Complement components and their receptors are expressed by activated leukocytes, macrophages, dendritic cells, mast cells, and NK cells. Production of receptors for C3, C3- and C5-peptides, and products of C3b degradation are upregulated during

inflammation (Lubbers et al. 2017; Li et al. 2011; Futosi et al. 2013; Van Lookeren Campagne et al. 2007).

Genome analysis of many representative species has allowed us to trace the evolutionary route of the complement system on the basis of the presence or absence of each complement gene (Nonaka and Kimura 2006). The genomes of invertebrates, including cnidarians, contain homologs of C3 and other components of the complement alternative and/or lectin-dependent pathways. However, many of them are merely predicted genes from the draft genome; their functions are not wholly known or can be suggested by structural homologies with mammals. The absence of complement genes in some species (*Drosophila melanogaster* and *Coenorhabditis elegans*) could be the effect of a secondary loss, presumably due to their short generation time.

The structural features shared between vertebrate C3, C4, and C5, and the similarity with the protease inhibitor α2-macroglobulin, have suggested that two lineages could have evolved from a common ancestor by gene duplication and divergence (Levasseur and Pontarotti 2011). Anyway, individual domains of complement components have been found in both protostomes and deuterostomes; in the latter the complement components appear to be established as a combination of pre-existing domains.

Ascidian Complement Lectin Pathway

Although both the alternative and lectin complement activation pathways are present in ascidians, here only the established lectin pathway is described (Fujita et al. 2004a, b; Nonaka 2014). In this pathway, the MBL/Ficolin-like-MASP complex bound to target cells directly activates the cascade, which can serve several functions, including agglutination, opsonization of cellular agents, activation of phagocytes, inhibition of microbial growth, cytotoxicity, and modulation of the inflammatory response (Raftos et al. 2001; Franchi and Ballarin 2017; Nonaka and Yoshizaki 2004; Nonaka and Satake 2010).

Homologs of the pathway key components (MBL, MASP, C3) have been identified in several ascidians (Vasta et al. 1999). C3 is a heterodimer made up of α- and β-C3-like chains. Collectins mediate recognition of PAMPs and stimulate the activation of the α-chain thiolester bonds that can directly bind to bacteria. MASPs cleave C3 into two fragments. The large C3b peptide mediates opsonization and the small C3a-like peptide is akin to the corresponding vertebrate anaphylatoxin (Marino et al. 2002; Pinto et al. 2003; Matsushita et al. 1998).

In the colonial *B. schlosseri*, complement component (C3-like, MBL-like, and Bf-like) genes are transcribed by hemocytes (mainly MCs) and the lectin activation pathway has been identified (Franchi and Ballarin 2016). The C3b receptor and the ficolin-like lectin associated with two MASPs were also found (Corey et al. 2016). The BsMBL gene is upregulated by zymosan, and opsonic activity appears to be C3 dependent (Franchi and Ballarin 2017).

C. intestinalis genome analysis has provided the most comprehensive picture of an almost complete set of genes homologous to the mammalian lectin complement pathway: MBL, ficolin, four MASP genes, two C3s (CiC3-1 and CiC3-2), three Bf/C2s, two α2-macroglobulin-like, and genes for C6/C7/C8/C9 proteins containing MAC/perforin domains (Azumi et al. 2003). The Ci-C6/C7/C8/C9 components exhibit protein structures similar to those of human late components (MAC); however, the activity of a cytolytic pathway needs to be established (Skjoedt et al. 2010; Marino et al. 2002; Nonaka and Satake 2010; Giacomelli et al. 2012). The deduced amino acid sequence of the CiC3-1 protein exhibits the above-reported structure and the thioester site is provided with a catalytic histidine and a convertase cleavage site. The anaphylatoxin-like CiC3a peptide is generated by MASP proteolytic cleavage of the CiC3α-chain N-terminus. As for mammalian C3a, the chemotactic function of CiC3a is localized at the C terminus, but the terminal Arg is not critical for the activity. The C3a fragment, which in ascidians may be chemotactic or opsonic, in *C. intestinalis* exerts chemotactic activity toward hemocytes, as shown by the attractive effect of the recombinant CiC3a. The inhibition with pertussis toxin also suggests that the receptor molecule mediating the chemotactic effect is the G protein–coupled receptor(s) (GPCRs) belonging to the rhodopsin family (Pinto et al. 2003; Melillo et al. 2006). GPCR-based signal transduction is ubiquitous in eukaryotic genomes. There is a highly compact set of GPCRs in the *Ciona* genome, and a wide survey refers to the presence of 169 putative receptors homologous with human GPCRs, indicating that they serve several functions shared with vertebrate signaling biology (Kamesh et al. 2008; Prasobh and Manoj 2009). Many *Ciona* GPCR receptors are highly divergent homologs of the chemokine receptor cluster genes.

The domain amino acid sequence of the cloned CiC3aR shows high homology with mammalian C3aR (Melillo et al. 2006). Differences concern the carboxyl-terminal tail and the third cytoplasmic loop; both are longer than the corresponding regions of C3aRs, and the shorter extracellular N-terminal sequence lacks the presumptive N-glycosylation site. As in mammals, the C-terminal end of the cytosolic tail contains many serine and threonine residues that represent presumptive phosphorylation sites.

In the *C. intestinalis* complement pathway, CiMBL, CiC3a, and CiC3a-R have a role in the proinflammatory process. Real-time PCR analysis showed that C3, constitutively expressed in the pharynx, is upregulated by LPS stimulation, while specific anti-CiC3 antibodies showed that the gene and lectin pathway are localized in hemocytes (granular amoebocytes) of the pharyngeal bars and in stigmata ciliated cells. CiC3a and CiC3b are present in the pharynx, and the amount of the CiC3a fragment increases following the LPS challenge. CiC3a-R is constitutively expressed only in granular and hyaline amoebocytes which, in chemotaxis and inhibition experiments, migrate in a directional way (Pinto et al. 2003; Melillo et al. 2006; Giacomelli et al. 2012).

The two CiC3-like genes seem to be diverged from a common ancestor of the vertebrate C3/C4/C5, and then duplicated into CiC3-1 and CiC3-2 in the *Ciona* lineage. Independent gene duplication and various diversification events have

occurred in distinct ascidian orders or cognate families. The phylogenetic analysis of the CiC3a-R amino acid sequence indicates that it does not cluster with any of the vertebrate C3aR and C5aR clades (Marino et al. 2002). The phylogenetic tree, based on the alignment of CiC3-1 and CiC3-2, including molecules of the α_2-macroglobulin superfamily, shows that the *Ciona* C3s form a cluster with *H. roretzi* C3. Thereby, the duplication event from which the CiC3-1 and CiC3-2 genes arose would have happened after the separation of the *Halocynthia* and *Ciona* ancestor.

The C3-like protein from *S. plicata* is closely related to *H. roretzi* C3 and *Ciona* C3s. Similar results have been obtained in *P. stolonifera*. Activation by LPS and zymosan in this species generates an 8.5-kDa proteolytic fragment that confers chemotactic activity toward hemocytes, as demonstrated by in vitro chemotaxis experiments. The C3 expression by *P. stolonifera* hemocytes is coincident with chemotactic activity (Raftos et al. 2002, 2003). A C3-like transcript has been found in *S. plicata* hemocytes challenged with LPS or carrageneen; the transcription was upregulated and the protein contained in vesicles promptly exocytosed. C3-containing granulocytes actively infiltrate the injured tissue and degranulate (Raftos et al. 2001, 2002). LPS treatment also stimulates the expression of a C3-like protein by *H. roretzi* cells in the stomach wall, which is engaged in antibacterial activity (Nonaka et al. 1999).

Even ascidian C3 receptors, homologous to mammalian complement receptors (integrin family), have been identified. In *H. roretzi*, two integrin-like proteins (αHr1 and αHr2 and two βHr1 and βHr2) have been found on the surface of a granulocyte population, and specific antibodies against a recombinant protein, reproducing the extracellular region of αHr1, inhibited C3-dependent phagocytosis, suggesting that in this ascidian, an ancestral form of CR3 and CR4 mediates phagocytosis (Miyazawa et al. 2001; Miyazawa and Nonaka 2004; Ewan et al. 2005). Both the βHr1 and βHr2 subunits are associated with the αHr1 subunit. The type of pairing found in ascidians, namely the same integrin α subunit (αHr1) paired with different integrin β subunits (βHr1 and βHr2), is different from the mammalian CR3 and CR4 pairing pattern and refers to ancestral forms of complement receptors.

Also, a C1q-like gene is expressed in ascidians; it is constitutively transcribed in the *C. intestinalis* pharynx, in *B. schlosseri* (Oren et al. 2013), and in *D. candidum* (Iwanaga and Lee 2005; Azumi et al. 2003, 2007). In mammals, C1q, as a complement recognition subcomponent, binds to a wide variety of targets (microorganisms, apoptotic and necrotic cells); the protein initiates C1r and C1s activation, and also forms a weak complex with MASP (Wallis 2007). In addition, it can bind pentraxins (CRP and SAP in humans), which belong to an ancient lectin family characterized by a unique structure of a disk-shaped cyclic pentamer of noncovalently bound identical subunits. Pentraxins function as soluble Ca^{2+}-dependent PRRs. They bind a variety of microbes, facilitate phagocytosis, and regulate the inflammatory response (Du Closs 2013). In addition, pentraxins share with collectins and ficolins the property of activating complement. A galactose-binding pentraxin-like protein has been isolated from the colonial *D. candidum* (Vasta et al. 1986; Quesenberry et al. 2003).

Genes for putative complement-control protein (CCP) featuring a mammalian CCP domain and genes for α2-macroglobulin (MASP-inhibitor) that are regulators of the complement activity have been identified (Pancer et al. 1995; Azumi et al. 2003). The α2-macroglobulin is one of the founding members of the larger thiol-ester protein superfamily, which includes a variety of similar protease-binding proteins with a wide phylogenetic distribution and shares a defined suite of structural and functional characters (Armstrong 2010).

PO and ProPO

Melanin is a pigment found in almost all animals; its role varies depending on the organism, and it is critical for survival. In vertebrates, the tyrosinase plays a crucial role in melanin biosynthesis, and components of the evolutionarily conserved tyrosinase family serve different functions (Cammarata and Parrinello 2009; Esposito et al. 2012). The survey of the *C. intestinalis* genome revealed one ortholog to human TYRP1, expressed in developmental stages.

Invertebrates, including ascidians, use phenoloxidase (PO) in place of tyrosinase for melanin biosynthesis, and the enzyme activity concurs with wound healing, sclerotization, pigmentation, and defense. Phenoloxidase and tyrosinase share two active sites that are copper dependent but vary in their remaining sequence and oligomeric organization. Phylogenetic analysis shows that the POs belong to the arthropod hemocyanin superfamily, and evolutionary origin from hemocyanin ancestors with enzymatic function has been suggested (Cerenius et al. 2008). Unlike arthropod PO, which is monophenoloxidase, ascidian PO is a bifunctional redox enzyme (*o*-diphenol:O_2 oxidoreductase) that catalyzes the ortho-hydroxylation of monophenol (i.e., tyrosine) forming *o*-diphenol and, then, the dehydrogenation of diphenols into *o*-quinones, which can polymerize producing melanin (Cammarata and Parrinello 2009).

PO and phenolic compounds have been identified in ascidian tunic cells, participating in tunic formation; in some species, melanin is also produced (Chaga 1980). In ascidians, as in arthropods, concomitant with prophenoloxidase (proPO) activation many immune reactions are performed, such as the generation of factors with antimicrobial, cytotoxic, opsonic, or encapsulation-promoting activities (Söderhäll and Cerenius 1998; Cerenius et al. 2008).

Ascidian hemocytes contain the proenzyme proPO, which is activated to PO by a serine protease cascade, in turn activated by PRRs after their binding to ligands (peptidoglycans, LPSs, bacterial carbohydrates, fungal β-glucans) (Jackson et al. 1993; Immesberger and Burmester 2004; Hata et al. 1998; Parrinello et al. 2003; Amparyup et al. 2012). Generally, the ascidian proPO is contained in MCs, although the activity has also been reported in other granulocyte types. The mechanism can be regulated by protease inhibitors, e.g., α2-macroglobulin, thereby avoiding overproduction of melanin, phenolic substances and ROIs that could lead to the disruption of self-tissues.

In *B. schlosseri*, soluble factors potentiate the activity of MCs and induce their degranulation. The MC vacuoles and granules contain both proPO and PO substrates (polyphenol tunichromes, quinones, DOPA-containing proteins), and the enzyme pathway produces quinones and cytotoxic ROIs (Nappi and Ottaviani 2000).

Among the ascidian PO enzymes, differences have been observed. Divalent cations are requested to enhance the L-dopa oxidation by *B. schlosseri* PO, whereas in *C. intestinalis* and *S. plicata*, the PO activity is not cation dependent. Although the ascidian PRRs involved in proPO activation are poorly known, there are differences regarding the ligands; β-1,3-glucans and oligosaccharides are active in *C. intestinalis* whereas they are inactive in *S. plicata* and *B. schlosseri* (Ballarin et al. 1994; Jackson et al. 1993; Arizza et al. 1995; Cammarata et al. 1999). In addition, following recognition of a harmful agent by hemocytes, molecular cross talk can be involved in the proenzyme activation (Lemaitre and Hoffmann, 2007; Cerenius et al. 2008).

Two C. *intestinalis* PO genes, CiPO-1 and CiPO-2, have been identified and cloned. They encode putative proteins clearly distinct from tyrosinase, lack a signal peptide, and display variations in their amino acid sequences (Immesberger and Burmester 2004; Cammarata and Parrinello 2009).

Upregulation of the ProPO System

The prophenoloxidase (proPO) pathway, fated to produce melanin, also releases intermediates involved in the inflammatory responses, in which melanin may not be the final product and the pathway is mainly related to the production of intermediate active factors, i.e., quinones or oxygen radicals.

The PO activity is expressed by activated *S. plicata* MCs and *C. intestinalis* URGs, and quinones that lyse in vitro erythrocytes and tumor cell lines are produced (Cammarata et al. 1997; Parrinello et al. 2003). Differently, in *B. schlosseri* nonfusion alloreaction, the cytotoxic activity is dependent on ROIs (Ballarin and Cima 1998; Ballarin et al. 2002). Indeed, in vivo, the enzymatic reduction of quinones forms toxic ROIs (Nappi and Vass 1993).

In several species, MCs are retained as the main proPO-expressing cell type, which contain substrate molecules and activating proteases, and are the effectors of the nonfusion reaction (Ballarin 2008; Smith and Söderhäll 1991). However, among botryllids, other cell types appear to be involved, such as diverse granulocytes, SRCs, and CCs (Scofield and Nagashima 1983; Shirae and Saito 2000).

In *C. intestinalis*, strong PO activity was found in cytotoxic URGs, whereas morula cells do not show any cytotoxic activity, although both are involved in inflammatory reactions (Parrinello 1996; Peddie and Smith 1993; Cammarata et al. 2008). In *Ph. mamillata* and *S. plicata* "hemocytes with large granules" display low PO activity whereas the typical morula cells do not show any PO-related activity (Cammarata et al. 1997; Parrinello et al. 2003). Although the activating PRR in *C. intestinalis* has not been identified, in crustaceans the proPO system is activated upon recognition of pathogens by pattern recognition proteins, including LPS- and

β-glucan-binding proteins (LGBPs). In the shrimp (*Paeneus monodon*) proPO system, a PmLGBP functions as a PRP for both LPS and β-glucan, and the transcription in hemocytes is enhanced by the microbial challenge (Amparyup et al. 2012).

In *C. intestinalis*, as an effect of LPS intratunic inoculation, the CiPO1 and CiPO2 transcription and the enzyme activity are enhanced by LPS in circulating hemocytes and in the tunic (Vizzini et al. 2015a). In the tunic matrix, the proPO activation pathway could depend on unknown proteases diverse from serine proteases; in fact the PO activity enhanced by LPS challenge could not be inhibited by trypsin inhibitors (Cammarata et al. 2008). A high proportion of hemocytes (CCs and URGs) express both POs in the pharynx and are spread in the connective tissue lining the epidermis. In addition, POs of different sizes are modulated in the tunic and pharynx, indicating that LPS inoculation challenges the proenzyme production by tunic cells and hemocytes, as well as the activation of the serine protease pathway (Trapani et al. 2015).

Differences have been found in the CiPOs transcription time courses and in the transcription level profiles of the two identified genes. The transcription of CiPO-1 is faster (1-4 h p.i.) and it is mainly expressed in two waves (the second at 24 h p.i.); CiPO-2 upregulation occurs later, reaching the maximum at 8 h p.i. These profiles could be indicative of distinct hemocyte populations (CCs, URGs) engaged in CiPO1 or CiPO2 production. The inflammatory role of PO is also indicated by the increased number of PO-containing URGs that populate the tunic matrix after the LPS stimulus (Cammarata et al. 2008). Phenoloxidase is also involved in the *H. roretzi* "contact reaction" in which hemocytes were mixed in vitro with allogeneic or xenogeneic hemocytes (Fuke 1980).

Peroxinectin

Peroxinectin is a component of the peroxidase–cyclooxygenase superfamily characterized by a C-terminal peroxidase domain and an integrin-binding motif (KGD: Lys-Gly-Asp) that can participate in cell–cell and cell–extracellular matrix interactions. This protein is involved in adhesion and migration mechanisms essential for immunity (Dong et al. 2009), granulocyte degranulation, microorganism immobilization, phagocytosis, encapsulation, and nodule formation (Johansson and Söderhäll 1989; Cerenius et al. 2008; Johansson et al. 1995; Johansson 1999; Kobayashi et al. 1990; Thornqvist et al. 1994; Hsu et al. 2006). Peroxiditic heme protein genes appeared very early in evolution, presumably recruited upon pathogen invasion to develop enzyme-dependent unspecific antimicrobial defense. To exert this activity, the enzyme requires the heme group linked in a suitable cavity, and oxidation products are responsible for killing microorganisms (Zederbauer et al. 2007a, b).

A *C. intestinalis* peroxinectin (CiPxt) has been cloned and sequenced from the pharynx, and the entire CiPxt sequence has been analyzed (Vizzini et al. 2013a). The peroxidase domain contains two His; one of them may be the proton acceptor involved in the peroxidase catalytic function, and the second one could be a heme binding site, while four cysteines indicate intrachain disulfide linkages. At the

C-terminal sequence, the putative KGD integrin–binding motif, the peptide signal at the N-terminal, and highly probable trypsin and chimotrypsin cleavage sites can be predicted. The three-dimensional model, which in part overlaps with human myeloperoxidase, shows the KGD in a loop in an external position. The secondary structure is mainly α-helical and each monomer has a central heme-containing core. The comparative analysis of the peroxidase domains showed significant homologies with peroxidase–cyclooxygenase superfamily members, including mammalian myeloperoxidase (MPO), eosinophil peroxidase (EPO), thyroid peroxidase (TPO) and invertebrate thyroid peroxidase (TPO) (*C. intestinalis* and *H. roretzi*), and peroxinectins (Pxt) from insects, crustaceans, and echinoderms. In vertebrates, the entire sequence of Pxt was not found. An increasing phylogenetic distance separates the peroxidase domain from chordata MPO, TPO, and EPO, and echinoid and crustacean Pxts. Although the *C. intestinalis* domain is included in the invertebrate Pxt subfamily, it forms, together with the echinozoa, a deuterostome cluster distinct from the arthropod group which, in turn, is distinguished into crustacean and insect clades; it is the closest to the mammalian group.

In ascidians, as in crustaceans, the Ptx may be involved in adhesion and migration mechanisms essential for immunity. The activity could be generated by the proteolysis associated with concomitant activation of the proPO system (Sritunyalucksana et al. 2001).

Peroxinectin Upregulation

In crustaceans, the Pxt gene can be upregulated by LPS or β-1,3-glucans and the transduction pathway regulating the expression of antimicrobial peptide genes (Dong et al. 2009; Liu et al. 2005, 2007).

The LPS inoculation enhances the level of CiPxt mRNA; the gene transcription is promptly and significantly boosted (4–8 h p.i.) reaching a maximum value at 12 h p.i., depending on the increased frequency of activated cells in the pharynx vessels and tunic, then it decreases (Vizzini et al. 2013a). CCs/MCs and SRCs inside the pharynx vessels appear to be the CiPtx–producing cells, and the riboprobe also labels the cytoplasm rim of URGs in the inflamed tunic matrix.

As reported above, LPS activates the proPO system through proteolytic cleavage that, as in other invertebrates, could activate properoxinectin into peroxinectin.

An increased number of CiPxt-positive compartment/morula cells and signet ring cells in the vessels can be related to LPS inoculation. Furthermore, a large number of CiPxt-expressing URGs intensely populate the inflamed tunic matrix (Vizzini et al. 2013a). URGs also express proPO, which is activated by proteolysis, thereby the same enzymatic cascade could cleave pro-Pxt, producing active Pxt.

The potential involvement of CiPxt in the *C. intestinalis* inflammatory response may be related to the defense role of mammalian myeloperoxidase and peroxidase of neutrophils, monocytes, and eosinophils (Klebanoff 2005; Wang and Slungaard 2006).

Cytokines

In mammals, cytokines form a large family of low-weight proteins including interleukins (ILs) and tumor necrosis factors (TNFs). They are mainly produced and secreted by endothelial cells, epithelial cells, granulocytes, resident macrophages, and NK cells in response to various harmful stimuli, including LPS. The same cytokine can be secreted by different cell types, can stimulate the production of additional cytokines, or can be pleiotropic; similar functions can be stimulated by different cytokines; and they can act synergically and regulate various and contrasting actions. Being proinflammatory, they are involved in cell proliferation, cell differentiation and activation, cell motility, chemotaxis, phagocytosis, apoptosis, and necrosis, as well as stimulating collagen synthesis in wound healing and tissue repair (Dinarello 2007; Meager and Wadhwa 2013). Cytokine-like activities have been reported in several invertebrate species, playing an essential role in defense (Ottaviani et al. 1995, 1996; Buchmann 2014; Hughes et al. 1990).

Many cytokines signal via the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, which is also frequently altered and constitutively active in a broad range of tumor cells.

TNFα-Like

Tumor necrosis factor-α (TNFα) is a major proinflammatory cytokine with pleiotropic and multifunctional properties (Goetz et al. 2004; Idriss and Naismith 2000; Bodmer et al. 2002). The signaling function of TNF receptor is also mediated by adapter protein factors (TRAFs) (Inoue et al. 2000).

In *C. intestinalis*, cDNA/EST derived from hemocytes and the draft genome sequence disclosed an ectodysplasin/TNF-like multigene family (Terajima et al. 2003; Shida et al. 2003) and in *C. savigny* a TNF factor ligand superfamily member (CsTL) has been identified (Zhang et al. 2008). A BLAST search revealed four *C. intestinalis* predicted genes related to TNF family members, including a TRAIL-like protein (TNF-related apoptosis-inducing ligand) of *D. rerio*, the bovine ectodysplasin A, the rabbit lymphotoxin-α precursor, and the feline Fas ligand. Since in mammals these members are type II membrane glycoproteins with limited homology to TNFα in the extracellular region, they have not been studied further. The CiTNFα gene with significant predicted similarity and identity to TNFα of the monkey *Aotes vociferans* and human TNFα was cloned and sequenced (Parrinello et al. 2008).

The CiTNFα deduced amino acid sequence shows that it is a type II transmembrane protein with an extracellular C-terminal domain containing sites for proteolytic cleavage, two conserved Cys residues that can form an intrachain disulfide bridge, and two potential N-glycosylation sites. The domain structure is similar to that of mammal and fish TNFα, supporting that CiTNFα is a component of the protein family. The putative molecular homology modeling process disclosed a conserved "jellyroll" structure consisting of two β-antiparallel sheets building a sandwich. Minor amino acid replacements are scattered, whereas major differences

(10 aa) form a very short α-helix. Frequent "hot" mutation points characterize the C-terminal sequence that, with the N-terminal sequence, forms the first two strands of the inner sheet. A high degree of CiTNFα genetic variability has been found in a *C. intestinalis* wild population, and mutation points with effects on the amino acid sequence are more numerous within the C-terminal region (Vizzini et al. 2017).

CiTNFα domain organization and 3′UTR comparative analysis display a relatively complex gene organization with seven exons and six introns. The gene organization shares regions with vertebrates, such as an interferon-γ-activated inhibitor of translation (GAIT) and a Musashi binding element (MBE) that may be sufficient for temporal regulation and translational silencing. The gene transcription occurs in different tissues (pharynx, ovary, stomach, intestine), but the expression is higher in the pharynx and intestine, which are mainly exposed to environmental challenge.

The phylogenetic tree unveils that CiTNFα protein and two predicted *C. savigny* TNFs form a cluster related to the vertebrate cluster, suggesting that the TNFα family domain and cytokine-like activities were present in ascidians. In this respect, it is noteworthy that *D. melanogaster* TNFα homologs, the Eiger A and B isoforms (Moreno et al. 2002), appear to be distant from the chordate group, and the earthworm CCF, reported as a functional analog (Silerova et al. 2006), also lies far from the TNF family.

In addition, the Ci-TNFα and Cs-TNF deduced amino acid sequences form a cluster with sea urchin TNF1A, separated from other invertebrates. Therefore, an evolutionary lineage traced back to invertebrate deuterostomes appears to diverge at the ascidian–vertebrate node.

Upregulation of the CiTNFα Gene

In mammals, activated neutrophils produce proinflammatory and immunoregulatory cytokines, including interleukins and TNF, and chemokines belonging to distinct protein families. Many germ line–encoded PRRs, colony-stimulating factors, cytokine receptors, and complement receptors have been shown to trigger cytokine production (Tecchio et al. 2014).

Ciona intestinalis CiTNFα is constitutively expressed and upregulated in vivo by LPS. In the pharynx, the CiTNFα gene is promptly upregulated, with an expression profile similar to that of mouse and fish TNFα (Parrinello et al. 2008, 2010; MacKenzie et al. 2003). The upregulation was already evident at 2–4 h p.i., decreased at 8 h p.i., then increased again until 24 h p.i. In spite of the subsequent decline, a low mRNA level was maintained, probably as an effect of the protracted inflammatory action differently exerted by LPS on the ascidian body wall tissues. The mRNA is transcribed by GAs and CC/MCs that during the inflammation enrich the tunic matrix, pharynx vessels, and the lacunar connective tissue. The nucleus of these cells was marked by the riboprobe, and a specific antibody localized the protein in the cytoplasm. URGs and various granulocyte transition forms, abundant in the inflamed tunic matrix, do not present any transcription signal. In the circulating hemolymph, also HAs are CiTNFα-expressing cells. Hemocyte-lysate and hemolymph plasma analysis unveiled that, just after the stimulation, the CiTNFα produced as hemocyte membrane-bound protein (43 kDa) is cleaved into a mature

soluble form (15 kDa). These findings further suggest that the CiTNFα exerts an important role in both local and systemic responses as a proinflammatory cytokine. In addition, the vascular epithelium is committed as shown by TNFα protein in the basal membrane lining vessel walls.

Surprisingly, some recognizable LLCs, collected in nodules associated with the vessel epithelium, appeared to express the cytokine; the mRNA was found in the large nucleus and the protein in the cytoplasm rim. Taking into account that activated LLCs did not appear to be directly involved in the inflammatory reaction, this finding leaves open the question on the LLC function and the possibility of an autocrine signaling.

In mammals, TNF receptors expressed by leukocytes are primarily involved in apoptosis and inflammation, but they can also take part in proliferation and differentiation (Ward-Kavanagh et al. 2016; Locksley et al. 2001). The *Ciona* genome contains eight TNFR-associated factor (TRAF)-related genes, which are the major signal transducers for the TNFR superfamily (Terajima et al. 2003), and three possible CiTNF receptors are capable of initiating signal transduction that culminates in caspase activation and programmed cell death (Chambon et al. 2007).

In botryllid ascidians, during the allorejection reaction, MCs produce and release molecules reacting with anti-TNFα antibodies. Indirect evidence also suggests that this cytokine is involved in the recruitment of MCs in the ampullae involved in allogeneic contacts (Cima et al. 2004; Ballarin 2008).

IL-1- and IL-17-Like Interleukins

Interleukins are a group of cytokines that function in the immune system; the majority of them are synthesized by a T lymphocyte population, monocytes, macrophages, and endothelial cells. They promote the development and differentiation of T and B lymphocytes and hemopoietic cells (Brocker et al. 2010). Interleukin-1 (IL-1) is a major immunoregulatory protein released by macrophages with many host defense–related properties.

Interleukin (IL-1)-like activities have been identified in a number of invertebrate species (Ottaviani et al. 1995, 1996; Beck et al. 1989b; Beck and Habicht 1991; Beck et al. 1993). In ascidian hemolymph, proteins sharing a number of IL-1-like physicochemical characteristics have been reported (Beck et al. 1989a, b, 1993). A fraction isolated from the *S. clava* hemolymph contains an IL-1β-like component that stimulates in a dose-dependent fashion the proliferative activity of granular amoebocytes, LLCs, and mouse thymocytes (Raftos et al. 1991b). An immunohistochemical study showed that molecules containing interleukin-1-like epitopes are expressed by endothelial tissue lining the pharyngeal wall. An IL-1-like functional activity may be indicated by the increased number of hemocytes in the vascular lacunae as a result of the cell proliferation response of challenged specimens. Accordingly, IL-1-receptor epitopes in cell nodules of the pharyngeal bar ansae have been found. Although the *C. intestinalis* genome does not reveal IL-1-like genes, human IL-1 molecular traits can be observed and an IL-1-receptor identified.

In botryllid ascidians, during the allorejection reaction, MCs produce and release molecules that cross-react with antihuman-IL-1α antibodies; in the nonfusion reaction this IL-1-like is involved in the MC recruitment into the ampullae (Ballarin 2008).

In mammals, interleukin-17 (IL-17) is a T cell–derived cytokine characterized by a Cys knot fold formed by two sets of paired β-strands stabilized by three disulfide interactions (Pappu et al. 2010). Several IL-17 family members are produced by NK cells and neutrophils (Michel et al. 2008; Weaver et al. 2007). The IL-17 induces proinflammatory effectors, neutrophil infiltration, and clearance of microorganisms; synergizes with other cytokines such as TNFα, may render cells cytotoxic, and participates in tissue injury (Tecchio et al. 2014; Gu et al. 2013).

Candidate IL-17 genes have been identified in invertebrate genomes (Roberts et al. 2008; Wu et al. 2013). In *C. intestinalis*, three IL-17-like genes (CiIL17-1, CiIL17-2, and CiIL17-3) have been found to be vertebrate orthologs (Vizzini et al. 2015b). Sequence and structural analysis of CiIL-17s show the same gene organization as the human IL-17A/F, formed with two introns and three exons, differing in the length of the introns which are longer in the human IL-17A/F. Four cysteines are strictly conserved in regions that correspond to functionally and structurally essential motifs. The three-dimensional model displays the Cys localization in β-sheets and supports the preservation of the disulfide linkage position in all of the IL-17 homologs. The sequence of the C-terminal region is critical for receptor binding in accordance with the presence, in the genome, of an IL-17 receptor homologous.

The phylogenetic tree unveils that the CiIL-17 genes share a common ancestor in the chordate lineages.

CiL-17 Gene Upregulation

In the pharynx from LPS-treated specimens, the transcription of the three CiIL-17-like mRNAs is upregulated in a short time (4-8 h p.i.) but their transcriptional profiles are slightly different (Vizzini et al. 2015b). CiL-17-1 and CiL17-3 are mostly upregulated at 4 h p.i., whereas the CiL-17-2 expression is challenged at 8 h p.i. These profiles indicate differential transcriptional activity of distinct hemocyte populations in the pharynx vessels. Riboprobes are contained in GAs with large granules, in CCs, and in the cytoplasm of a cell type provided with a large vacuole resembling the SRC or URG transition stage.

The riboprobe-containing hemocyte populations increase in density following the LPS stimulus, while the relative numbers of hemocytes that express each transcript vary in accordance with the time courses, further suggesting a modulated response in distinct cell populations.

The role of the CiIL-17 has not been ascertained, and hypotheses concern the known activity in the mammalian inflammatory reaction and findings from other ascidians. The recombinant *Botryllus* IL-17 significantly enhances, in a dose-dependent manner, the cellular cytotoxicity of allogeneic effector cells (Cima et al. 2016; Corey et al. 2016). In the colony, during the generation change, the gene for an IL-17 ortholog is overtranscribed and probably involved in modulation of the cellular events occurring during the take-over phase of the life cycle (see below). The IL-17-like could mediate cross talk between MCs and phagocytes.

TGF-β (CiTGF-β)

Transforming growth factor-β (TGF-β) belongs to a family of regulatory cytokines that have pleiotropic functions in a broad range of cell lineages involved in numerous physiological and pathological processes and immune responses (Li et al. 2006). TGF-β signaling elicits diverse cellular responses that are primarily mediated through Smad transcription factors, the key of cytokine signaling pathways (Shi and Massagué 2003; Massagué and Gomis 2006).

In *C. intestinalis*, the CiTGF-β is structurally related to the protein family, synthesized as a long proprotein composed of a hydrophobic signal peptide, an N-terminal prodomain, and a C-terminal active peptide with a cleavage site for generating the C-terminal active fragment (Vizzini et al. 2016a). The tridimensional model shows a cysteine knot motif, and the secondary structure is characterized by two α-helices and seven β-sheets. In addition, an RGD motif (tripeptide Arg-Gly-Asp) may be a potential binding site. Like other family members, the prodomain shows a low degree of conservation for correct processing and secretion of the mature dimeric complex. The active peptide exhibits conserved cysteine residues engaged in intrachain disulfide bonds.

Comparative analysis of the TGF-β genes discloses an ancestral bilateria repertoire consisting of two TGF-β type II and three type I receptors (Huminiecki et al. 2009). In ascidians, the ancestral TGF receptor repertoire includes three type II receptors and at least two R-Smad.

CiTGF Upregulation

In the LPS-induced *C. intestinalis* inflammatory reaction, the CiTGF-β gene is promptly upregulated (1–4 h p.i.), suggesting its engagement in the first phase of the response (1–4 h), then a second transcription wave (48 h p.i.) seems to correspond to time-elapsed activation of distinct cell populations. This transcription profile foreshadows the CiTGF-β potential function as a proinflammatory cytokine. Within the vessel lumen, tightly packed hemocyte clusters, mainly formed by granulocytes and URGs, express the mRNA. It is of interest to mention that in mammals, TGF-β has a role in differentiation of T helper 17 cells and in IL-17 production (Lohr et al. 2006), and some functional relationship between CiTGF-β and CiIL-17 in the early inflammatory reaction may be hypothesized (Vizzini et al. 2016a).

LPS Challenges Gene Upregulation in the Vessel Epithelium and Epidermis

In mammals, the vessel endothelium functions as an interactive barrier between blood and tissue, and it is the primary target for inflammatory agents. As a response, the endothelial cells express chemokines that initiate recruitment at sites of tissue inflammation and activation of circulating leukocytes (Trepels et al. 2006; Bierhaus et al. 2000). LPS induces gene upregulation, producing adhesion molecules, while

the endothelial permeability is enhanced and proinflammatory mediators are secreted.

In *C. intestinalis*, LPS challenges the pharynx epithelium as well as the epidermis lining the tunic (Vizzini et al. 2008, 2013a; Parrinello et al. 2015a, b). Although a systematic histological study, taking into account the various timing phases, has not been carried out, at an early stage (a few hours p.i.) of the response, galectins (CiLgals-a and CiLgals-b), CiTNFα, CiPO2, CiPxt, and CiMBL, as well as the CiCAP modulatory factor, are upregulated to various extents in traits of the vessel epithelium.

Also, the transcription of the Ci–type IX collagen α-chain is upregulated by LPS inoculation. Hemocytes circulating, scattered in the connective tissue under the epidermis and in the tunic matrix, produce the mRNA within 4 h p.i. Morula cells, amoebocytes (granular/hyaline), and URGs express the collagen. In the URGs the protein is confined in the cytoplasmic rim lining the granule. In a slower time (about 24 h p.i.), the epithelial cells of the epidermis that outline the tunic are mainly involved in collagen expression. The epidermis and infiltrating hemocytes could have an active role in tunic-repairing processes. In addition, the proliferative activity of the epidermis for the renewal of the tunic cells (Di Bella et al. 2005, 2015) could be stimulated by the cytokine-like enhanced expression. In this respect, traits of the epidermis usually monolayered in naïve ascidians appeared to be multilayered during the encapsulation process (Di Bella et al. 2005; Parrinello and Patricolo 1984).

As in vertebrates, the *C. intestinalis* inflammatory reaction results in a regulated pattern of proinflammatory factors and tissue remodeling.

CiLgals, CiPOs, and Pxt Are Upregulated in the Endostyle

The ascidian endostyle is a glandular ciliated groove that lies along the middle ventral wall of the pharynx. It is the first trait of the digestive system and extends to the esophagus, and its function concerns feeding as well as a thyroid-like activity. Histologically, it has been divided into nine functional units called “zones” numbered bilaterally from midventral to dorsolateral. Zones 1, 3, and 5 are supporting elements involved in catching and transporting food. Zones 1–4 produce the mucus, a complex of mucoproteins and mucopolysaccharides that capture food particles, including bacteria, conveyed from the pharynx ciliated epithelium (Petersen 2007; Parrinello et al. 2017). The bottom of the groove (zone 1) is lined with a longitudinal row of very long cilia that move the mucus to the sides of the endostyle and then lateral cilia push it toward the gut.

Endostyle zones express both CiLgals. Specific antibodies and riboprobes showed that cells of zones 2 and 3 constitutively produce CiLgals-a and CiLgals-b. Both zones are further activated by LPS inoculation, and the CiLgals-a and -b production is enhanced. In zone 4, the LPS stimulus induces CiLgals-b mRNA transcription and the protein is localized in taller epithelial cells, suggesting differential zone expression and function (Parrinello et al. 2015a). Vesicles are involved in galectin production and transport. Although data on the galectins function in the

endostyle are not available, it is reasonable to suggest that, as in the stomach, they could be involved in activity related to microorganisms with which this structure is in continuous contact.

Other zones appear to be challenged by LPS. The CiPO1, CiPO2, and CiPxt riboprobe signals were found in zones 7, 8, and 9 (Vizzini et al. 2013a, 2015a). These zones consist of low epithelial cells similar in their features. They are the thyroid-equivalent components of the endostyle that contain iodoproteins and express thyroid-specific genes that are developmentally regulated. In these zones, peroxidase activity has been reported, while a specific thyroid peroxidase (TPO) has been identified in zone 7 (Fujita and Sawano 1979; Ogasawara et al. 1999). A relationship between the hemocyte CiPOs and CiPxt activities and the peroxidase of thyroid-like tissues may be only hypothesized. However, it is known that in humans, proinflammatory cytokines cause thyroid inflammatory disorders (Ajjan et al. 1996). Furthermore, thyroid cells can express functional sensors for exogenous and endogenous damage signals, and they are also capable of launching innate immune responses without the assistance of immune cells (Kawashima et al. 2013). These findings open a new front in ascidian biology.

In the Pharynx, LPS Challenges Immune Regulatory Mechanisms

Posttranscriptional regulation of mRNA processing is well known to play a fundamental role in determining the outcome of gene upregulation, also affecting initiation and resolution of the inflammatory reaction (Anderson 2010). Furthermore, in almost all eukaryotes, a polyadenylation process intervenes just after the gene transcription, affecting qualitatively and quantitatively the dynamic of mature mRNA for translation. In vertebrates, an alternative polyadenylation (APA) mechanism is operational in inflammation. The mRNA metabolism is controlled and protein isoforms with distinct functions, or mRNAs differing in the length of their 3′ untranslated regions (3′UTR), are produced (Di Giammartino et al. 2011). The 3′UTRs serve as traits for binding to factors (i.e., microRNA, RNA-binding proteins) that control the regulation. Through variations of the 3′UTRs, APA regulates the stability, tissue localization, and translation efficiency of the target mRNAs. In the CR-APA (coding region-APA-alternative polyA), sites are located in internal intron/exon organization and APA events can produce different protein isoforms. Alternatively, APA sites are located in the 3′ untranslated region (3′UTR, UTR-APA), resulting in transcripts with 3′UTRs of different lengths but encoding the same protein. CR-APA can affect gene expression qualitatively (Elkon et al. 2013), whereas UTR-APA has the potential to quantitatively affect the expression (Fox 2015).

In *C. intestinalis*, LPS could stimulate a CR-APA event in the CAP gene (Bonura et al. 2010; Vizzini et al. 2016b; Parrinello et al. 2016). The catabolite activator protein (CAP) that belongs to the superfamily of cysteine-rich secretory protein (antigen 5 and pathogenesis-related1), is a transcriptional activator (dimeric) with a

DNA-binding domain at the C terminus that modulates immune responses (Gibbs et al. 2008). Two cAMP molecules bind dimeric CAP and function as allosteric effectors by increasing the affinity for DNA.

In silico analysis showed that CiCAP is characterized by the CAP superfamily motifs (CiCAP1, CiCAP2, and CiGLIPR1). The CiCAP2 deduced amino acid sequence shows an N-terminal domain with high homology to that of vertebrates, a C terminus homologous to the collagen-binding adhesion of *Streptococcus mutans*, and glycosylation sites. In the phylogenetic tree, the CiCAPs appear to be closely related to the human GLIPR1 protein (glioma pathogenesis-related protein1). A GAIT element (gamma interferon inhibitor of translation element) was found in the 3′UTR of CiCAP-2 mRNA. The GAIT is a cis-acting RNA element found in several immune-related mRNAs, involved in specific translation control (Vyas et al. 2009).

The CiCAP-2 gene is challenged by LPS through a CR-APA site. In the pharynx, it is rapidly upregulated (1–4 h p.i.), and the mRNA level is in agreement with the increased hemocyte populations in the pharyngeal vessels. Genes expressing cytokines are regulated at transcriptional, posttranscriptional, and translational levels, and in humans, the biosynthesis of TNFα is mainly regulated at the posttranscriptional level (Jensen and Whitehead 2001; Karpova et al. 2001). A computational analysis of *C. intestinalis* revealed several post-transcriptional CiTNFα regulatory elements (Vizzini et al. 2013b, 2016b).

Overall, these findings suggest that the initiation phase of the ascidian inflammatory reaction can be controlled by both transcriptional and posttranscriptional regulation.

Various Agents Challenge Encapsulation

Inflammatory responses can be caused by irritant agents and tunic injury. The typology of the cellular reaction, the cell types involved, and the resulting effects can depend on the nature and size of the injuring agent, as well as on the interactions between the tunic and vascular system. Small particles (e.g., carmine, colloidal carbon, trypan blue, colloidal thorium dioxide) injected into the tunic or the vascular system are phagocytized and cleared, whereas a capsule surrounds larger objects. Tissue damage accompanies the inflammatory response and damage signals could be involved in encapsulation responses, and their potential role in the modulation of inflammation cannot be excluded (Kaczmarek et al. 2013; Pradeu and Cooper 2012). However, since invertebrate experimental procedures can hardly be performed in aseptic conditions, contamination with microbial molecules cannot be excluded. In addition, following tissue stress or cell death, different cellular stimuli (e.g., TNF, Fas ligand, TRAIL ligand, dsRNA), IFN-g, ATP depletion, and pathogens) have been shown to induce necrosis (Vanlangenakker et al. 2012).

Glass fragments that injure the *M. manhattensis* tunic and branchial tissues are enveloped by infiltrating hemocyte populations. MCs migrate from the hemolymph and collect in the wound edges to form a capsular multilayered structure containing

strands of tunicin (Anderson 1971). The tunic matrix becomes filled with material released from degranulating MCs, and fibrous material envelops the wound. A similar response was observed in *P. stolonifera* injured tunic (Wright and Cooper 1983). It has been suggested that in naïve ascidians, MC intracellular content contributes to tunic matrix formation.

In *C. intestinalis* the tunic inflammatory response has been stimulated by inoculating particulate materials or soluble proteins (erythrocytes, colloidal carbon, bovine serum albumin, limpet hemocyanin). High concentrations of various erythrocyte types form an agglutinated mass and a large capsule becomes visible to the naked eye (from hours to days) through the transparent tunic (Fig. 3: 1, 2). The inoculation of different erythrocyte types did not show any specificity of the response. Although a similar reaction has been observed by inoculating proteins, the erythrocyte mass entrapped in the tunic matrix stimulates the strongest reaction. Histochemical analysis of capsules identified abundant neutral polysaccharides and proteins in the tunic matrix (Parrinello 1984, unpublished data). Just after the inoculation of any agent type, the tunic matrix is enriched by a massive hemocyte infiltration, mainly granulocytes, CCs, and URGs (Fig. 3: 4), while univacuolated cells release their vacuolar content to form the capsular barrier (Fig. 3: 1, 6). Because of the absence of vessels in the *Ciona* tunic, the recruited activated hemocytes come from the hemolymph in the pharynx vessels and connective tissue, crossing the epidermis (Di Bella and De Leo 2000). As an effect of the LPS-induced activation, URGs and CCs express the CiC3a-1 chemotactic fragment as a product of proteolytic cleavage of CiC3-1 (Pinto et al. 2003).

Granulocytes with small granules degranulate while URGs and vacuolated cells release their contents, encasing the inflamed tissue (Parrinello 1981; Parrinello et al. 1984; Parrinello and Patricolo 1984) (Fig. 3: 9–11). Electron microscopy observations showed granulocytes with large granules and vacuolated cells with various granules and vacuolization features (Parrinello et al. 1990; De Leo et al. 1996, 1997). The identification of the classic raspberry-like MCs was doubtful, conversely vacuoles and granule contents disclosed various features in their electron transparency or density, indicating maturation pathways before the release. In this respect, antimicrobial peptides may be released by URGs and CCs/MCs. In *C. intestinalis*, two putative gene families coding for antimicrobial peptides (Cimam-A and Cipap-A) have been identified (Fedder and Leippe 2008). Two synthetic peptides, representing the cationic core region of AMPs, displayed in vitro potent antibacterial and antifungal activity permeabilizing the target plasma membrane. Immunogold electron microscopy showed that the Cimam-A AMP is contained in the URG granule and among the vacuoles/granules, or at the cytoplasmic periphery of CCs/MCs.

During the inflammatory response challenged by erythrocytes, an extraordinary number of URGs and CC/MCs populate the tunic matrix along with several cells that appear to be advanced inflammatory stages of granular or vacuolated cells. In particular, part or all of the electron-dense content of the URGs becomes electron transparent, and vacuoles differing in size contain both electron-transparent and/or electron-opaque materials. Although direct evidence is lacking, the antimicrobial peptide release could be associated with the degranulation and vacuolization

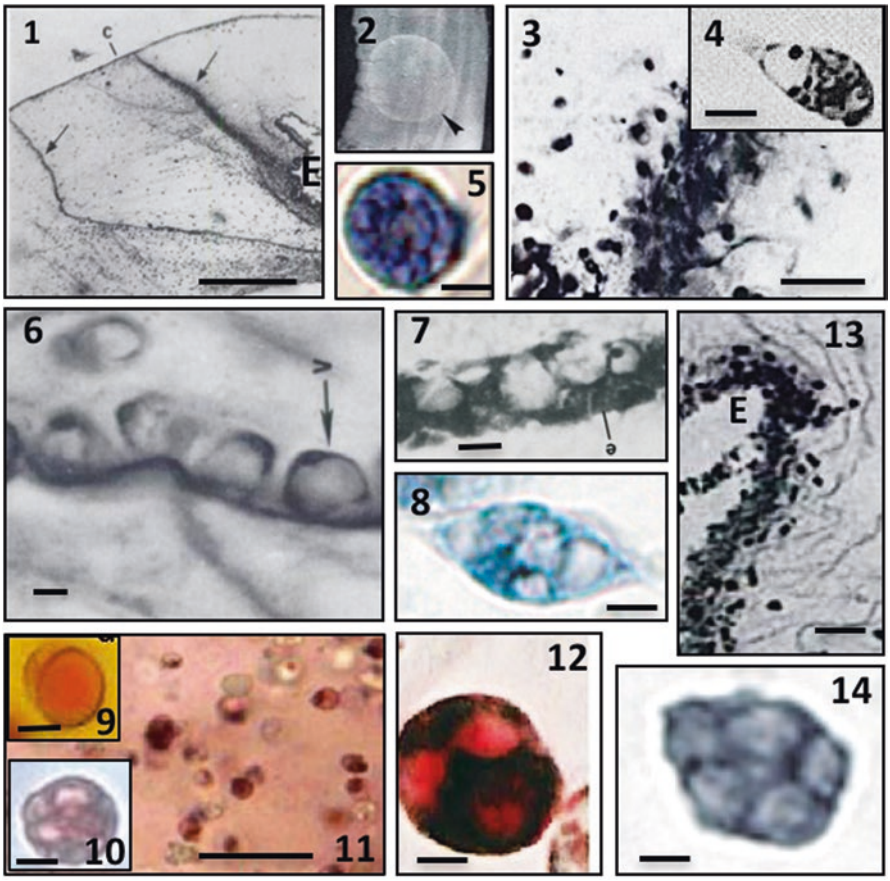

Fig. 3 Encapsulation in the tunic of *Ciona intestinalis* inoculated with erythrocytes. **1** Histological section of the capsule, in which infiltrated hemocytes (granulocytes and vacuolated cells) construct the capsule (Mallory's stain). **2** Outside view of the capsule at 12–24 h post-inoculation. **3** Enlarged view of granulocytes and vacuolated cells. **4** Granulocyte. **5** Compartment/morula cells labeled with anti-CiTNFα-specific antibody. **6** Vacuolated hemocytes (SRCs) that release unstained (Mallory's stain) material (v). **7** Activated epidermis cells, which contribute to the reaction by exocytosis of material from large vacuoles (e) (Mallory's stain). **8** Vacuolated hemocyte labeled with anti-CiPO2 antibody. **9–12** Transition stages of URGs (**9**) and compartment/morula cells (**10**, **11**) containing various PO localizations and levels (DOPA-MBTH cytochemical stain). **13** Granulocytes envelop the erythrocyte mass (E). **14** Compartment/morula cells labeled with anti-CiLgals-a antibody. Bars 5 μm: 4, 5, 6, 9, 10, 12, 14; bar 20 μm: 3, 11; Bar 25 μm: 13; bar 50 μm: 1

processes (Di Bella et al. 2011). These finding further suggest that microbial stimuli could be involved. Also the epidermis was challenged and epithelial cells presented large vacuoles (Fig. 3: 7), the larger size and vacuolization are indicative of intensive releasing activity implementing the capsule structure. In addition, as an effect of proliferative activity, traits of the monolayered epidermis became multilayered,

and activated cells could separate from the tissue to reach the tunic matrix (Di Bella et al. 2005).

Production of collagen could also contribute to capsule structure. CCs scattered in the tunic and contained in the hemolymph, as well as the epidermis cells, express the Ci–type IX collagen 1α-chain, and the gene is promptly upregulated by LPS. This discovery, associated with type I collagen-like identified by cross-reacting antibodies, suggest that a collagen-rich granulation tissue is formed (Vizzini et al. 2001, 2002, 2008).

In specimens from some batches, after a prolonged period (many days) of unresolved inflammation, the capsule degenerates, a gelatinous blister is formed, and a large wound in the reacting area is formed. Although no data have been reported, in this reaction, environmental microbia could affect the wound, further challenging the degenerative response. Findings on tunic cell behaviors and fate were obtained by TEM observations. Numerous cell populations around the irritant agents formed a carpet in the tunic matrix sections, and they displayed various levels of membrane dissolution, while granules and vacuoles were spread in the extracellular matrix (Fig. 3: 1, 11, 13) (Parrinello 1981; De Leo 1992; De Leo et al. 1996, 1997; Parrinello et al. 1990). Both URGs and vacuolated cells undergo cell wounding, and their content could be involved in the capsule degeneration and/or in reparative processes (Fig. 3: 9–12). URGs, from circulating hemolymph of naïve ascidians, are known to release in vitro hemolytic substances, and displayed contact-dependent lytic activity toward various cell targets independently of their origin (sheep, rabbit, human erythrocytes, and tumor cell lines) that could indicate a missing-self recognition mechanism (Parrinello et al. 1995; Parrinello et al. 1996; Parrinello 1996). This activity, also related to sphingomyelin as a target, appeared to be dependent on phospholipase A2 (Arizza et al. 2011). In addition, activated URGs are PO-expressing cells and an excess of cytotoxic products from the enzyme activity could contribute to the tunic lysis and cell necrosis. In *S. plicata* the phenoloxidase-dependent cytotoxic activity of granulocytes with large granules (which may be morula cells) against erythrocytes and a tumor cell line has been demonstrated (Cammarata et al. 1997).

The causes of the necrotic events have not been ascertained; however, loss of membrane integrity and release of intracellular components can challenge and amplify the inflammatory response with the possible involvement of damage signals (Kaczmarek et al. 2013).

Inflammatory Events Characterize Colonial Ascidian Take-Over and Alloreactions

The ability for intraspecific self/nonself recognition is manifest in colony fusion or nonfusion reaction of genetically compatible or incompatible colonies, respectively, that come in contact at their growing edges. These responses occur in several botryllid species. In the Japanese species *Botryllus primigenus* and the cosmopolitan species *B. schlosseri*, the outcome of the contact is genetically controlled by a highly

polymorphic (fusibility/histocompatibility) FuHc locus, the alleles of which are codominantly expressed (see references in Ballarin (2008)). In *B. schlosseri*, this locus is responsible for colony fusion/rejection in a simple Mendelian ratio. The great majority of botryllid colonies in nature are heterozygous and fusion occurs when at least one allele is shared by two contacting colonies that fuse their tunics and anastomize their vascular systems (chimera). In *B. schlosseri* chimerae, one of the partners reabsorbs the other one (losing partner) during one of the blastogenetic cycles (take-over) mimicking the zooid resorption that cyclically occurs in a normal colony. Zooids of a precedent generation undergo apoptosis and are cleared by phagocytosis while buds produce a new generation (Rinkevich 2002, 2005). As mainly shown in *B. primigenus* and *B. schlosseri*, nonfusion of incompatible colonies is initiated by a partial fusion of the tunics followed by a rejection reaction at the facing marginal ampullae (vascular endings) where points of rejection are formed along the contact borders. Active, soluble histocompatibility factors diffuse between both the facing partners.

Cell recruitment by chemotaxis, extravasation, cell degranulation, and induction of cytotoxicity—characteristic mechanisms of the inflammatory reaction—lead to necrosis and colony separation. MCs are the effector cells; they migrate through the ampullar epithelium into the tunic, degranulate, releasing polyphenols, PO, and intermediate products of the enzymatic pathway, NOIs and ROIs, and so cause cytotoxicity.

There are no unique results for genomic characterization of the *B. schlosseri* FuHc locus and for the allorecognition factor candidates (McKitrick and De Tomaso 2010; Voskoboynik et al. 2013a, b; Rinkevich et al. 2012). In any case, none of the genes with potential roles in the allorecognition response are directly related to the MHC gene. Among the candidates, the polymorfic Hsp40-L gene has been included. The Hsp40-L deduced amino acid sequence shows that most of the polymorphisms of the protein occur in the last 100 residues of the C terminus (client-binding domain), which presumably provides specificity for target proteins. Polymorphism of Hsp40-L correlates with fusion/rejection outcomes and a transcript was detected in the epithelial cell layer of the ampullae (Nydam et al. 2013). Several Hsps have been found to function like MHC molecules by binding antigenic peptides, being expressed within cells and on cell surfaces, and mediate T cell activation (see references in Bartl et al. 2003). Evidence from a set of chaperones, besides processing of antigen, indicates that an Hsp might have been a MHC predecessor.

Although the mechanism of recognition remains to be elucidated, potential functional relationships between disparate allorecognition systems have been proposed (Taketa and De Tomaso 2014). From different approaches it emerges that the candidate FuHc gene (also termed "*Botryllus* Histocompatibility Factor" (BHF)) (Voskoboynik et al. 2013a, b) is upregulated in opposing colonies and the transcript associated with the rejection response. The enhanced expression was found in hemolymph, ampullae, buds, and endostyle, as well as in larva and sperm, supporting the histocompatibility-related function, and it is intriguing that separate lineages of somatic and germ line stem cells or pluripotent stem cells differentiate according to the niche in which they land (Laird et al. 2005).

The complexity of the colonial ascidian recognition system is further increased by the mechanism reminiscent of the missing-self effector arm of vertebrate NK cells. Screening for genes differentially expressed during allorecognition in *B. schlosseri* indicates the involvement of the CD94/NKR–like gene (McKitrick and de Tomaso 2010; Taketa and De Tomaso 2014; Voskoboynik et al. 2013a, b), which may be one of multiple genes within the FuHc locus.

The Take-Over

Inflammatory components are involved during the *B. schlosseri* blastogenetic cycle.

Each colony is a clone derived from a founder "oozoid," which is the outcome of the metamorphosis of tadpole-like and swimming larvae. The founding oozoid begins an asexual budding process forming the fertile hermaphrodite colony, and cyclical budding produces blastogenetic generations (Brown et al. 2009). In *B. schlosseri*, zooids connected by a common vasculature have a short life-span (about 1 week), then cease their main physiological activity and die in a massive wave of apoptosis (take-over) (Lauzon et al. 1992). In the colony, the zooids are joined peripherally by "primary buds," which migrate into the vacated region left by the resorbed individuals. Secondary buds are connected to the new zooids, and so multiple individual generations can develop.

The resorbed zooids are selectively removed via phagocytosis in a programmed cell clearance homeostatic process (Cima et al. 2010; Lauzon et al. 2013; Elliot and Ravochandran 2010). The recognition of apoptotic cells involves both phosphatidylserine (eat me signal) and CD36 (Cima et al. 2003). In mammals, CD36 is a membrane glycoprotein present on mononuclear phagocytes that functions as a scavenger receptor and participates in internalization of apoptotic corpses, thus contributing to the inflammatory reaction (Moodley et al. 2003). On the basis of morphological and histoenzymatic properties, two distinct phagocytic cell populations (amoebocytes and macrophage-like cells) are the effector cells (Ballarin and Cima 2005; Voskoboynik et al. 2004). MCs, which are effector cells in allorejection, are not involved. Circulating professional phagocytes producing BsRBL are actively involved; they produce the lectin that interacts with the surface of the apoptotic cells and facilitate the corpses' removal. The rise in the frequency of phagocytes is related to the massive apoptosis and to the enhanced mRNA transcription during the blastogenetic cycle (Ballarin et al. 2013).

Chimerae

Vertebrates do not undergo transplantation reactions naturally. Compound ascidians are the phylogenetically closest group in which transplantation reactions can occur in nature, even if durable and lasting success is very rare (Rinkevich 2002).

Botryllid colonies that share one FuHc allele can fuse, the ampullae undergo anastomosis, and a single chimeric colony is formed (Ballarin 2008). Following a fusion,

one chimeric partner can be eliminated in a process of allogeneic "resorption" in which an inflammatory cell-based rejection is mainly mediated by cytotoxic MCs and phagocytes that remove the remains of the "losing" partner. This reaction is a delayed allogeneic response put into effect to prevent the risk of somatic/germ cell parasitism in genetically nonhomozygote partners (Rinkevich and Weissman 1987).

The resorption is similar to the nonfusion reaction of allogeneic incompatible colonials (see below), including recruitment by chemotaxis, extravasation, cell degranulation, and activation of phagocytic and cytotoxic programs in which the BsRBLs are presumably involved (Corey et al. 2016; Rinkevich and Weissman 1992; Rinkevich 2005; Ballarin and Zaniolo 2007; Ballarin et al. 2013). In *B. schlosseri*, the resorption is due to PO-dependent cytotoxicity by MCs and phagocytosis by HAs and MLCs, which infiltrate the regressing zooids and cause a necrotic lesion in the ampullar epithelium, whereas LLC populations do not appear to be directly involved (Rinkevich et al. 1998).

The allogeneic resorption process and the blastogenetic take-over share the massive phagocytosis, but in a chimera, losing partner elimination is an integrated function of cytotoxic (MCs) and phagocytic programs.

Interestingly, there is a relationship between the blastogenetic cycle and the cytotoxic program. Microinjections for cell transfer showed that the maximal effector response of MCs occurs when these cells are isolated during the developmental period of take-over, indicating a presumptive licensing effect on MCs (Corey et al. 2016). Comparative transcriptome analysis disclosed that pathways induced during take-over are also used in the allogeneic resorption setting. The transcriptome profiles showed a significant overlap at the intersection of the transcription profiles of take-over and chimera resorption. Several genes that express mediators, known to play a role in acute inflammation, were significantly upregulated in both processes, such as the transcription factor NF-KB, a member of the Janus kinase (JAK) family, two members of the TNF receptor–associated signal transduction family, and a member of the interferon regulatory transcription factor family. In both processes, genes usually activated during the inflammatory response are upregulated, including components of the lectin complement activation pathway (C3, CFB), the C3b receptor CR1, MBL-like lectin, MASP1 and MASP2, thrombin factor VIII, factor XI, plasminogen, and kallikrein family proteins. CFB is a component of the alternative pathway that binds C3b; the kallikreins belong to a group of serine proteases involved in plasminogen proteolysis, producing plasmin (Yousef and Diamandis 2003). Furthermore, a network of genes involved in phagocytosis is shared, including tyrosine kinase and phosphatidylserine receptors (Linger et al. 2008). Others genes are related to proteolysis, programmed cell death, and phagocytic clearance.

The upregulation of a member of the IL-17 family (BsIL-17), related to the significant augmentation of the MC cytolytic activity, suggests that during the "take-over," cell death programs render MCs cytotoxic, eliminating the chimeric partner in collaboration with activated phagocytes (Corey et al. 2016). In mammals, macrophages stimulated by IL-17 express many proinflammatory cytokines and chemokines (Zhang et al. 2011). Also, BsRBLs expressed by activated phagocytes are

involved in the take-over, contributing in enhancing MC and phagocyte activities (Ballarin et al. 2013).

According to Corey et al. (2016), the disparate immunogenic pathways induced during take-over may be also used in the setting of allogeneic resorption.

As reported above, self-renewing stem cells with competitive phenotypes may originate cell lineages involved in chimerae rejection. In this respect, the cell death program that render MCs effector cells may be traceable to a distinct cell lineage. Finally, the effector system is reminiscent of missing-self recognition that involves differential expression of cell surface germ line–encoded receptors (Taketa and De Tomaso 2015).

Inflammatory Events in Nonfusion Allogeneic Rejection

Allorecognition, rejection, and the FuHc locus form a complex system that has been well studied for *B. schlosseri.* When allogeneic intraspecific lab-reared colonies are artificially brought in contact at their cut surfaces, the initial fusion of the tunic occurs, soluble histocompatibility factors diffuse, and then allorecognition and consequent irreversible rejection are manifested (Ballarin 2008). Following the initial tunic fusion of the partners, the epithelium permeability of the facing ampullae increases, the MC frequency inside the contacting ampullae is enhanced, and the MCs migrate into the tunic through the epithelium of the ampullar tips and degranulate, releasing their contents, including the PO (Oren et al. 2008). Among botryllids there are differences in the mechanism of nonfusion reaction, and variations in allorejection reactions include the ratio of MCs to total hemocytes, and the levels of PO activity, which varies among the examined species (Shirae and Saito 2000). In *B. scalaris*, phagocytes crowd inside the fused ampullae and stimulate the aggregation of hemocytes into large clusters, which are encapsulated by other phagocytes, plugging the lumen of the fused ampullae, and blood flow is interrupted. In contrast to other botryllid species, granular amoebocytes contain low levels of PO, and no signs of selective recruitment or degranulation of MCs were observed (Shirae et al. 1999).

The missing-self model could involve the BsCD94-1. In the allorejection, a population of granulocytes bears this receptor but no MCs express the receptor. The labeled granulocytes were found inside the vessels, ampullae, and tunic, and, according to the previous findings, a large number of MCs accumulate at the tip of the interacting ampullae, but none of them express BsCD94-1 (Khalturin et al. 2003). The mechanism of allorecognition and the precise roles of the competent cells, including cooperative effects, remain to be defined.

In the *B. schlosseri* nonfusion reaction, melanin, the end product of the PO pathway, is synthesized and accumulated as brownish color dots between the interacting ampullae where a clot is formed by fibers and clumped dead cells (mainly MCs; points of reactions, PORs). In this botryllid the cytotoxicity has been related to the induction of oxidative stress, such as with superoxide dismutase and NOS, while the PO pathway has a role in the formation of the melanized necrotic mass. Finally, the

interacting ampullae are destroyed, the vascular continuity between the two partners is interrupted, and the allogeneic colonies separate.

Upon MC activation, proinflammatory cytokines (IL-1α-like, TNFα-like, IL-17-like) are released, and complement-like components (BsC3, BsBf, ficolins, MASPs) are expressed. The cytokine-like molecules contribute to cell recruitment and modulate cellular events, inducing phagocytes to produce BsRBLs with opsonic activity, while the BsC3 pathway can be opsonic. Molecular and morphological studies substantiate the existence and activation of vertebrate-like blood-based coagulation components that are retained to be homologs to proteolytic coagulation factors (Oren et al. 2007). High similarity with thrombin, coagulation factors V and IX, lower similarity with fibrinogen/fibronectin, and a plasminogen were found. EGF-like and von Willebrand factor–like domains, serine proteases, and protease inhibitors were also identified. These coagulation-related genes are differentially upregulated during the allorejection processes and a role in clot formation has been suggested. The fibrinogen-like is confined to vacuoles within a specific SRC population circulating in the interacting ampullae and in CCs scattered inside the vasculature. Most of these reacting cells appeared to be attached to each other, forming small aggregates (Oren et al. 2008). The expression of a von Willebrand factor was exclusive of a macrophage-like cell population, located mainly within the vasculature and ampullae, thus indicating a systemic response.

In mammals, the hemostatic feature of coagulation is activated immediately upon injury. Platelets bind to collagen exposed by a damaged blood vessel, strengthened by the von Willebrand factor, ensuring the formation of primary hemostatic plugs.

Inflammatory Arm in Solitary Ascidian Tissue Transplantation

In solitary ascidians, naturally occurring fusion or allorejection similar to that described in colonial organisms cannot be hypothesized. Transplantation experiments provide results that are not always homogeneous, probably related to the ascidian species and experimental procedure differences.

In *M. manhattensis*, pharynx auto- and allogeneic grafts do not fuse with host tissues, and both present characters of an inflammatory reaction (Anderson 1971). The implant becomes infiltrated with morula cells that degranulate, causing graft necrosis without any encapsulation.

In *S. plicata*, most first-set integumentary allografts are rejected whereas the majority of autografts remain viable (Raftos et al. 1987a, b, 1988). The late phase of the tunic allograft rejection present cellular and molecular components of the host inflammatory response. Hemocytes actively infiltrate the grafted tissue and aggregate around it, then dense boundaries of extracellular material are formed between the graft and the surrounding tunic matrix; thereby the transplanted tissue appears to be encapsulated. Phagocytes undertake phagocytosis, and degranulating granulocytes may be cytotoxic. Vascular components within allografts are destroyed; the allograft undergoes nonspecific gradual necrosis and it detaches.

Among the cell types observed in first-set and secondary allografts, LLCs are implicated. They invade incompatible allografts and surround the tissue prior to rejection. This influx coincides with the destruction of the grafted tissues, while the other cell types undertake nonspecific responses, presumably stimulated as an inflammatory reaction to the injury. According to Raftos et al. (1987a, b), the allograft response of *S. plicata* exhibits a memory, with a second allograft being lost far more rapidly than the first-set allograft, while a third-party grafting indicates specificity. This graft rejection has been imputed to the role played by the LLCs. However, the identification of specific markers on LLCs could contribute to better clarification of the dynamics and mechanisms of the response, also taking into account the differences among species and the limited role exerted by these cells in inflammation.

In *Styela clava*, in vitro allogeneic cytotoxicity has been reported (Kelly et al. 1992).

In *C. intestinalis* allogeneic tunic transplants, a persistent inflammatory response is characterized by granulocytes and phagocytes, whereas morula cells were not observed (Reddy et al. 1975). Because of the high mortality of the treated ascidian specimens, the secondary immune response was not examined. The direct involvement of LLCs as effector cells, based on morphological observations of the reacting tissues, remains unclear, while the involvement of URGs and the proPO system activation may be suggested.

While more detailed insight is awaited, these findings suggest that independently from the involvement of LLCs as initiators of the allorejection and being eventually responsible for specific recognition, the effector mechanisms seem to be associated with the inflammatory reactions.

Anyway, the responses of the urochordate *S. plicata* to tunic grafts and *B. schlosseri* allorejection confirm the existence of a histocompatibility system and suggest that ascidian innate immunity may have adaptive features (Kvell et al. 2007).

Are Damage Signals Involved in Inflammatory Reactions?

The "danger theory" (Matzinger 1994; Gallucci and Matzinger 2001; Matzinger 2002), by which intracellular molecules (damage-associated molecular patterns (DAMPs)) released by injured tissues can activate the immune system, comes from the observation that a systemic inflammatory response syndrome can be caused by a sterile injury. Many reports on vertebrates, mainly mammals, have identified danger signals and their presumptive receptors, while DAMP molecules have also been associated with tissue repair (Pandolfi et al. 2016; Hirsiger et al. 2012). The real meaning of the theory, in explaining several responses of the innate and adaptive immune systems, has been debated and basic search criteria have been indicated (Kono and Rock 2008).

The initial danger theory was not intended to account for innate immunity that is the natural, nonspecific, nonanticipatory, and nonclonal but germ line–encoded response by invertebrates. Several observations suggest that in invertebrates, the inflammatory response may be triggered by damage done to the host; however, there are difficulties in examining the experimental models, including wild-type ascidian individuals.

Except for lab-reared organisms (maybe botryllids), results exclusively due to endogenous damage signals from a trauma excluding contamination with bacterial products may be doubtful, and in all cases the requested criteria cannot usually be respected.

Therefore, while taking into account these restrictions and waiting for them to be overcome, the evaluation of the danger signals and their effects in ascidian organisms is mainly based on the knowledge of the already identified DAMPs, their presumptive receptors, the signaling pathways involved, and their potential effects, aided by genome and transcriptome analyses.

In addition, according to Pradeu and Cooper (2012), the more appropriate term "damage signal" (not opposed but associated with self/nonself recognition) can be used.

The endogenous stress signals can be released in response to a variety of tissue trauma resulting from environmental temperature, chemicals, xenobiotics, radiation, oxygen deprivation, and food constraints (Bianchi 2007). Molecules released immediately after nonprogrammed cell death or secreted from immune cells without them dying are retained DAMPs that can recruit and activate innate immune cells. Heat shock proteins (Hsps) are perhaps the most diverse DAMPs (Calderwood et al. 2012); they reside in several cellular compartments and can exert functions with regard to immunity and inflammation. In addition to their roles in promoting correct protein folding, Hsps are involved in initiating innate immune responses to cellular stress. These proteins, lacking a signal peptide, are released from damaged cells outside the classical ER–Golgi system and probably bind to one or more of the Toll-like receptors (TLRs) inducing (Hps60/70) the production of NO, TNFα, and IL-12 via TLR4 (Asea et al. 2002; Vabulas et al. 2002).

A conserved Hsp70 chaperone system (with eight members) has been identified in the *Ciona* genome (Wada et al. 2006); they are similar to but simpler than those in humans. In addition, the genome contains 36 genes for J-proteins, a gene for a J-like protein, and three genes for BAG multifunctional (including apoptosis) family proteins. Several reports have disclosed that *C. intestinalis* Hsps are stress inducible, and the degree of the induction was different from gene to gene. Under heat stress (a 10 °C upshift), the transcriptional profiles showed that the expression of six Hsp70 genes, eight J-protein family genes, and two BAG family genes were enhanced. Endoplasmic reticulum (ER) stress (brefeldin A treatment) increases the mRNA levels of four Hsp70 genes and four J-proteins (Wada et al. 2006; Fujikawa et al. 2010). The J-protein and BAG families are major groups of co-chaperones of the HSP70 proteins and are responsible for the functional diversity and modulation of the chaperone system.

In *S. plicata* the Hsp70 gene has been characterized and amplified; the examined deduced amino acid sequence is part of a large clade including *C. intestinalis* Hsp70, and the transcriptional profile has been performed from specimens living in environmentally stressing conditions (Pineda et al. 2012). The results showed that the protein expression increases with higher seasonal stress levels, as did monitoring of stress responses in a salt marsh population exposed to wide temperature and salinity fluctuations. The Hsp70 expression varied over time, with higher stress levels recorded in summer and winter. In addition the interaction between temperature and salinity was significant in enhancing Hsp70 expression, and mortality events were

related to drastic changes in abiotic factors that overwhelmed the observed stress response mechanisms.

Although no data on possible modulation of the innate immune response by environmental factors have been reported, these findings suggest that also in ascidians, Hsp70 may be a DAMP candidate.

Some insights into endogenous DAMPs that challenge the immune system come from the ascidian larva and metamorphosis that may be retained as a model for inflammatory gene expression in the presumable absence of sepsis. The nonfeeding "urodele" larva that characterizes most ascidian species, after a very short swimming period (from a few minutes to many hours, according to the species) in which it becomes "competent" (able to initiate metamorphosis), settles onto the substrate and the papillae used for the adhesion and the tail are rapidly resorbed. According to Cloney (1982), in many ascidians, within a few minutes following onset of the metamorphosis phase, the sensory vesicle is withdrawn, and the axial complex sensory organ and visceral ganglion are destroyed by phagocytes over a period of a few days.

In nature, during the short swimming period in the large seawater volume, bacterial product contamination is unlikely. Nevertheless, several components of the inflammatory machinery appear to be activated. In the competent larva, hemocytes and mesenchymal cells undergo a variety of targeted migrations across the epidermis into the larval tunic, and after settlement, they reach the expanding tunic (Cloney 1982; Cloney and Grimm 1970). Furthermore, cells migrate across the epidermis in both the trunk and the anterior papillary region.

In *Boltenia villosa* (Davidson and Swalla 2002) and *C. intestinalis* (Chambon et al. 2007) precompetent and competent larvae, genes are activated and multiple transcripts for each gene that match proteins involved in immunity have been found. In addition, similarity between two species belonging to different orders can be observed.

In *B. villosa*, two selectins, hemocytin, pentraxin, Bv-LRR (leucine-rich repeat domain), von Willebrand factors (Bv-vWa1), Bv-Sccp2 (selectin 2), and Bv-MASP were first detected in precompetent larvae and were distinctly upregulated during larval competence. Bv-Ccp2 complement factor (complement control domain) is first detected in competent larvae and is then highly upregulated within 1 h after settlement. Bv-Ptx (pentraxin) and Bv-Ccp3 are mainly upregulated just after settlement. A trypsin-like serine protease is also transcribed with differently modulated expression patterns. The majority of the immune-related transcripts display dynamic patterns of temporal expression. Bv-VWa1, Bv-Ptx, and Bv-Ccp3 show distinct peaks of expression followed by declining levels. The expression patterns of immune-related transcripts is modulated in the main development stages. Bv-LRR and Bv-Ccp2 genes are upregulated during larval or postlarval development, whereas Bv-MASP and Bv-Sccp2 show increasing transcription during larval competence and then remain relatively stable. Bv-Ptx is also expressed in the area of the resorbing sensory vesicle, indicating that this lectin could be involved in phagocytosis of the vesicle. In the precompetent larvae, none of the immune-related transcripts were detected, whereas transcripts are often expressed in the mesenchyme of

precompetent larvae, as shown for Bv-Crn (coronin), which is known as actin-binding protein, widespread in eukaryotes, that can be associated with phagocytic activity (Rybakin and Clemen 2005). Bv-Sccp2 and Bv-Ccp3 transcripts were localized in both the epidermis and in nearby hemocytes. Interestingly, although the Bv-HspBP2 (Hsp-70 binding protein) gene expression was used for housekeeping, the expression is not observed in the ampullae and displays a distinctive pattern concentrated around the bases of the ampullae, suggesting that this stress-related gene is modulated during metamorphosis.

According to Davidson and Swalla (2002), akin to the extravasation of leukocytes across endothelia, complement signaling and selectins may be involved in the cell migration and initial adherence crossing the epithelia, while innate immune signaling may coordinate the resorption of larval tissues (Vestweber and Blanks 1999). The expression of the transcripts in the migrating hemocytes, and in the areas of the epidermis across which they migrate, support this hypothesis. In addition, these activities can be associated with differential transcription of putative extracellular matrix–modifying genes such as tenascin-c-like, thrombospondin-like, arylsulfatase-like, and tenascin-x-like. These gene transcriptions indicate that, in parallel with vertebrate processes, their products could be involved in the restructuring and repair of transforming tissues during metamorphosis.

In the *Ciona intestinalis* larva, programmed cell death during metamorphosis correlates with Ci-ERK and Ci-JNK activation, which plays a proapoptotic role. In the tail, this activation precedes the wave of apoptosis, suggesting that the phosphorylated form of Ci-ERK transduces the death-activating signal in tail tissues (Chambon et al. 2002, 2007; Tarallo and Sordino 2004). The screening of genes regulated by Ci-ERK and Ci-JNK identified gene transcripts known to be involved in innate immunity localized in the papillae, nerve cord, visceral ganglion, and sensory vesicle (Chambon et al. 2007). In particular, seven genes (including CiFicolin and Ci-von Willebrand factors) controlled by Ci-JNK could be involved in phagocytosis of the visceral ganglion and sensory organs. The modulation of gene expression such as that of Ci-Sccp (complement control domain) could also enhance cell–cell communication. Complement control proteins regulate the complement system activation and are also involved in directing the complement toward unwanted material such as cell debris (Kirkitadze and Barlow 2001).

In addition, within a few hours from hatching, the mesenchyme cells of *C. intestinalis* competent larvae produce (transcript and protein) CiTNFα (Parrinello et al. 2010). These cells, coming from mesenchyme pockets located in the posterior part of the trunk, migrate along the epidermis to reach the papillae, as well as crossing the epidermis, and populate the matrix of the larval tunic. The cytokine-producing cells are compartment/morula cell-like in accordance with the producing hemocytes identified in the pharynx challenged by LPS. The CiTNFα protein was also found along the sensory vesicles, where it may be involved in the vesicle resorption. The same migrating cell type that reach the same regions in the larval body expresses the CiPO2 gene and produces the protein (Parrinello et al. 2015b). Presumably distinct mesenchyme cell populations may be involved in the expression of CiTNFα and CiPO2. Although in competent larvae the functions of the cytokine and

phenoloxidase are not known, these findings disclose that they are shared with the pharynx and tunic inflammatory responses.

Nitric oxide (NO), a pluripotent physiological messenger produced by oxidation of L-arginine catalyzed by the enzyme NO synthase (NOS), is involved in regression of the tail, which is controlled by caspase-dependent apoptosis. Notably, the NO/cGMP signaling pathway, together with the stress-inducible protein HSP90, have been shown to be involved in the metamorphosis of the ascidians *B. villosa* and *Cnemidocarpa finmarkiensis* (Bishop et al. 2001).

Finally, in *C. intestinalis*, NO regulates tail regression and the NO synthase (NOS) gene is always transcribed, reaching the maximum level in late larvae just before tail resorption (Comes et al. 2007). NO regulates metamorphosis in a dose-dependent manner, since any increase or decrease of NO levels result in delay or acceleration of tail resorption. NOS is expressed in the anterior part of the trunk at the early–middle larval stage and in the posterior part of the sensory vesicle at the middle larva stage.

In conclusion, the activation of immune-related genes during the dramatic body reorganization in metamorphosing tadpole larva may be also dependent on endogenous signals that induce transcription necessary for the resorption of some larval tissues. Of course, the origin of the signals is unclear and it remains to be established whether they come from degenerating tissues, or whether other signals could activate the immune-related genes. Therefore, if all of this is true, the larvae of protochordates may be a reliable model to study the damage theory.

Concluding Remarks

Ascidians, like other invertebrates, are a heterogeneous taxon. Many species develop tadpole larvae. All are marine but are widespread in diverse living environments, and they can have distinct lifestyles (colonial or solitary, benthonic, or pelagic). Their populations are geographically isolated, and all of them have proceeded along their own evolutionary routes. Their habitats are typically laden with infectious agents: viruses, bacteria, fungi, protists, and metazoan parasites, associated with a given environment. Moreover, they are filter-feeding organisms that have elaborate obligatory relationships with harmful agents of which they can be tolerant (symbiosis) or can activate defense reactions. There are certainly important immunological commonalities among the ascidian species, but their diversities may be related to their distinct evolutionary lineages as well as possibly depending on diverse selective pressures; thereby, populational/species differences can be found.

Although recombinatory mechanisms for generating adaptive specific recognition are lacking, they have developed a variety of molecular and cellular pathways leading to self-protection that shows the inflammatory basis of their innate immunity. As established for vertebrates, cellular and molecular mechanisms participate in a protective response, eliminating pathogens and repairing damaged tissues. A complex of genes, phylogenetically and functionally linkable to the mammalian inflammatory reactions (Fig. 2), can be upregulated (PRRs, self-receptors,

cytokine-like molecules, the lectin complement cascade, and signaling and immune regulatory pathways). Inflammatory cells can be recruited to the injured site, responding to chemokines, disclosing recognition capability for self or nonself, and acting as effector cells. Genomic findings and structural analyses of deduced amino acid sequences highlight structural and/or functional conservation of immune-related domains, modules and motifs, and more or less large variations can lead to molecular divergence in taxa that are phylogenetically distant or close, allowing observation of intrapopulational polymorphism (e.g., CiTNFα). With respect to recognition of nonself, both humoral and cell membrane–associated pattern recognition molecules are capable of binding determinants characteristic of broad groups of pathogens. The germ line PRRs show wide glycan-binding interaction that is further amplified through molecular microheterogeneity and subunit oligomerization. In some cases, their functional role appears to be turned for binding proteins. In addition, the germ line immunoglobulin variable region–containing chitin-binding proteins (VCBPs) with regionalized hyperpolymorphism represent a wide nonself recognition mechanism that in part compensates for the absence of the jawed vertebrate somatic rearrangement.

It is of interest that immune-related genes can have a role in developmental and larval stages, presumably producing multifunctional proteins or responding to damage signals (Zucchetti et al. 2008; Parrinello et al. 2010, 2015a, b).

Another interesting subject concerns the missing-self mechanism that is conserved in mammals. Cytotoxic NK lymphocytes contribute to immune responses and homeostasis through germ line–encoded activating and inhibitory receptors that, in concert, regulate their activities. Receptors recognize self MHC class I that acts as inhibitory, becoming activated when its specific ligands are absent or altered. The *B. schlosseri* allorecognition system is reminiscent of the missing-self mechanism in which a BsCD94/NKR–like gene homolog of the vertebrate NK receptor has been reported. In addition, a CD94/NKR–like gene has been also identified in *C. intestinalis*. In both species, this gene is expressed in hemocytes, indicating that, as in mammals, ascidians may be provided with both self and nonself recognition mechanisms. Sensing the self and/or recognizing the nonself lead to immune gene upregulation, and orchestrated responses are performed.

In this respect, the gamete self-incompatibility of the hermaphroditic species displays that another mechanism accompanies the self or nonself recognition; it is intriguing that it is related to the immune system or has evolved independently. In the ascidian fertilization process, gamete allorecognizable receptors from polymorphic loci are evolutionarily selected to interact with alloligands but ignore self-ligands. Some data indicate that immune-related genes may be involved in oocytes and development, i.e., the *C. intestinalis* Hsp70 and the *Botryllus* histocompatibility factor. The CiHsp70 and CiLgals are constitutively expressed during oogenesis in the follicle cells of the oocytes, suggesting involvement in self-sterility (Marino et al. 1998; Parrinello et al. 2018).

In invertebrates, the presence of circulating cells with reliable immunocompetencies, as in jawed vertebrate lymphocytes, has not been shown. In ascidians, lymphocyte-like cells appear to be stem cells and may be retained as a primordial form of vertebrate lymphocytes because they can sense various stimuli including

cytokine-like molecules, enhance their proliferative activity, and differentiate into effector cells. In spite of the large number of studies, a shared hemocyte differentiation lineage has not been reported, but granulocytes with various features and morula cells, which are activated granulocytes, are pivotal in inflammatory reactions. Inflammatory cells may have originated from LLCs and, in distinct species, may assume several functional and morphological features even when they have the same activity. The CC/MCs that express immune-related products appear to be mature/activated hemocytes. Lymphocyte-like cells could be merely retained pluripotent stem cells; conversely, in allograft rejection of solitary ascidian species and nonfusion allorejection in colonial ascidians, their potential immunocompetence has been indicated, and they represent an intriguing topic in studying protochordate immunoevolution.

The necrotic events that occur during the inflammatory reaction mainly in the tunic could be a source of damage signals that presumable intervene, challenging a complex network of responses. Nonfeeding ascidian larvae and the metamorphosing events could be a suitable model to examine the "damage theory." The challenges and the expression of immune-related genes occur in the competent larval stage when direct contact with microorganisms has not yet reasonably happened.

In addition, immune-related genes active during the inflammatory response may exert multifunctional roles. In *C. intestinalis* the genes for CiLgals- and -b are upregulated in oogenesis, and galectins are expressed in accessory cells of the immature oocytes as well as in the ooplasm and nucleus (Parrinello et al. 2018). Furthermore, the CiTNFα-like gene is expressed in swimming larvae, while the transcription of a phenoloxidase gene (CinPO2) is modulated in the development stages and larva.

In conclusion, the extraordinarily sophisticated ascidian inflammatory reaction and gene upregulation may represent the evolutionary pivotal basis of chordate innate immunity, from which the complexity of the vertebrate immune mechanisms originated.

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Part II

From Cephalochordates to Vertebrates

Cephalochordata: Branchiostoma

Zhan Gao and Shicui Zhang

The cephalochordate amphioxus, a basal chordate discovered by Pallas in 1774, is the best available stand-in for the proximate invertebrate ancestor of vertebrates. It has a vertebrate-like body plan, including a notochord, a hollow dorsal neural tube, a post-anal tail, segmented muscle blocks, gill slits, and posterior direction of blood flow in the dorsal vessels and anterior direction of blood flow in the ventral vessels (Kowalevsky 1867; Rähr 1979). However, this animal is much less complex than vertebrates as it has a genome (17% that of the human genome) uncomplicated by extensive genomic duplication (Gibson-Brown et al. 2003; Putnam et al. 2008) and lacks lymphoid organs and free circulating blood cells (Gans et al. 1996; Metchnikoff 1891; Möller and Philpott 1973a, b; Silva et al. 1995). Thus, amphioxus is an ideal model for gaining insights into the origin and evolution of the immune system in vertebrates. Over the past decade, great progress has been made in the study of amphioxus immunity. In this chapter we focus on the recent progress of immunity study in amphioxus.

Immune-Related Organs and Cells

In mammals, the immune system comprises organs and tissues that can be functionally divided into two categories: the primary lymphoid organs and the secondary lymphoid organs. The former are bone marrow and thymus, and the latter include lymph nodes, spleen, and mucosa-associated lymphoid tissue such as gut-associated

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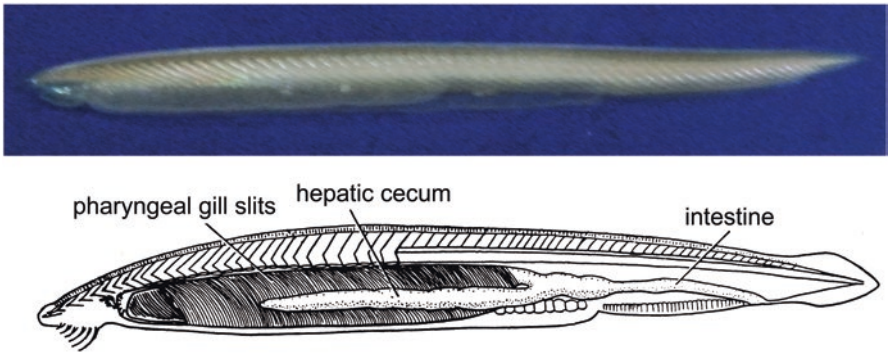

Fig. 1 Amphioxus. The digestive system, including the pharyngeal gill slits, hepatic cecum, and intestine, in amphioxus is regarded as the major defense line. Top: photograph of *Branchiostoma japonicum*; below: diagram of the digestive system of amphioxus

lymphoid tissue and bronchus-associated lymphoid tissue. Emerging evidence also suggests that the liver in all vertebrates is an immunological organ that plays an important role in innate immunity (Gao et al. 2008; Markiewski et al. 2006; Racanelli and Rehermann 2006), especially in acute-phase response (APR). In amphioxus, the digestive system—including the pharyngeal gill slits, hepatic cecum (also known as the digestive diverticulum), and intestine—is regarded as the major line of defense (Fig. 1). The pharynx is an elongated tube with side walls perforated by nearly 200 oblique vertical slits, and this number increases as the animal gets older. The slits are separated by bars containing skeletal rods and further subdivision is provided by cross-bars. The function of the pharyngeal gill slits is to extract food particles consisting of microscopic organisms from the water current. Because of such a feeding strategy involving siphoning seawater, the gill slits are in continuous contact with both dietary and seawater microbiota, and should thus act as the first line of defense immunity in amphioxus. In jawed vertebrates, T cells develop in specialized thymopoietic tissue located in the pharynx (Boehm et al. 2012). In agnatha lamprey larvae, T-like lymphocyte bearing type A variable lymphocyte receptor (VLR) develops in the thymus-like lympho-epithelial structure (called thymoid) in the tip of the gill filament and the neighboring secondary lamellae (Bajoghli et al. 2009). Interestingly, putative lymphocyte-like cells have been reported as being seen in the pharynx in amphioxus (Huang et al. 2007). Therefore, further studies on whether lymphocyte-like cells indeed develop from the pharynx will contribute to a description of the thymus-equivalent organ in the basal chordate and add new evidence to aid understanding of the immune functions of amphioxus pharynx.

Among the vertebrate secondary lymphoid tissues, the gut epithelium is a structure comprising a single layer of cells and is physiologically thought to possess fully developed immunological capabilities (Weitman et al. 2013). The structure of amphioxus gut shares some similarities with the vertebrate gut. They both have absorptive cells and goblet cells in the mucous membrane that contribute to

digestion and absorption of nutrients. Moreover, a large blood vessel lined with endothelial cells containing many lysosomes has been observed in amphioxus gut (Han et al. 2010). Active phagocytosis and secretion of immune effectors in the epithelial cells of amphioxus gut indicate that, in addition to the digestive and absorptive function, the gut also plays a role in immune defense (Han et al. 2010; Huang et al. 2007).

The liver is the major source of complement components, including factor B (Bf)/C2 family proteins in vertebrates, and participates in the immune response as an important line of host defense against invading pathogens (Gao et al. 2008; Morgan and Gasque 1997). Amphioxus has a hepatic cecum, the pouch that protrudes forward as an out-pocketing of the digestive tube and extends along the right side of the posterior part of the pharynx, which has long been considered to be the precursor of the vertebrate liver (Müller 1844; Welsch 1975). He et al. (2008) mapped the cellular distribution of Bf/C2 in the hepatic cecum in amphioxus, supporting the equivalence of the hepatic cecum to the vertebrate liver. Also, the presence of Bf/C2 in the humoral fluids of amphioxus, as evidenced by immunoblotting, suggests that the protein synthesized in the hepatic cecum can be secreted into the blood, circulating via the bloodstream throughout the body. In addition, by investigating the expression patterns of many vertebrate liver-specific genes in amphioxus, Wang and Zhang (2011) proved that the hepatic cecum in amphioxus is the "prehepatic" organ homologous to vertebrate liver and plays an important role in APR, indicating that, like the vertebrate liver, the hepatic cecum in amphioxus is also an immunological organ. Thus, amphioxus gut, including the cecum, should act as both a physical barrier and a site of continuous immunological interaction, similar to the liver and gut-associated lymphoid tissue found in vertebrates such as fish and mammals. Studies focusing on the complex symbiotic interactions in gut tissue of amphioxus will certainly shed more light on the basic biology of gut immune homeostasis and may reveal basic mechanisms of dysfunctional gut immunity.

In vertebrates, innate immunity depends chiefly on a group of proteins and phagocytic cells that recognize the features of pathogens and are rapidly activated for defense against invaders. In mammalian species, monocytes, macrophages, neutrophils, tissue dendritic cells, and mast cells are important innate immune cells in defense against pathogens (Rabinovitch 1995). Specialized phagocytes (hemocytes) play an indispensable role in the innate immune systems in lower invertebrates, such as *Drosophila* (Lemaitre and Hoffmann 2007) and *Ciona intestinalis* (Rowley 1982). *Drosophila* hemocytes are considered to be the functional equivalent of the monocytes/macrophages in vertebrates (Lemaitre and Hoffmann 2007). Rhodes et al. (1982) claimed to have observed a small number of free and fixed phagocytes in the coelomic cavity of amphioxus. These cells typically had a cleft nucleus, lysosome-like bodies, and, often, cilia and rootlet structures. However, other researchers failed to find phagocytes using either light or electron microscopy in amphioxus (Silva et al. 1995). Thus, the existence of phagocytes in amphioxus remained controversial until the identification of macrophage-like cells in amphioxus gut mucosa (Han et al. 2010). With bacterial infection, these macrophage-like cells became attached to, and encapsulated, the bacteria via phagocytosis to form a phagosome, which fused with a

lysosome to form a phagolysosome-like structure, leading to degradation of the bacteria. Moreover, the identification of two macrophage migration inhibitory factors (MIFs), important cytokines involved in the regulation of macrophage function, provides further molecular evidence for the existence of macrophage-like cells in amphioxus (Du et al. 2004, 2006). In addition to macrophage-like cells, some monocyte-like cells containing the typical large reniform nucleus have also been observed in gut mucosa of amphioxus (Yuan et al. 2015a).

In jawed vertebrates, B and T lymphocytes are responsible for the specificity of adaptive immunity responses. The former are derived from the bone marrow and are responsible for humoral immunity, whereas the latter mature in the thymus and are primarily responsible for cell-mediated immunity. In lampreys, primitive jawless vertebrates, other types of lymphocytes that clonally express somatically diversified antigen receptors called VLRs are responsible for adaptive immunity responses (Guo et al. 2009; Mayer et al. 2002). These cells are shown to be akin to the T and B cells of jawed vertebrates. Notably, a cluster of cells with morphological similarities to vertebrate lymphocytes, containing large, dark staining nuclei and a thin rim of cytoplasm, have been identified in amphioxus pharynx (Huang et al. 2007). Electronic microscopy reveals the detailed structure of these cells, which contain a large nucleus with a peripheral rim of heterochromatin adjacent to the nuclear envelope, surrounded by a thin layer of cytoplasm. Moreover, the sizes of these lymphocyte-like cells increased remarkably when amphioxus was challenged by pathogenic bacteria (Huang et al. 2007).

Aside from the morphological changes, paralogs/orthologs implicated in the process of lymphocyte activation, regulation, and maturation, such as the Ikaros family zinc finger protein 1 (IKZF1, also known as Ikaros/LYF-1), early B cell factor 1 (EBF1), and ETS (E-twenty six) family transcription factors, have also been identified in amphioxus (Huang et al. 2008; Yu et al. 2005). All these transcription factors play essential roles in the activation and differentiation of vertebrate lymphoid cells. Moreover, research on the genome reveals that amphioxus possesses several molecules involved in antigen presentation, such as proteasome (proteasome, macropain) subunit β-type (PSMB) 7/10, PSMB5/8, PSMB6/9, and gamma-interferon (IFN)-induced lysosomal thiol reductase (GILT) (Huang et al. 2008; Liu et al. 2007; Yu et al. 2005). Amphioxus also has almost all the molecular repertoires involved in the downstream cascades of protein tyrosine kinases, including the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and protein kinase C pathways (Bertrand et al. 2009). In addition, a death receptor (DR)-mediated extrinsic apoptosis pathway, which was long considered to be vertebrate specific and to have emerged along with adaptive immunity (Micheau and Tschopp 2003; Collette et al. 2003), has been identified in amphioxus (Yuan et al. 2010a). Collectively, these findings suggest the possible occurrence of hematopoiesis and lymphocyte development pathways in cephalochordates.

Innate Immune Recognition and Signaling Network

Many innate immune-related gene families have undergone lineage-specific expansion in amphioxus. The greatest expansion took place in pattern recognition receptors (PRRs), which include at least 39 Toll-like receptors (TLRs), 73 nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs), 144 scavenger receptors (SRs), and 717 C-type lectin-like receptors (CTLR) in the amphioxus *Branchiostoma floridae* (Huang et al. 2008; Yu et al. 2007a). In addition, the amphioxus genome has 57 Toll/interleukin (IL)-1 receptor (TIR) adaptor-like models, 17 tumor necrosis factor (TNF) receptor (TNFR)-associated factor (TRAF) models (Yuan et al. 2009b), 41 initiator caspase models, and 332 death-fold domain (DFD)-containing models (Huang et al. 2008), suggesting a large intracellular intermediate signal transducing network not seen in other genomes.

Toll-Like Receptor Signaling Pathway

TLRs are the first PRRs that have been identified and are conserved throughout the entire animal kingdom, and have important immune functions (Roach et al. 2005). TLRs are type I membrane proteins, which can be separated into two structural types: the vertebrate-type (V-type) and the protostome-type (P-type) (Hibino et al. 2006). A typical V-type TLR consists of an LRRNT–(LRR)n–LRRCT (leucine rich repeat [LRR] N-terminal–(LRR)n–LRR C-terminal) ectodomain for ligand recognition, a transmembrane region, and a cytoplasmic TIR domain for signaling, whereas a typical P-type TLR differs from a V-type TLR in that its (LRR)n stretch is divided by an additional LRRCT–LRRNT motif (Huang et al. 2008; Yuan et al. 2009a). All vertebrate TLRs are V-type, while most protostome TLRs are P-type. Amphioxus has an expanded V-type TLR family (at least 36 TLRs), 12 P-type TLRs, and 40 TIR-containing adaptors (Huang et al. 2008).

The amphioxus TLR system has been functionally characterized. After engagement of TLRs by their cognate ligands, two pathways can be triggered in amphioxus: the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway and the TLR adaptor molecule (TICAM)-dependent pathways (Yang et al. 2011; Yuan et al. 2009a) (Fig. 2). Amphioxus MyD88 can interact with TLR1 and mediate the activation of nuclear factor (NF)-κB through its death domain (DD) and middle region. This MyD88-dependent pathway can be negatively regulated by TRAF2a (Yuan et al. 2009b) and sterile alpha- and armadillo-motif-containing protein (SARM) (Yuan et al. 2010b). In contrast, amphioxus TICAM specifically mediates the activation of NF-κB by interacting with TNFR-interacting serine–threonine kinase 1b (receptor interacting protein [RIP] 1b). This TICAM-dependent activation of NF-κB is negatively regulated by RIP1a (Li et al. 2011), TRAF2a, and SARM (Yuan et al. 2009b; Yang et al. 2011). Amphioxus RIP1a can compete with RIP1b for the interaction with TICAM, while the TRAF2a and SARM can directly bind with TICAM, leading to its inability to recruit downstream molecules. Since a homolog of TICAM could not been identified in species lower than amphioxus, the

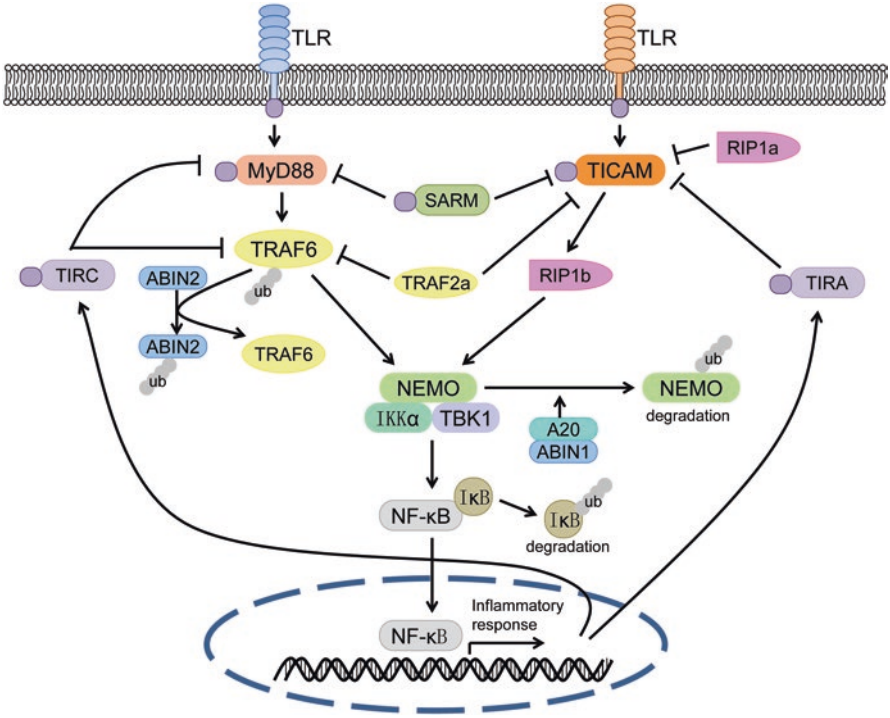

Fig. 2 Putative Toll-like receptor (TLR) signaling network in amphioxus. After engagement of TLRs by their cognate ligands, the myeloid differentiation primary response gene 88 (MyD88)-dependent and TLR adaptor molecule (TICAM)-dependent pathways are triggered. When MyD88 is recruited to specific TLRs, it associates with tumor necrosis factor receptor-associated factor (TRAF) 6, resulting in activation of IκB-related kinases (IKKs) and nuclear factor (NF)-κBs. This pathway can be inhibited by TRAF2a, sterile alpha- and armadillo-motif-containing protein (SARM), and TIR adaptor C (TIRC). In contrast, when TICAM is recruited to specific TLRs, it associates with receptor interacting protein (RIP) 1b to activate NF-κBs. This TICAM-dependent pathway can be negatively regulated by RIP1a, TRAF2a, SARM, and Toll/interleukin (IL)-1 receptor (TIR) adaptor A (TIRA). The expressions of TIRA and TIRC are both controlled by NF-κB, causing an effective feedback regulation of amphioxus NF-κB signaling. Moreover, amphioxus A20 binding inhibitor of NF-κB (ABIN) 2 can compete with TRAF6 for the ubiquitin chains, while ABIN1 can physically link A20 to NF-κB essential modifier (NEMO) to facilitate ubiquitination of NEMO, leading to the inhibition of NF-κBs

TICAM-dependent signaling in amphioxus is thus thought to be the most primitive MyD88-independent pathway during evolution. Recently, ubiquitination has also been shown to be a conserved strategy in regulating amphioxus NF-κB activation (Yuan et al. 2014a). Amphioxus A20 binding inhibitor of NF-κB (ABIN) 2 can compete with TRAF6 for the K63-linked ubiquitin chains, whereas ABIN1 physically links A20 to NF-κB essential modifier (NEMO) and facilitates the A20-mediated K48-linked polyubiquitination and degradation of NEMO, leading to the inhibition of NF-κB (Yuan et al. 2014a). Besides MyD88, TICAM, and SARM,

which have significant homology with their vertebrate counterparts, many TIR-bearing genes throughout the genome seem to be amphioxus-specific, such as TIR adaptors bearing both the TIR domain and caspase activation and recruitment domain (CARD) (Huang et al. 2011b; Huang et al. 2008). These TIR adaptors with unidentified domain architectures may serve as regulators in amphioxus TLR signaling (Peng et al. 2015).

Nucleotide Oligomerization and Binding Domain-Like Receptor Signaling Pathway

NLRs are a family of intracellular sensors that have important functions in apoptosis, inflammation, and intracellular innate immunity (Motta et al. 2015). They consist of a central NACHT domain for oligomerization, a C-terminal LRR region for ligand recognition, and N-terminal domains for signaling, such as CARD, PYRIN, or baculovirus inhibitor of apoptosis repeat (BIR) domain (Saleh 2011). The *Drosophila* and *Caenorhabditis elegans* innate immune systems seem to function without NLRs, whereas early diverging metazoans and some fish have large NLR repertoires. The sea urchin genome possesses 203 NLRs, the majority of which consist of an N-terminal DD, a central NOD domain, a nucleotide oligomerization and activation domain, and a C-terminal LRR region (Hibino et al. 2006). The amphioxus genome has at least 92 NLR genes (Huang et al. 2008). Various N-terminal domains can be found in amphioxus NLRs, such as death effector domain (DED), CARD, and CARD + TIR, as well as multiple DDs and TIR + DDs.

Although homologs of caspase and RIP adapter with DD (CRADD), RIP kinase 2 (RIPK2), and ASC are present in apoptosis-associated speck-like protein containing a caspase recruitment domain, the IL-1 proteins and IL-1β-converting enzyme (ICE)-like caspases are absent. The signaling of vertebrate NOD/NALP proteins requires interactions of their CARD/PYRIN domains with downstream adapters such as CRADD, ASC, and RIPK2 for the activation of NF-κB, as well as the processing of IL-1 proteins by ICE-like caspases; therefore, the presence of different kinds of N-terminal domains and the absence of several key proteins involved in downstream signaling suggest that amphioxus NLR signaling may be quite different from that of vertebrates.

Antiviral Mechanisms

Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), including RIG-I, melanoma differentiation-associated protein 5 (MDA5), and LGP2 (Laboratory of Genetics and Physiology 2), are DExD/H box RNA helicases that recognize cytoplasmic pathogen-associated molecular patterns (PAMPs) coming from viral genomes. RLRs have similar structures: an N-terminal CARD, a central DExD/H box RNA helicase domain, and a C-terminal repressor domain. LGP2 lacks the CARDs and was thought to be a regulator of RLR signaling (Beutler et al. 2007; Satoh et al. 2010). After ligand engagement, RLRs recruit downstream adaptor molecule

mitochondrial antiviral signaling (MAVS; also called IPS-1, VISA, and Cardif) to trigger signaling cascades (Kawai et al. 2005; Seth et al. 2005; Xu et al. 2005). MAVS is composed of an N-terminal CARD domain, through which it interacts with a RIG-I or MDA5 CARD domain. MAVS then recruits the TRAF2/3/6 and TANK (TRAF family member associated NF-κB activator) complex and finally activates the IFN regulatory factor (IRF) 3/IRF7 transcription factors to induce expression of type-1 IFN and other pro-inflammatory factors (Takaoka and Taniguchi 2008).

Several RLRs are found to be present in amphioxus. However, unlike vertebrate RLRs, which use a CARD–CARD domain structure for receptor oligomerization and interaction with downstream adaptor MAVS for signal transduction, amphioxus RLRs have other types of domain combinations, such as CARD + TIR domains, DD, and DED, suggesting that the activation and association of amphioxus RLRs with downstream adaptors function via another type of domain–domain interaction (Huang et al. 2008). Among them, an LGP2 homolog (BjLGP2) and BjLGP2-triggered signaling pathway have been identified in the amphioxus *B. japonicum* (Liu et al. 2015b). BjLGP2 is structurally characterized by the presence of an N-terminal DExD/H box RNA helicase domain with ATP-binding motif and ATPase motif, an intermediate HELICc (Helicase superfamily C-terminal domain containing protein) domain with RNA-binding motif and a C-terminal RD domain with an RNA binding loop and two Zn^{2+}-binding motifs. It is predominantly expressed in the hepatic cecum and hind-gut, and is upregulated following challenge with poly(I:C). BjLGP2 can enhance the expression of IFN and IFN-inducible genes in flounder gill (FG) cells upon poly(I:C) challenge. It can also significantly induce the expression of the antiviral genes *ifn-i* and *Mx* as well as the signal transduction relevant genes MAVS, NF-κB, and IRF-3 in FG cells upon lymphocystis disease virus (LCDV) challenge. Moreover, BjLGP2 inhibits the replication of LCDV in FG cells and the gene transcription of Singapore grouper iridovirus in grouper spleen cells. As the downstream elements MAVS-like protein, TANK-binding kinase (TBK) 1, IKKi, IRF3, and IRF7 have all been found to be present in amphioxus (Liu et al. 2015b), amphioxus LGP2 is thus highly likely to play an antiviral role via a RLRs signal transduction pathway similar to that of the signaling pathway of vertebrate RLRs (Fig. 3).

RNA interference (RNAi) machinery has been exploited for antiviral defense in both *Drosophila* and *C. elegans* (Mussabekova et al. 2017). Some essential elements of RNAi machinery are also found in amphioxus, including DICER, AGO1 (Argonaute), and *Drosophila* R2D2-like molecules. This suggests that, as in *Drosophila* and *C. elegans*, RNAi may also play a role in amphioxus antiviral immunity (Yuan et al. 2014b).

Transcription Factors and Their Direct Upstream Kinases

Most transcription factors and their direct upstream kinases are present in amphioxus, including NF-κB, nuclear factor of activated T cells (NFAT), IRF, Ikaros, PU.1/Spi, IκB-related kinase (IKK)/TBK, MAPK/c-Jun N-terminal kinase (JNK),

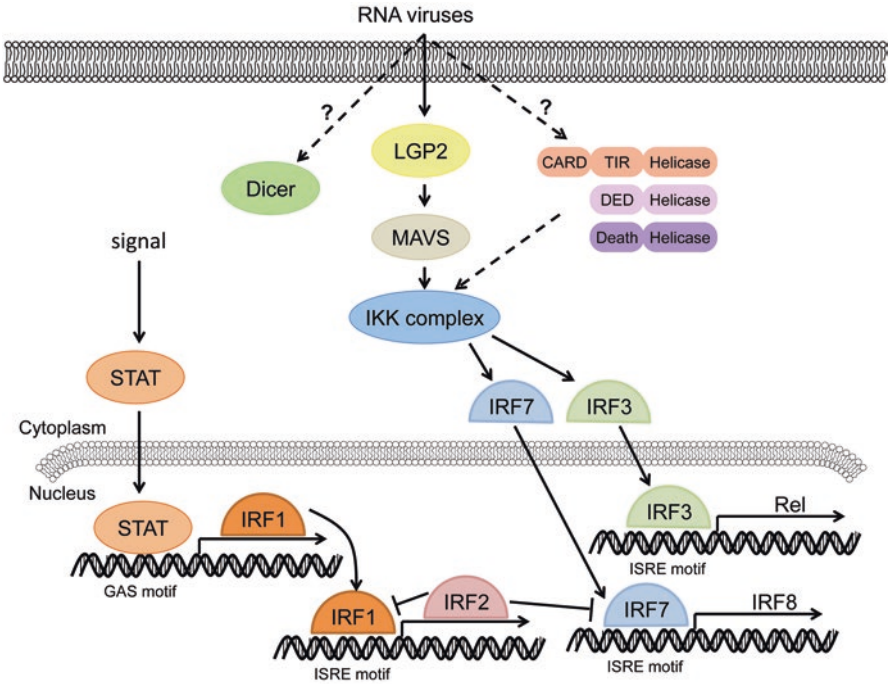

Fig. 3 Putative antiviral mechanism in amphioxus. RNA interference (RNAi)-related proteins (e.g., DICER) and several retinoic acid-inducible gene-I-like receptor (RLR)-like proteins (e.g., LGP2 [Laboratory of Genetics and Physiology 2]) are both found to be present in amphioxus. When LGP2 recognize double-stranded RNA (dsRNA) from viruses, it recruits adaptor mitochondrial antiviral signaling (MAVS), which can trigger signal transduction to nucleus through the TBK1 (TANK-binding kinase 1)/IKKi and interferon regulatory factor (IRF) 3/IRF7 pathway. Amphioxus contains nine IRF members, which constitute an archaic IRF signaling framework. In this framework, IRF2, IRF8, and Rel are identified as target genes of IRF1, IRF7, and IRF3, respectively, suggesting a dynamic feedback regulation among amphioxus IRF and nuclear factor (NF)-κB. Moreover, the IRF1 can be activated by signal transducers and activators of transcription (STATs). The putative pathways mediated by the RNA helicase domain-containing adaptors with previously unidentified protein architectures require further confirmations. A solid arrow indicates that the pathway has experimental evidence, a dashed arrow indicates no experimental support, and a question mark indicates that the existence of the item is not verified. TANK tumor necrosis factor receptor-associated factor family member associated nuclear factor-κB activator

and activator protein 1 (AP-1) (Huang et al. 2008). These transcription factors and kinases have not undergone expansion, and remain similar to those in vertebrates in both structure and number.

Nuclear Factor-κB Family Members

Fruit flies and humans have three and five NF-κB homologs, respectively (Song et al. 2012; Yuan et al. 2013). In contrast, amphioxus possesses two NF-κB homologs, Rel and p105. Upon activation, the p105 is cleaved into the mature form p58 in

amphioxus, which further forms homodimers or heterodimers with Rel to create mature NF-κB complexes for target gene transcription, as in fruit flies and humans (Yuan et al. 2013).

Interferon Regulatory Factor Family

Amphioxus contains nine IRF members (Yuan et al. 2015b). When challenged by specific pathogens, signal transducers and activators of transcription (STATs) are activated to induce the transcription activator IRF1, which then activates transcription of the transcription repressor IRF2. When IRF3 and IRF7 were activated by TBK1, IRF7 binds to the promoter of IRF8 whereas IRF3 binds to the promoter of Rel, leading to the subsequent expression of IRF8 and Rel for the application of IRF3/7-based responses to immune demands (Fig. 3).

Cytokines and Their Receptors

Cytokines have different names based on their sources, such as lymphokines, secreted by lymphocytes, and monokines, secreted by monocytes/macrophages; some cytokines are referred to as ILs, and others are known as chemokines. The most well-known are TNF-α, IL-1, and type-1 IFNs. TNF-α is involved in systemic inflammation and is a member of a group of cytokines that stimulate APR. It is produced mainly by activated macrophages. The term IL describes a variety of polypeptides that act specifically as mediators between leukocytes, whereas type-1 IFN can interfere with virus replication and also link innate immunity with adaptive immunity. Although homologs for IL-1 receptors, TNFs, IL-17, and macrophage MIFs are present in amphioxus genome (Huang et al. 2008), most of the vertebrate cytokines are absent, including most ILs, all IFNs, chemokines, colony-stimulating factors (CSFs), and their cognate receptors. The reason for this can be either a true absence of these genes or the inability of similarity searches to identify such fast-evolving genes.

It is interesting that although IFNs are lacking in amphioxus, it possesses some IFN-stimulated genes involved in the antiviral defense, such as viperin (Lei et al. 2015). Viperin (*v*irus *i*nhibitory *p*rotein, *e*ndoplasmic *r*eticulum-associated, *in*terferon-inducible) is a cellular protein which appears to use a variety of different antiviral mechanisms, from interacting directly with viral and host proteins essential for viral replication to interaction with host organelles such as the endoplasmic reticulum (ER), lipid droplets, and mitochondria (Helbig and Beard 2014). Amphioxus viperin has features in common with those of vertebrate viperins, including the presence of the SAM (S-adenosyl-L-methionine) superfamily domain with the characteristic CNYKCGFC motif, syntenic conservation, and predicted 3-dimensional structure. The cells transfected with amphioxus *viperin* are able to kill LCDV or inhibit its propagation, and co-incubation of recombinant viperin with white spot syndrome virus (WSSV) markedly attenuates its infectivity (Lei et al. 2015). These suggest that amphioxus viperin, like that of vertebrates, is also capable of promoting resistance against viral infection. Vertebrate viperin could be induced

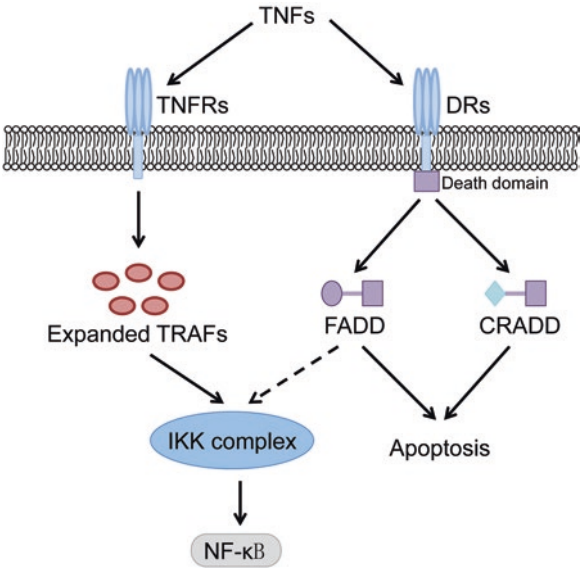

Fig. 4 Putative tumor necrosis factor (TNF) system in amphioxus. Transduction of TNF signals requires the interaction between TNF receptor (TNFR) cytoplasmic tails (with or without death domain) and the downstream adaptors. The TNFRs with death domain (death receptors [DRs]) recruit adaptor Fas-associated death domain (FADD) or caspase and receptor interacting protein adapter with death domain (CRADD), which can trigger caspase-dependent apoptosis. While the TNFRs without death domain recruit adaptor TNFR-associated factors (TRAFs), an expanded family containing 17 homologs in amphioxus results in activation of nuclear factor (NF)-κB. In addition, amphioxus FADD1 is also capable of activating NF-κB

in either an IFN-dependent or IFN-independent pathway. Given the lack of IFNs in amphioxus, it is thus proposed that amphioxus viperin may only be induced in an IFN-independent pathway.

Tumor Necrosis Factor System

In vertebrates, the TNF system mediates activation, proliferation, differentiation, and homeostasis of immunocytes, participates in the development and maintenance of long-lived or evanescent lymphoid tissues, and implements the clearance of cancerous, aged, and diseased cells (Hehlgans and Pfeffer 2005). There are approximately 20 TNF and 20–30 TNFR genes in a given species of vertebrates. In contrast, only one TNF (called *eiger*) and one TNFR have been identified in *Drosophila melanogaster*, and four TNFs and seven TNFRs in sea urchin (Robertson et al. 2006). However, the amphioxus genome contains 21 TNF and 31 TNFR genes, which is comparable to vertebrates (Fig. 4) (Huang et al. 2008). Amphioxus TNF proteins can be divided into two major lineages: the TNF-related apoptosis inducing ligand (TRAIL)/Fas ligand (FASLG)-related and the Ectodysplasin-A (EDA)/Eiger-related lineages. The TRAIL/FASLG-related lineage includes ten TRAIL genes and three FASLG/TNFA-like genes, whereas the EDA/Eiger-related lineage has 11 TNF genes homologous to insect Eiger, and vertebrate EDA and TNFSF13.

The TRAIL-related lineage was first created by tandem duplication from the ancient EDA/Eiger-related lineage and subsequently underwent substantial function divergence before whole-genome duplications.

TNFR can be divided into two types, i.e., TNFR with (DR) and without a cytoplasmic DD (TNFR-noDD). DRs can activate caspase-dependent apoptosis, while TNFR-noDDs can act as DR antagonists or activate NF-κB and JNK pathways. The amphioxus genome contains 14 DRs and 22 TNFR-noDDs (Huang et al. 2008). Sequence analysis of the DD indicates that only two amphioxus DRs show some similarity to the vertebrate DR genes *NGFR* and *EDAR*. The rest of the amphioxus DRs are more similar to each other than to vertebrate DRs, suggesting that most amphioxus DRs have undergone a lineage specific expansion. As for amphioxus TNFR-noDDs, their TNFR repeats are too divergent to be used for reliable phylogenetic analysis.

Transduction of TNF signals requires the interaction between TNFR cytoplasmic tails and the downstream adapters. Humans have six TRAFs and four DFD adapters (*FADD*, *TRADD*, *CRADD*, and *EDARADD*) for this purpose, while the sea urchin draft genome contains only one *FADD*, one *CRADD*, and four TRAF adapters (Robertson et al. 2006). The amphioxus genome contains a set of homologs of *FADD*, *CRADD*, and *EDARADD*, a family of 17 TRAFs, and a total of 332 DFD-containing models (Huang et al. 2008). If a substantial proportion of these genes participates in the TNF system, it would represent the most complicated TNF signaling network ever known in the animal kingdom.

The TRAF family can be classified into three major groups: two ancestral groups, TRAF4 and 6, and the newly evolved group comprising TRAF1, 2, 3, and 5 (Grech et al. 2000). All the TRAF proteins share a similar structure but differ from one another in physiological function. In amphioxus, the TRAF1/2 and 3 lineages are clearly expanded by lineage-specific duplication and rearrangement, whereas TRAF4 and 6 remain relatively stable in the genome and protein structure (Yuan et al. 2009b). Amphioxus TRAF1/2 and 3 molecules display various expression patterns in response to microbial infection, and some of them can attenuate the NF-κB activation mediated by human TRAF2 and 6. Amphioxus TRAF4 has two unique functions: activation of NF-κB pathway and involvement in somite formation. Amphioxus TRAF6 is conserved in activating the NF-κB pathway for antibacterial defense, but the mechanism appears different from that in humans because the amphioxus TRAF domain cannot inhibit but instead activates the NF-κB at a level comparable to that observed with full-length TRAF6, contrasting to the dominantly negative role of the human TRAF domain (Yuan et al. 2009b).

Migration Inhibitory Factor

Macrophage MIF was first described as a cytokine some 50 years ago (Bloom and Bennett 1966), and emerged in mammals as a pleiotropic protein with pro-inflammatory, chemotactic, and growth-promoting activities. Of less importance in mammals is an intrinsic but non-physiologic enzymatic activity that points to MIF's evolution from an ancient defense molecule (Sparkes et al. 2017). Two MIF complementary DNA (cDNA) clones have been isolated from amphioxus (Du

et al. 2004). The amphioxus *MIF* gene is present as multi-copies per haploid genome, which is very unusual compared with *MIF* gene in vertebrates given the known genome duplication theory. Amphioxus MIF has both tantomerase and redox activities, but fails to utilize reduced glutathione (GSH) to reduce insulin instead of dithiothreitol (DTT), strikingly different from MIF in mammalian species (Du et al. 2006).

Expansion and Reshuffling of Death-Fold Domains

The signal transduction of PRRs and cytokine receptors requires a cytosolic protein interaction network composed of various adapters or intermediate transducers. DFDs, including DD, CARD and DED, are basic building blocks for homotypic interactions. They are widely present in NLRs, RLRs, DRs, apoptotic proteins, and other signal transducers, and broadly participate in TLR/IL-1 receptor (IL-1R), NLR, TNF, RLR, and apoptosis pathways, as well as cross-talk among them. The human genome contains about 60 DFD genes, while the sea urchin genome contains 116 DFD genes (Robertson et al. 2006). Amphioxus genome contains 332 DFD-containing models (NLRs and DRs excluded). Human DFD proteins consist of 16 distinct architectures, of which amphioxus has at least 14. In contrast, the amphioxus DFD repertoire has at least 40 domain combinations not seen in human, and this architectural complexity is generated by dynamic domain reshuffling (Huang et al. 2008). Since a novel domain combination may create a novel signaling pathway, the increased architectural complexity may lead to more complicated signaling networks.

Immune Effector Molecules

Genomic annotation of the amphioxus genome reveals the presence of an extraordinary complexity and diversity of immune effector genes (Huang et al. 2008). In this section we briefly describe the immune functions of these immune effectors.

Galectins

Galectins are a family of proteins characterized by their binding specificity for β-galactoside sugars, and they have been identified in vertebrates, invertebrates, and protists (Shoji et al. 2003; Houzelstein et al. 2004). Galectins can regulate immune and inflammatory responses (Cummings and Liu 2009). The vertebrate galectin cysteine-rich domains (CRDs) are always encoded by three exons with two subtypes and are defined by the exon-intron structures F4-CRD and F3-CRD. The F4-CRD–linker–F3-CRD gene structure is shared by all vertebrate tandem galectins, one ascidian (*C. intestinalis*) galectin (Houzelstein et al. 2004), and sea urchin (*Strongylocentrotus purpuratus*) galectin (RL-30). A F4-CRD–linker–F3-CRD-type

tandem galectin (Gal-L) and its alternatively spliced mono-CRD isoform (Gal-S) are also identified in amphioxus, and the recombinant proteins of the two isoforms both have β-galactoside binding activity (Yu et al. 2007b). This alternative spliced form is also found in human galectin-8 (Bidon et al. 2001). The amphioxus Gal-L-N-CRD and Gal0-L-C-CRD are 63% homologous to each other, and this phenomenon is also present in *S. purpuratus* RL-30. The shared exon–intron organization, alternatively spliced form, and high homology between N-CRDs with C-CRDs in amphioxus and sea urchins strongly supports that all the chordate CRDs originated from a common ancestral CRD of echinoderm or from more primitive species by a mechanism of gene duplication and divergence (Yu et al. 2007b; Houzelstein et al. 2004).

The amphioxus *Gal-L* is mainly expressed in the immunity-related tissues such as hepatic cecum, intestine, and gill, whereas *Gal-S* is ubiquitously expressed in all the tissues examined. The expression of *Gal-L* is elevated after acute challenge with various microorganisms, but Gal-L only binds to specific bacteria (Yu et al. 2007b). Like some mammalian galectins, amphioxus Gal-L and Gal-S are present both inside and outside cells. These suggest that amphioxus galectins may function like their vertebrate homologs, being involved in the inflammatory response by cross-linking β-galactoside glycojugates or glycoprotein receptors on cell surfaces to mediate cell–cell or cell–matrix interactions, responding to the invading pathogens and triggering signal transduction pathways.

C-Type Lectins

C-type lectins are the largest and most diverse family of lectins found in animals. It has been reported that C-type lectins from *C. elegans*, *D. melanogaster*, and vertebrates are highly divergent from each other (Dodd and Drickamer 2001). There are over 1200 C-type lectin gene models in the amphioxus genome (Huang et al. 2008). C-type lectin domains (CTLDs) capable of carbohydrate binding usually have sugar-binding motifs (mostly EPN [Glu-Pro-Asn]/QPD [Gln-Pro-Asp] + WND [Trp-Asn-Asp]). Various sugar-binding motifs are found in amphioxus CTLDs, such as EPN, QPD, EPS [Glu-Pro-Ser], EPK [Glu-Pro-Lys], EPE [Glu-Pro-Glu], EPD [Glu-Pro-Asp], QPS [Gln-Pro-Ser], and QPN [Gln-Pro-Asn]. The variety of derived motifs may suggest diversified sugar-binding specificity.

Half of amphioxus CTLDs consist solely of a CRD domain, and three of them—AmphiCTL1, 2 and 3—have been functionally characterized (Yu et al. 2007a). *AmphiCTL1* is dramatically upregulated in amphioxus challenged with *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and zymosan. AmphiCTL1 protein is exclusively detected in the inner folding tissues of the hepatic cecum. Recombinant AmphiCTL1 possesses hemagglutinating activity, which preferentially binds to Gram-positive bacteria and yeast, but has little binding activity toward Gram-negative bacteria. It aggregates *S. aureus* and *S. cerevisiae* in a Ca^{2+}-dependent manner and specifically binds to insoluble peptidoglycan (PGN) and glucan, but not to lipopolysaccharide (LPS), lipoteichoic acid (LTA), and mannan. AmphiCTL1 can directly kill *S. aureus* and *S. cerevisiae* via binding to the cell wall

polysaccharides such as PGN and glucan. In contrast, *AmphiCTL2* and *AmphiCTL3* are mainly expressed in the intestine and skin, and their expression is upregulated by the challenge with Gram-positive and -negative bacteria and yeast. Given the abundant presence of C-type lectins with a similar structure to AmphiCTL1 in the genome, amphioxus may have already prepared a defense network against almost all possible invading microorganisms.

In amphioxus, CTLDs are in most cases associated with collagen, sushi/short consensus repeats (SCR)/complement control protein (CCP), and epidermal growth factor (EGF) domains. Interestingly, a novel C-type lectin containing EGF and low-density lipoprotein receptor (LDLR) domains has been identified in the amphioxus *B. japonicum* (BjCTL). *BjCTL* is significantly upregulated in amphioxus challenged with Gram-negative and -positive bacteria (Qu et al. 2016). BjCTL is a typical Ca^{2+}-dependent carbohydrate-binding protein capable of agglutinating and binding to both Gram-negative and positive bacteria. In addition, it can specifically bind to LPS, LTA, and PGN, which is primarily attributable to CRD domain and can be inhibited by galactose.

Intelectins

Intelectins are lectins expressed in humans and other chordates (Yan et al. 2013a). Several lines of evidence suggest that intelectins recognize microbes and may function as an innate immune defense protein. An ascidian intelectin has been shown to act as an opsonin functioning in phagocytosis by hemocyte (Abe et al. 1999), and an amphioxus intelectin has been shown to be able to agglutinate bacteria (Yan et al. 2012, 2013b). In zebrafish and rainbow trout, intelectin gene expression is stimulated upon microbial exposure (Lin et al. 2009; Russell et al. 2008). In mammals such as sheep and mice, intelectin gene expression is also upregulated upon parasitic infection (Datta et al. 2005; French et al. 2008).

There are 22 intelectin homologs in amphioxus, and two of them—AmphiITLN71469 and AmphiITLN239631—have been characterized (Yan et al. 2012, 2013b). AmphiITLN71469 consists of a fibrinogen-related domain in the N-terminus and an intelectin domain in the C-terminus. *AmphiITLN71469* is mainly expressed in the gut and skin, and is significantly upregulated in response to *S. aureus* challenge, but only modestly to *E. coli* treatment. Recombinant AmphiITLN71469 can strongly agglutinate Gram-positive bacteria in a Ca^{2+}-dependent manner, but has lower agglutination activity against Gram-negative bacteria, possibly because AmphiITLN71469 has a higher affinity to PGN than to LPS (Yan et al. 2012). Another intelectin homolog, *AmphiITLN239631*, consisting of a collagen–fibrinogen–intelectin domain structure, is ubiquitously expressed in all the tissues examined and also participates in the response to *S. aureus* and *E. coli*. Like AmphiITLN71469, recombinant AmphiITLN239631 can also agglutinate both Gram-positive and Gram-negative bacteria in a Ca^{2+}-independent manner, which is thought to be a result of the binding of AmphiITLN239631 to PGN and LPS (Yan et al. 2013b). However, the bacterial binding properties and bacterial agglutinating

degrees of AmphiITLN239631 are both lower than AmphiITLN71469. The differences, combined with their different tissue distributions, may suggest the functional diversity between the two amphioxus intelectins.

Peptidoglycan Recognition Proteins

PGN recognition proteins (PGRPs) are pattern recognition molecules that are conserved from insects to mammals and can bind bacteria and their cell wall component PGN (Royet and Dziarski 2007; Dziarski and Gupta 2010). *Drosophila* has 13 PGRPs that function as either sensors or effectors. Sensor PGRPs recognize pathogens and activate innate signaling pathways such as Toll, immune deficiency (Imd), and prophenoloxidase pathways, whereas effector PGRPs have either bactericidal or amidase activities. PGRP amidases can hydrolyze PGN to reduce its immunostimulatory activity. Mammals possess four PGRPs, all of which serve as effectors (Dziarski and Gupta 2006, 2010). Amphioxus has 17–18 PGRP genes, none of which reliably clusters with insect or mammalian PGRPs (Huang et al. 2011b). Sequence analysis indicated that all amphioxus PGRPs have Zn^{2+} binding and amidase active sites, suggesting their potential amidase activity. There are two types of PGN: the meso-diaminopimelic acid-type (Dap-type), which is found in all Gram-negative bacteria and Gram-positive bacterium *Bacillus*, and the L-lysine-type (Lys-type), which is found in most Gram-positive bacteria. It has been proposed that PGRPs that prefer to bind to the DAP-type possess a GW-R motif, whereas PGRPs that prefer to bind to the Lys-type have an NF-V motif (Dziarski and Gupta 2006). In amphioxus, most PGRPs bear the GW-R motif and none bears the NF-V motif, but other variants such as GY/F-R, NY/W-R, and PY-R exist, suggesting a certain degree of recognition diversity for amphioxus PGRPs. Many amphioxus PGRP genes are stimulated during the gut immune responses, with the peak expression level several times higher than that of GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Those PGRPs with extremely high expression levels are likely effectors. One PGRP among the highly expressed PGRPs and bearing a non-conserved binding motif, named PGRP1, has been functionally characterized (Huang et al. 2011b). Recombinant PGRP1 can bind both DAP- and Lys-types of PGN, with higher affinity toward the Lys-type, and can lyse the cell wall of *E. coli*. These findings suggest that PGRPs may be one of the major effectors in the gut mucosal immunity given their high expression levels.

A short PGRP gene (*pgrp-s*) possessing a domain combination of chitin-binding domain (CBD)–PGRP has been identified from *B. japonicum* (Yao et al. 2012). The *pgrp-s* is predominantly expressed in the hepatic cecum, hind-gut, and muscle in a tissue-specific manner. The recombinant PGRP-S and truncated protein with the CBD domain deleted (P86/250) both show affinity to Dap-type PGN, Lys-type PGN, and chitin. Consistently, they are also able to bind to *E. coli*, *S. aureus*, and *Pichia pastoris*. Moreover, both PGRP-S and P86/250 display enzymatic activity of amidase, capable of hydrolyzing Dap-type and Lys-type PGNs. Like vertebrate PGRPs, PGRP-S is directly microbicidal, capable of killing *E. coli*, *S. aureus*, and

P. pastoris, whereas P86/250 can only inhibit the growth of *E. coli* and *S. aureus*, and its anti-*P. pastoris* activity is also significantly reduced, indicating that the N-terminal CBD domain is necessary for the antifungal activity of amphioxus PGRP-S (Yao et al. 2012). Notably, this domain combination of CBD–PGRP has only been found in amphioxus, which might result from domain shuffling when a great expansion of the amphioxus immune gene repertoire occurred and possibly broadened its recognition spectrum.

Gram-Negative Bacteria-Binding Proteins

Gram-negative bacteria-binding proteins (GNBPs) can bind LPS and β-1,3-glucan, thus belonging to PRRs. GNBPs can be divided into two groups, of which group A is restricted to *Drosophila* and has lost the key residues for glucanase activity (Zhang et al. 2007), whereas group B is present in various invertebrates and has predicted glucanase activity. GNBPs have been lost in jawed vertebrates, but five GNBPs are found in amphioxus, suggesting the presence of GNBPs in the chordate ancestor. One of the GNBP genes, designated as *AmphiGNBP*, has been characterized (Jin et al. 2012). AmphiGNBP contains a conserved β-1,3-glucan-recognizing and -binding domain, and two extra WSC (cell Wall integrity and Stress response Component) domains which are unique in AmphiGNBP protein. The two WSC domains of AmphiGNBP protein coupled with the expansion of amphioxus immunity repertoire might have undergone intensive domain shuffling during the age of the Cambrian explosion. *AmphiGNBP* is mainly expressed in immune tissues such as hepatic cecum and intestine, and its expression is affected after LPS stimulation.

Chitin-Binding Proteins

Chitin is the second most abundant biopolymer in nature and can be found in fungi, algae, and protostomes. Mammals have a set of dedicated chitin binding proteins (CBPs) for digestion and immune regulation (Lee 2009). There are abundant CBPs in arthropods, with roles in digestion, development, structural formation, and host defense (Arakane and Muthukrishnan 2010). In ascidian, the Ig domain-containing CBPs, that is, V region-containing CBPs (VCBPs) and bacteria, are found to be co-localized to chitin-rich mucus along the intestinal wall. VCBPs first influence the bacterial biofilm formation, and then the overall settlement and colonization of bacteria in the ascidian gut (Dishaw et al. 2016).

The CBPs identified in amphioxus genome include three PGRPs, three chitinases, seven VCBPs, and 12 multiple chitin binding domain-containing proteins (Huang et al. 2011b; Wang et al. 2015). The CBD of PGRP not only has the chitin binding activity, but also enables the PGRP to gain a fivefold increase of amidase activity towards the Lys-type PGNs, leading to a significantly broadened substrate spectrum (Wang et al. 2015). Amphioxus chitotriosidase-like protein, containing a

catalytic domain and a CBD, shows both binding and hydrolyzing activities towards chitin as well as antifungal activity, all of which depend upon the CBD (Xu and Zhang 2012). Multiple chitin binding domain-containing proteins represent one of the most abundant expressed families in the gut immune response (contributing to 1.25% of the total transcripts in the bacteria-challenged cDNA library). Moreover, chitin binding domains are also found in other candidate immune genes such as membrane attack complex (MAC)/perforin domain (MACPF) proteins, mannose-binding lectin (MBL)-associated serine protease (MASP)-like proteases, and C-type lectin receptors (CLRs). The presence of the chitin binding domain-containing *MASP* gene may represent a shortcut activation pathway to the complement cascade against the chitin-containing microbes (Huang et al. 2011b).

Apextrins

Apextrin was initially found to be an embryonic development-related protein. Later, the potential immune function of apextrin was found because of the high upregulation of apextrin expression after bacterial challenge (Dheilly et al. 2011). Amphioxus has nine apextrin-like gene models, and two of them have been cloned in *B. japonicum* (BjALP1 and BjALP2) (Huang et al. 2014). Both BjALP1 and BjALP2 can interact with bacterial PGN and the minimal PGN motif muramyl dipeptide (MDP) via ApeC, the C-terminal domain of apextrins. BjALP1 is a mucosal effector secreted into the gut lumen to agglutinate the Gram-positive bacterium *S. aureus* via PGN binding. Neutralization of secreted BjALP1 by anti-BjALP1 monoclonal antibodies can cause serious damage to the gut epithelium and rapid death of the animals after bacterial infection. BjALP2 is an intracellular PGN sensor that binds to TRAF6 and prevents TRAF6 from self-ubiquitination and hence from NF-κB activation. MDP can compete with TRAF6 for BjALP2, which releases TRAF6 to activate the NF-κB pathway. BjALP1 and BjALP2 therefore play distinct and complementary functions in amphioxus gut mucosal immunity. Specifically, BjALP1 functions in the extracellular space to reduce the harmful effect of pathogenic microbes, whereas BjALP2 functions as a PRR that serves as a sentinel for intracellular bacterial invasion (Fig. 5).

Lipopolysaccharide-Binding Protein

LPS-binding protein (LBP) is a serum glycoprotein belonging to the family of lipid-binding proteins, which include bactericidal/permeability-increasing protein (BPI), phospholipid ester transfer protein, and cholesterol ester transfer protein (Kirschning et al. 1997). LBP can bind LPS and then transfer LPS to CD14 (Wright et al. 1990), resulting in activation of the TLR signaling pathway (Kopp and Medzhitov 1999). In contrast, BPI, which shares a similar structure with LBP, exerts strong antibacterial activity, neutralizes LPS, and acts as an opsonin (Elsbach 1998). Though many LBP and BPI-like molecules have been found in invertebrates, functional analyses

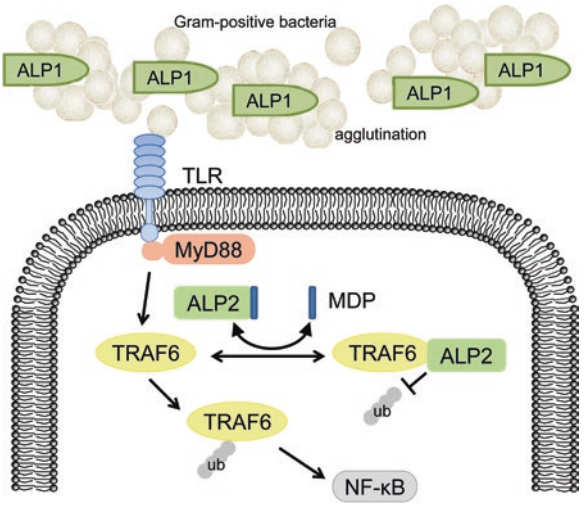

Fig. 5 Proposed model of the synergic function of apextrins in amphioxus. During the immune response a large number of BjALP1 proteins are secreted to form a mucosal cushion with other mucosal effectors to protect the epithelium surface, whereas intracellular BjALP2 regulates the immune response and homeostasis by monitoring the peptidoglycan (PGN)/motif muramyl dipeptide (MDP) concentration

have not been conducted. A member of LBP/BPI family has been identified and characterized from amphioxus (Xu 2011). The functions of this molecule are more like BPI, and thus it is named Amphi-BPI. It is mainly expressed in the hepatic cecum, intestine, and ovary, and is upregulated by LPS stimulation. Recombinant Amphi-BPI protein possesses LPS binding and bactericidal activities.

Antimicrobial Peptides

Antimicrobial peptides (AMPs) are endogenous antibiotics that are widely distributed in nature as ancient components of innate immunity. They are often cationic and amphipathic molecules that interact with microbial membranes, and kill microbes by direct disruption of cellular components, including the microbial membrane and DNA (Park and Hahm 2005), and thus the acquisition of resistance against AMPs is very rare compared with conventional antibiotics (Hancock and Sahl 2006). AMPs have attracted great attention for overcoming multidrug-resistant microbes. To date, only two AMPs, a big defensin (Teng et al. 2012) and a novel AMP (Liu et al. 2015a), have been identified in amphioxus.

Identifying novel AMPs in databases largely depends upon the existence of a sufficient sequence homology and a query sequence from a known AMP. However, homology between orthologous AMPs is extremely low. Fortunately, AMPs generally include signal sequences and proregions that tend to be significantly more conserved than mature AMPs or full-length AMPs themselves. This advantage, i.e., signal sequence conservation, has been successfully employed to search for and identify novel AMPs from databases within the same lineages of amphibians, fish, and amphioxus (Tessera et al. 2012; Juretic et al. 2011; Liu et al. 2015a). An AMP named BjAMP1 has been identified from the databases of *B. japonicum* using the signal sequence of jawless hagfish HFIAP-1, a known AMP of the cathelicidin

family. *Bjamp1* encodes a protein with features typical of AMPs, and its expression is remarkably upregulated following challenge with LPS and LTA. Moreover, the synthesized putative mature AMP (mBjAMP1), consisted of the C-terminal 21 residues of BjAMP1, underwent a coil-to-helix transition in the presence of TFE (2,2,2-trifluoroethanol) or SDS (sodium dodecyl sulfate), agreeing well with the expectation that BjAMP1 was a potential AMP. mBjAMP1 can also interact with LPS and LTA. Importantly, mBjAMP1 can directly kill a broad spectrum of microbes via membrane active mechanism, while it is non-cytotoxic to mammalian cells. These data render this new AMP a promising template for the design of novel peptide antibiotics against MDR microbes.

Lysozymes

Lysozymes are a superfamily of ubiquitous enzyme catalyzing hydrolysis of the β-(1,4)-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in PGN, the major bacterial cell wall polymer. Lysozymes identified from organisms ranging from bacteriophages to humans have been classified into six types: chicken-type (c-type) lysozyme, goose-type (g-type) lysozyme, invertebrate-type (i-type) lysozyme, plant lysozyme, bacterial lysozyme, and phage lysozyme. They share the same enzyme activity but differ significantly in amino acid sequence and biochemical properties. Vertebrates possess only c- and g-types, while invertebrates have c-, g-, and i-type lysozymes (Callewaert and Michiels 2010; Van Herreweghe and Michiels 2012). In amphioxus *B. japonicum*, both c- and g- as well as i-type lysozymes have been identified, and this is the first time all three types of animal lysozyme have been discovered in a single species (Liu et al. 2006; Xu et al. 2014). The existence of the biologically active i-type lysozyme in amphioxus clearly indicates that the i-type lysozyme gene is retained at least in Protochordata. Therefore, the i-type lysozyme cannot be considered to be restricted only to invertebrates any more. How and when the i-type lysozyme was lost during chordate evolution remains elusive and demands further study.

Most organisms are able to produce different types of lysozymes and/or multiple forms of the same lysozyme, and it is presumed that these may have complementary or even different functions. The analysis of the tissue-specific expression pattern of lysozyme genes in amphioxus shows that *AmphiLysG* and *AmphiLysC* are predominantly expressed in the hepatic cecum and hind-gut, and *AmphiLysI* is abundant in the gill and notochord. The analysis of tissue-specific localization of lysozyme proteins in amphioxus shows that AmphiLysC is mainly localized in the hepatic cecum, and hind-gut, and AmphiLysI in the gill, both of which are consistent with their gene expression patterns, but AmphiLysG is primarily distributed in the muscle and notochord, which is different from its gene expression pattern. The different distributions of all three types of lysozymes in *B. japonicum* suggest that they play complementary and/or different functions. All three lysozymes in *B. japonicum* have the capacity to degrade the *Micrococcus lysodeikticus* cell wall, but their specific activity in terms of enzyme units per milligram protein differs from each other, which is in an order of AmphiLysI > AmphiLysC > AmphiLysG.

Other Effector Molecules

In addition to the immune effectors list in sections "Galectins" to "Lysozymes", quite a few unclassifiable effector molecules are found to play roles in amphioxus defense, including avidin (Guo et al. 2017), calreticulin (Liu et al. 2013), alanine aminotransaminase (Jing and Zhang 2011), transferrin (Liu et al. 2009), and creatine kinase (An et al. 2009). These molecules have been shown to display either antibacterial activity capable of inhibiting bacterial growth or opsonin activity capable of enhancing phagocytosis. We are sure that more new immune effectors will be identified with time from amphioxus.

Complement System

The complement system, which helps antibodies and phagocytic cells to clear pathogens from an organism, is a central component of innate immunity and a link between innate and adaptive immunity. The mammalian complement system has three activation pathways (classical, alternative, and lectin) and two terminal pathways (opsonic and cytolytic). In all three activation pathways, C3-convertase cleaves and activates C3, causing a cascade of further cleavage and activation events. Amphioxus *C3-like* gene was identified by Suzuki and colleagues in 2002, which is the first molecular evidence for complement in amphioxus (Suzuki et al. 2002). The first functional characterization of complement activity in amphioxus was performed by Zhang et al. (2003). They showed that the humoral fluids of amphioxus have alternative complement pathway-mediated hemolytic activity. The content of C3 in amphioxus humoral fluid ranges from 0.79 to 1.47 mg/ml, which is close to the C3 concentration in human sera (Zhang et al. 2003). Amphioxus C3 can be cleaved into C3a and C3b (Huang et al. 2011a; Gao et al. 2013), suggesting that the cleavage mechanism is conserved in amphioxus C3.

The complement system of amphioxus has been extensively studied. The amphioxus genome contains multiple copies of a number of complement-related genes, such as 50 *C1q-like*, 41 *ficolin-like*, two *MASP*, two *C3-like*, three *Bf/C2*, five *C6-like*, and 427 *CCP*-containing models (Huang et al. 2008). The alternative complement-mediated killing of *Vibrio* species by the humoral fluids of amphioxus has been demonstrated (Li et al. 2008). Alternative complement activity was also shown to be present in the egg cytosol of amphioxus (Liang et al. 2009). In addition, the lectin pathway and a C1-mediated complement system have also been demonstrated in amphioxus. All these suggest that the complement system plays an important role in the protection of amphioxus (at each stage of development) against pathogenic attack.

C1q-Activated Pathway

In mammals, the classical pathway is initiated by the binding of C1q to immunoglobulins (IgG or IgM) within immune complexes, and its associated C1r and C1s,

which leads to the subsequent cleavage of C4 and C2, followed by C3 activation. From an evolution perspective, the classical complement pathway appears to have emerged only in the jawed vertebrates, when the adaptive immune system (AIS) was established in the cartilaginous lineage. However, in the jawless fish the lamprey, C1q has also been shown to be able to directly interact with the type B VLR (VLRB), the counterpart of Igs in the jawed vertebrates, and to form complexes on the surface of target cells, resulting in complement activation and subsequent cell lysis (Wu et al. 2013). This raises the possibility that the complement system analogous to the conventional classical pathway of the jawed vertebrates is present in lampreys.

Since there is no evidence to show the presence of antibody in amphioxus, the C1q-like proteins in amphioxus may initiate the complement activation in a new fashion. A C1q-like member in the amphioxus *B. japonicum*, named *BjC1q*, has been functionally analyzed both in vivo and in vitro (Gao et al. 2014). It is predominantly expressed in the hepatic cecum, hindgut, and notochord, and is significantly upregulated following challenge with bacteria or LPS or LTA. The recombinant proteins of BjC1q and its globular head domain can specifically interact with LPS and LTA, but BjC1q displays little lectin activity. BjC1q can assemble to form high molecular weight oligomers, and bind human C1r, C1s, and MASPs as well as amphioxus serine proteases involved in the cleavage of C4/C2 and C3 activation (Gao et al. 2014). Although lacking the ortholog genes of C1r and C1s, amphioxus has MASP1 and MASP3 (Endo et al. 2003), which have the ability to cleave C3 (Huang et al. 2011a). Importantly, BjC1q can interact with human IgG as well as an amphioxus Ig domain-containing protein, resulting in the activation of the classical complement pathway. These suggest that amphioxus possesses a sort of functional C1q-mediated complement pathway.

Alternative Pathway

Activation of the alternative pathway is spontaneous and depends on autohydrolysis of the C3. In mammals, hydrolyzed C3 yields C3b, and this change in shape allows the binding of Bf, followed by the subsequent cleavage of Bf by factor D (Df) to form a C3 convertase (C3bBb). In addition, the stabilization of C3bBb complex needs properdin. The alternative pathway is regulated by several different kinds of regulatory proteins, such as factor I (If) and factor H (Hf). The key genes involved in the alternative pathway, including *C3*, *Bf-like*, *properdin-like*, and *Hf-like* genes, have been identified from amphioxus (Gao et al. 2017; He et al. 2008; Cai et al. 2014), although no homologs have been reported for *Df* and *If* genes.

Bf and C2 are thought to be gene duplication products. Mammalian Bf and C2 have identical modular structures and similar function. The frog *Bf* gene can be clearly distinguished from *C2* based on their deduced amino acid sequences (Nonaka et al. 1997). In contrast, the *Bf/C2* genes in zebrafish, sharks, lampreys, or invertebrates are difficult to identify as *Bf* or *C2* (Nonaka and Kimura 2006). Amphioxus *Bf/C2* encodes a mosaic protein consisting of CCP-EGF_CA-CCP-CCP-vWFA-SP

structure (He et al. 2008). The EGF_CA domain is an extra domain, which is not present in any other Bf/C2 family proteins from invertebrates and vertebrates, but the function of this domain is unclear. In addition, this protein is mainly localized in the hepatic cecum, and the expression of this gene is significantly upregulated following challenge with LPS, suggesting that it is an immune-related gene.

In mammals, properdin is critical in the stabilization of alternative pathway convertases, and it is a pattern recognition molecule that binds to certain microbial surfaces, apoptotic cells, and necrotic cells (Kemper et al. 2010). Amphioxus properdin has features similar to mammalian properdin, including the N-terminal signal peptide, a truncated TSR0, and TSR1–8 containing the WXXWXXW and RXR motifs, which are implicated in adhesion to blood cells and binding to polysaccharide. It plays similar roles to vertebrate properdins, such as bacterial recognition, binding to LPS and LTA, and regulation of the alternative pathway (Gao et al. 2017).

Lectin Pathway

The lectin pathway is similar to the classical pathway, except that it uses pattern recognition molecules, including MBL, ficolin, collectin liver 1 (CL-L1), collectin kidney 1 (CL-K1), and collectin placenta 1 (CL-P1), instead of antibody to target activation (Hansen et al. 2016). These lectins or lectin-like molecules primarily, but not exclusively, use the collagen domains for MASPs binding. For example, ascidian GBL, a glucose-binding C-type lectin without collagen, can recruit MASPs and activate C3 (Sekine et al. 2001). There are 1215 CTL models in the amphioxus genome, at least 66 of which have COL-CTLD structures (Huang et al. 2008). However, no clear orthologs for MBL or GBL have been identified thus far in amphioxus. It is notable that lamprey C1q possesses lectin activity, and can activate MASP-A and C3 (Matsushita et al. 2004), whereas neither of the two C1q-like proteins identified in amphioxus so far have lectin activity (Gao et al. 2014; Yu et al. 2008), forming a sharp contrast to lamprey C1q.

There are 41 ficolin-like models identified in the amphioxus genome (Huang et al. 2008), and a homolog of ficolin, termed BjFCN1, has been functionally characterized in *B. japonicum* (Huang et al. 2011a). BjFCN1 shows a Ca^{2+}-dependent lectin activity, and can bind LTA and Gram-positive bacteria. BjFCN1 can form a complex with the N-terminal portion (CUB-EGF-CUB) of BjMASP1/3. Combined with the fact that the C-terminal portion (protease domain) of BjMASP1/3 can accelerate the cleavage of BjC3, it is thus possible that amphioxus possesses a ficolin-MASP1/3-C3 pathway.

Terminal Pathway

The terminal pathways need the participation of C3, C5, C6, C7, C8, and C9 to perform opsonic or cytolytic function. In amphioxus, only C3-like and C6-like molecules have been identified (Suzuki et al. 2002). In mammals, several complement

activation fragments perform opsonic function, such as C3b, which tags pathogens, immune complexes, and apoptotic cells for phagocytosis. Amphioxus C3 has been shown to function as an opsonin (Pan et al. 2011), as sea urchin and sea ascidian C3 proteins do (Nonaka et al. 1999; Clow et al. 2004). Moreover, amphioxus C3a has been shown to have antibacterial activity and is capable of inducing macrophage migration and enhancing macrophage phagocytosis and respiratory burst responses (Gao et al. 2013). These indicate that opsonization may be an important role that C3 plays in invertebrates.

In vertebrates, the cytolytic pathway includes at least four C6-like proteins (C6/C7/C8/C9), which assemble to form the MAC on the targeted cells. These proteins feature a MACPF required for membrane perforation. C6 is the longest protein containing the "prototypic" structure $(TSP1)_2$-LDLa-MACPF-EGF-TSP1-$(CCP)_2$-$(FIMAC)_2$, while the other proteins contain fewer domains. In particular, C9 adopts the shortest structure, TSP1-LDLa-MACPF-EGF. The amphioxus genome has 29 MACPF genes, five of which encode C6-like proteins. They all adopt the same structure, $(TSP1)_2$-LDLa-MACPF-EGF-TSP1, and lack the C-terminal CCP or factor I-MAC (FIMAC) domains, which are required for interacting with C5 in vertebrates. The functions of amphioxus *C6-like* genes have not been determined. The complement-mediated cytotoxic pore-forming mechanism in amphioxus deserves to be further studied, which is still an unsolved question in all invertebrates.

Expression and Regulation of Complement Genes

Most complement genes in mammals are primarily expressed in the liver (Morgan and Gasque 1997). In amphioxus, only a few complement genes have been examined at the expression level; among them, the *C1q*, *Ficolin*, *MASP1/3*, *C3*, and *Bf/C2* genes are strongly expressed in the hepatic cecum, which is the "pre-hepatic" organ homologous to vertebrate liver (Han et al. 2006; Li and Zhang 2010; Wang and Zhang 2011). However, accumulating data show that complement genes in amphioxus are expressed at multiple sites outside the hepatic cecum, such as the gill and gut, which are also immunological organs. The extra-hepatic cecum expression of complement genes suggests that the amphioxus complement system not only functions in the circulatory system but also plays a role at the local sites after the onset of infection or injury. Moreover, C3 and Bf-like proteins were detected in the fertilized eggs (Liang et al. 2009) and *BjFCN1*, *BjMASP1/3*, and *BjC3* genes were detected in the ovary (Huang et al. 2011a), suggesting the maternal provision of complement-relevant molecules that may protect amphioxus eggs and embryos against invading pathogens before full maturation of the immune system. Recently, a conserved microRNA (miR-92d) was shown to be involved in the regulation of complement pathway by targeting C3 in amphioxus (Yang et al. 2013), suggesting that the expression of complement genes are subject to multiple-level regulation.

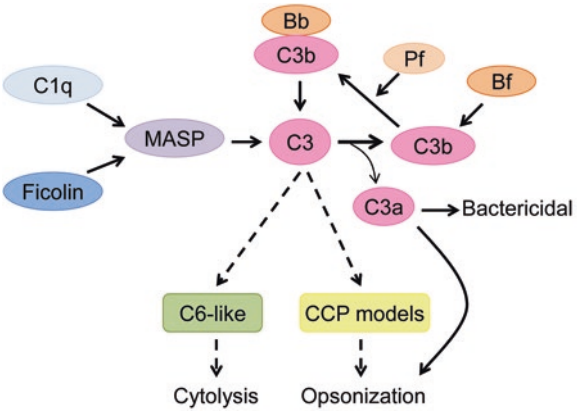

Fig. 6 Putative complement system in amphioxus. The identification of C1q, ficolin, mannose-binding lectin-associated serine protease (MASP), C3, factor B (Bf), Pf (properdin), and C6 demonstrated that the basic framework of the complement system has been established in amphioxus. Amphioxus complement systems are activated and amplified by the formation of C3 convertases through the C1q-mediated, ficolin-mediated, and alternative pathways. Besides, amphioxus genome contains abundant complement control protein (CCP)-containing models, and their functions in the regulation of amphioxus complement remain to be determined. A solid arrow indicates that the pathway was supported by experimental data, but a dashed arrow indicates that no experimental support is present

Outline of the Amphioxus Complement System

Based on the descriptions given in this section, the basic framework of the complement system depending on the key components can be outlined in amphioxus (Fig. 6). The C1q-MASP-C3 and ficolin-MASP-C3 pathways have been established. The alternative pathway has also been proved by the facts that C3, Bf, and properdin participate in the hemolytic and bacteriolytic activity of humoral fluids. It is worth mentioning that the lectin pathway is much expanded in amphioxus because of the presence of a number of effective PRRs such as ficolins and collectins. The terminal pathway of amphioxus seems to contain opsonization and cytolysis. Both the opsonization and bactericidal activity of C3a have been observed, whereas the cytolysis pathway mediated by MAC remains to be further studied. In addition, the amphioxus genome contains abundant CCP containing models, and their functions in the regulation of amphioxus complement remain to be determined.

Oxidative Burst System in Amphioxus

Oxidative burst occurs in the activated mammalian phagocytes, which rapidly release a large amount of reactive oxygen species (ROS) into the phagosome to kill ingested bacteria (Forman and Torres 2002). In oxidative burst, nicotinamide

adenine dinucleotide phosphate (NADPH)-oxidase (NOX) 2 (also called gp91phox), together with p22phox, p47phox, p67phox, p40phox, and GTPase RAC, produces H_2O_2, and then myeloperoxidase converts H_2O_2 into ROS. Furthermore, NADPH-oxidase NOX1, NOX4, dual oxidase (DUOX) 1 and DUOX2, lactoperoxidase, eosinophil peroxides, and thyroid peroxidase (TPO) also produce ROS and play a role in innate immunity (Rada and Leto 2008; Leto and Geiszt 2006; Klebanoff 2005).

NOX enzymes are membrane-bound enzyme complexes widely distributed in different species, and are broadly implicated in host defense, signaling, and biosynthesis. There are seven NOX family members in mammals, including NOXs 1–5, DUOX1, and DUOX2. Among them, NOX2, also known as gp91phox, is the prototype NADPH-oxidase. NOX2 requires the assembly of at least five additional components for its activation, including p22phox, p47phox, p67phox, p40phox, and GTPase RAC.

Amphioxus has all the key elements of oxidative burst, and the ROS production is indispensable for efficient antibacterial responses in amphioxus (Yang et al. 2014; Huang et al. 2011b). Amphioxus has two DUOX proteins that form a co-ortholog pair with vertebrate DUOXs 1 and 2, and has a single NOX2 that corresponds to vertebrate NOXs 1, 2, and 3. Vertebrate NOXs 1, 2, and 3 are close paralogs, which were possibly created by two rounds of whole-genome duplication in early vertebrate evolution (Putnam et al. 2008). Amphioxus also has p22phox, p47phox, p67phox, p40phox, and RAC. Amphioxus NOX enzymes and cytosolic factors are co-localized in the epithelial cells of the gill, intestine, and hepatic cecum and can be upregulated after exposure to microbial pathogens. Relative to humans, amphioxus has a complete gene set for the NOX system, and this NOX system functions as the classical phagocytic respiratory burst machinery (Fig. 7). This phagocytic respiratory burst machinery participates in the initiation of the phagocytic process of the gut epithelial lining cells in amphioxus (Yang et al. 2014; Huang et al. 2011b).

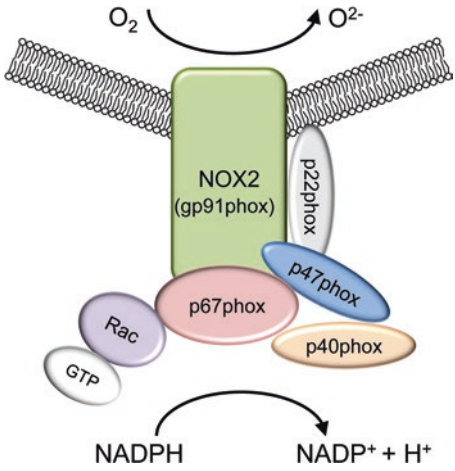

Fig. 7 The scheme of the supercomplex of the oxidative burst machinery in amphioxus. , nicotinamide adenine dinucleotide phosphate-oxidase (NOX) 2 and p22phox are transmembrane components. Rac, p47phox, p67phox, and p40phox are cytosolic subunits that are relocated to bind with NOX–p22phox on activation

Apoptotic Network

Apoptosis, which eliminates damaged or unnecessary cells, is an essential mechanism in all animals. In vertebrate cells, the apoptotic response is mediated through either the extrinsic or intrinsic pathway. These two pathways are also present in amphioxus.

Extrinsic Apoptotic Pathway

In vertebrates, the extrinsic apoptotic pathway is initiated by the binding of extracellular death ligands to DRs, such as the binding of TRAIL to DR4 or DR5. As members of the TNFR superfamily, the DRs further recruit the cytosolic adaptor Fas-associated DD (FADD), which interacts with procaspase-8 through DEDs to form an oligomeric death-inducing signaling complex. The activated caspase-8 released subsequently cleaves the effector caspase-3, which finally executes apoptosis.

DRs consist of TNFR1, Fas, DR4, and DR5 in humans. They are type I transmembrane proteins and are characterized by a set of extracellular CRDs and a cytoplasmic tail that contains a DD. Amphioxus has 14 DRs and hundreds of DD-containing adaptor proteins (Huang et al. 2008). Among them, one DR protein (DR1) and two FADD homologs (FADD1 and FADD2) have been identified from the amphioxus *B. japonicum* (Yuan et al. 2010a). Amphioxus DR1 contains four extracellular CRDs and a cytoplasmic tail that has a DD and a TRAF6-binding site. The DR1 is a receptor of amphioxus TRAIL, and its cytoplasmic region can interact with FADD1, CRADD, and TRAF6. Amphioxus FADD1 and FADD2 both contain a C-terminal DD and an N-terminal DED, but they may have evolved independently. In HeLa cells, *FADD1* over-expression results in the formation of death effector filamentous structures in the cytoplasm and the activation of the NF-κB pathway, whereas FADD2 protein is restricted to the nucleus, although its DED induces apoptosis when it is in the cytoplasm.

The caspase family members can be divided, with respect to function, into two major groups. The members of the first group include the initiator caspases (caspase-2, -8, -9, and -10) and the effector caspases (caspase-3, -6, and -7), which can directly lead to apoptosis, while the members of the second group include caspase-1, -4, and -5, which are involved in the maturation of pro-inflammatory cytokines. The amphioxus genome contains at least 45 caspase genes, including 18 genes related to both caspase-9 and caspase-2, 15 to caspase-8/-10, five to caspase-3/-6/-7, and seven to unknown caspase genes (Huang et al. 2008). Amphioxus caspase-8 with conserved protein architecture is involved in the FADD–caspase-8 mediated pro-apoptotic extrinsic pathway, while caspase-3-like may mediate a nuclear apoptotic pathway. Also, caspase-1/-2 can co-localize with FADD2 in the nucleus and be recruited to the cytoplasm by amphioxus apoptosis-associated speck-like proteins containing a caspase recruitment domain, indicating that caspase-1/-2 may serve as a switch between apoptosis and caspase-dependent innate immune response in amphioxus, and more generally in invertebrates (Xu et al. 2011). Collectively, these data suggest that amphioxus possesses the TRAIL–DR–FADD–caspases extrinsic apoptosis pathway, which was previously thought to be unique to vertebrates (Fig. 8).

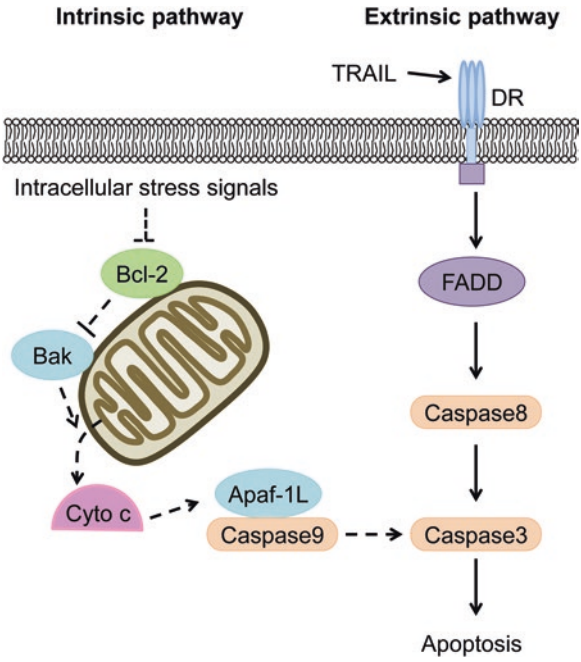

Fig. 8 Putative apoptosis system in amphioxus. The apoptosis system can be divided into the intrinsic and extrinsic pathway. The intrinsic pathway is triggered by death stimuli generated within the cell, leading to the release of mitochondrial cytochrome C. The extrinsic pathway is initiated by the binding of extracellular death ligands to death receptors (DRs)

Intrinsic Apoptotic Pathway

In vertebrates, the intrinsic apoptotic pathway is triggered by death stimuli generated within the cell, such as DNA damage, leading to the release of mitochondrial cytochrome c, which associates with caspase-9 and apoptotic protease activating factor 1 (Apaf-1) to form an apoptosome. The apoptotic response is regulated by the Bcl-2 family, which has both pro-apoptotic and anti-apoptotic members to regulate apoptosis and which lead to the release of cytochrome c and other apoptosis-inducing mitochondrial proteins (Youle and Strasser 2008). Amphioxus contains homologs of the three proteins, Bcl-2, Apaf-1, and caspase, which are directly involved in the intrinsic apoptotic pathway (Fig. 8) (Zmasek et al. 2007).

In vertebrates, Bcl-2 domain is found in the pro-apoptotic Bcl-2 family proteins Bak, Bok, and Bax, which promote mitochondrial permeabilization, and in the anti-apoptotic proteins Bcl-2 and Bcl-xl, which inhibit apoptosis by heterodimerizing with the pro-apoptotic proteins. There are seven putative proteins with the Bcl-2 domain in amphioxus. The pro-apoptotic protein Bak has a single amphioxus ortholog, and one gene is most closely related to mammalian Bok. The three mammalian anti-apoptotic Bcl-2 paralogs (Bcl-2, Bcl-xL, Bcl-2 L2) are also represented by a single amphioxus homolog (Zmasek et al. 2007).

The Apaf-1 family in animals is defined by the NB–ARC domain. Apaf-1 is represented by a single ortholog each in humans, fruit fly, and nematode. Amphioxus has an Apaf-1 homolog with similar CARD–NB–ARC–WD40 domain composition to human Apaf-1. Moreover, amphioxus has multiple Apaf-1-like proteins with

novel domain combinations; for example, the single CARD domain is replaced by pairs of CARD domains, DD, and TIR domain, suggesting that amphioxus may have unique apoptotic signaling pathway.

Some Raw and Basic Elements of the Adaptive Immune System

To trace the origin of the AIS, one can try to discover when and how lymphocytes appeared during evolution or seek orthologs of the genes encoding Ig, T cell receptor (TCR), major histocompatibility complex (MHC), and recombination activating gene (RAG) 1/2 in invertebrates. Little evidence shows the presence in invertebrates of the AIS mediated by lymphocytes bearing variable receptors in jawed vertebrates, but the characterization of the pro-MHC region, RAG1/2 genomic locus, and variable receptors suggests the presence of some basic components of an AIS in amphioxus, which are available for recruitment to an AIS during chordate evolution.

Pro-major Histocompatibility Complex Region

One element of AISs, a primitive paralogous region of human MHC regions, has been found in amphioxus (Abi-Rached et al. 2002). The human MHC is located in the 6p21 chromosomal region. Numerous genes that map around this location have paralogs at one, two, or three other chromosomal locations on the 9q33–34, 19p13.1–p13.3, and 1q21–q25 regions (Kasahara et al. 1997). The similarity between these four chromosomal regions suggests the linkages may have adaptive significance and/or they may be echoes of segmental or genome duplication and chromosome reorganization in a primate ancestor. By cloning nine human anchor genes located in the MHC region from a *B. floridae* cosmid library, Abi-Rached and coworkers first identified the corresponding paralogous MHC region in amphioxus. With the help of two amphioxus (*B. floridae* and *B. belcheri*) genomes, additional anchor genes in this region were identified and compared with linkage relationships in four paralogous MHC regions in humans (Fig. 9), further suggesting that the ancestral organization of the human MHC region was retained in the basal chordate amphioxus (Yuan et al. 2014b).

Recombination Activating Gene (RAG) 1 and RAG2 Genes

Co-option of RAG1 and RAG2 for antigen receptor gene assembly by V(D)J (variable, diversity, joining) recombination was a crucial event in the evolution of jawed vertebrate adaptive immunity. A homolog of the RAG1 core domain and its N-terminal domain has been found in amphioxus (Kapitonov and Jurka 2005), and its core domain can degrade both DNA and RNA (Zhang et al. 2014). Recently, a RAG2 homolog along the same scaffold of RAG1 has been identified in amphioxus

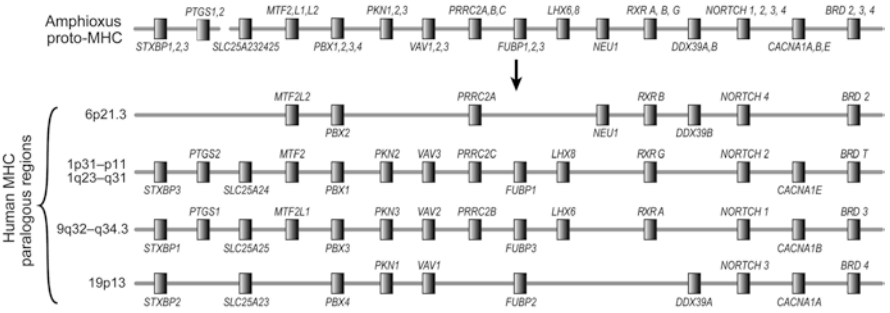

Fig. 9 Comparison of amphioxus proto-major histocompatibility complex (MHC) region with four paralogs of human MHC regions. The proto-MHC region in amphioxus contains numerous genes with linkage similar to four paralog MHC regions in humans, suggesting that the ancestral organization of the human MHC region has been retained in the basal chordate amphioxus. The arrow indicates whole-genome duplication

(Huang et al. 2016), but it lacks the C-terminal plant homeodomain (PHD) finger of mammalian RAG, which has been shown to suppress RAG transposase activity (Elkin et al. 2003). Amphioxus RAG1-like and RAG2-like proteins are encoded by a transposable element superfamily, *ProtoRAG*, which meets the structural criteria for the long-sought RAG transposon (Huang et al. 2016). A typical *ProtoRAG* is flanked by 5-bp target site duplications (TSDs) and a pair of terminal inverted repeats resembling V(D)J recombination signal sequences. Between the terminal inverted repeats reside the tail-to-tail-oriented and intron-containing RAG1-like and RAG2-like genes. Amphioxus RAG1/2-like proteins can mediate terminal inverted repeat-dependent transposon excision, host DNA recombination, transposition, and low-efficiency terminal inverted repeat rejoining using reaction mechanisms similar to those used by vertebrate RAGs (Fig. 10). These data suggest that *ProtoRAG* represents a molecular "living fossil" of the long-sought RAG transposon.

Fig. 10 (continued) to the RAG1 NBD. These two proteins also contain a series of repeats (gray) but in different locations in their N-terminal regions. The core region (amino acids 1–352 in the mouse protein; light blue) of mouse RAG2 protein consists of six kelch repeats which fold into a six-bladed beta propeller, and the non-core plant homeodomain (PHD) (dark blue). Amphioxus and sea urchin contain RAG2-like proteins, but amphioxus RAG2L is missing the PHD finger. (**c**) Amphioxus RAG1L/2L are capable of terminal inverted repeat (TIR)-dependent DNA cleavage, transposition, and self-resealing. DNA cleavage by amphioxus RAG1L/2L and mouse RAG1/2 exhibits striking mechanistic similarities. In both systems, DNA cleavage occurs by a nick-hairpin mechanism adjacent to the sequence 5′-CAC-3′, the only perfectly conserved portion of recombination signal sequences (RSSs). Furthermore, RAG and amphioxus RAG1L/2L have similar divalent metal ion requirements, rely on a parallel group of acidic catalytic residues, generate 5-bp target site duplications (TSDs), and prefer CG-rich transposition target sites

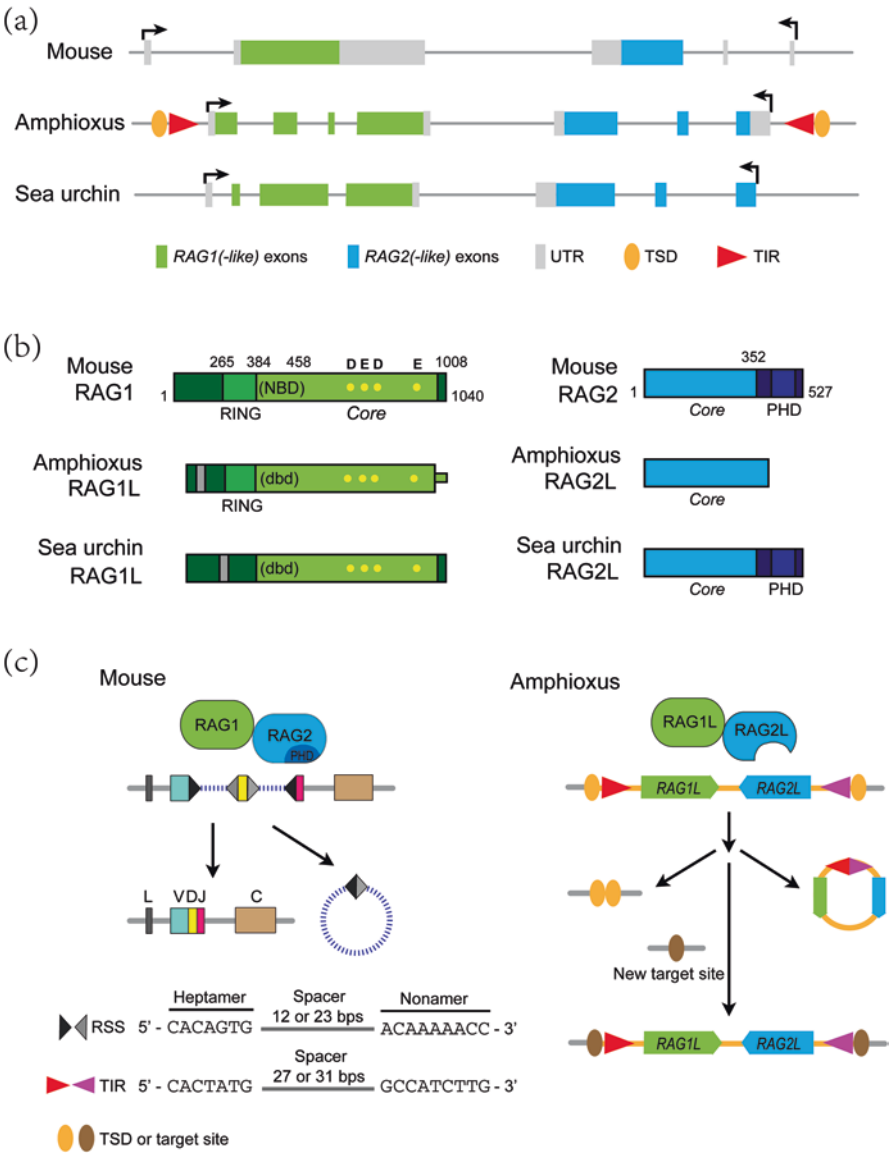

Fig. 10 Recombination activating gene (RAG) and RAG-like genes. (**a**) Genomic organization of the RAG1/2 loci in mouse, amphioxus, and sea urchin. (**b**) The catalytically active core region (amino acids 384–1008; light green) of mouse RAG1 protein contains DNA-binding regions, such as the nonamer-binding domain (NBD), as well as the acidic catalytic residues (D600, E662, D708, and E962; yellow dots). The N-terminal non-core region of RAG1 contains a RING/zinc finger (RING) that coordinates four zinc atoms. Amphioxus RAG1L contains sequence similarity that extends into the N-terminal non-core region of RAG1, spanning almost all of the RING/zinc finger region, but similarity is higher in the core, while sea urchin RAG1L contains only limited sequence similarity to the RAG1 RING/zinc finger region. These two proteins might contain a DNA-binding domain (dbd) positioned similarly to the NBD, but do not have sequence similarity

Variable Receptors Possibly Involved in Alternative Adaptive Immunity in Amphioxus

Although antigen receptors such as Igs, TCRs, and VLRs have not been identified in amphioxus, two Ig superfamily (IgSF) members with high polymorphism have been well-studied. The first is the VCBPs, secreted proteins with two Ig V-type regions at the N-terminus and a chitin binding domain at the C-terminus (Cannon et al. 2002). The presence of the chitin binding domain implies a role of these globulin-lectin proteins in immune defense, which was supported by their specific expression in the gut as a protection against bacterial infection (Dishaw et al. 2011). Amphioxus VCBPs constitute a multigene family (comprised of VCBP 1–5); VCBP 1, 2, 4, and 5 are encoded in a single, contiguous gene-rich chromosomal region and VCBP 3 is encoded in a separate locus (Litman et al. 2007). The VCBPs exhibit extensive haplotype variation, including copy number variation, indel polymorphism, and a markedly elevated variation in repeat type and density (Litman et al. 2007; Cannon et al. 2004). Detailed structural insights into VCBPs has revealed that the hyperpolymorphic positions are localized on the β-sheet surfaces of the folded V domains (Fig. 11a), which are the sites of the highest variability in the V domains of Ig and TCR (Hernandez Prada et al. 2004, 2006). Thus, VCBPs may reflect an important transition between non-rearranging innate pattern-recognition molecules and the conventional adaptive immune receptors.

The other well-studied IgSF member in amphioxus is V and C domain-bearing protein (VCP), a membrane-bound IgSF member with an Ig V domain and an Ig C2 domain in its extracellular region and an intracellular immunoreceptor tyrosine-based activation motif (ITAM) for lymphoid signaling (Yu et al. 2005). Unlike antigen receptor molecules in vertebrates, no J region is found in amphioxus VCP, but some canonical residues of other V-type domains in vertebrates are conserved in VCP, especially the residues that are critical for the formation of Ig-folding structures. Similar to VCBPs, VCP is highly diversified in amphioxus population and responds to bacterial challenges with a broad spectrum of bacterial binding such as PRRs (Yuan et al. 2015a).

A large repertoire of LRR-containing genes, including a group of VLR-like proteins, has been found in amphioxus genome (Huang et al. 2008; Cao et al. 2016). Structural analyses show that one of the VLR-like proteins forms a crescent-shaped structure of five LRRs (Fig. 11b), and a negatively charged patch at the concave of LRR solenoid structure might be responsible for antigen recognition (Cao et al. 2016). This VLR-like protein binds to the surface of Gram-positive bacteria via a couple of acidic residues at the concave.

It is clear that although no direct evidence for the presence of AIS has been found, the identification of pro-MHC region, RAG1/2 genomic locus, and some raw elements of AIS, including molecular structures, high levels of polymorphism, and signaling mechanisms, show the existence of some basic components of AIS in amphioxus that can be available for recruitment to AIS during chordate evolution. It is possible that the evolution of AIS may have arisen from two major events: the transfer of the RAG transposon and two rounds of whole-genome duplication.

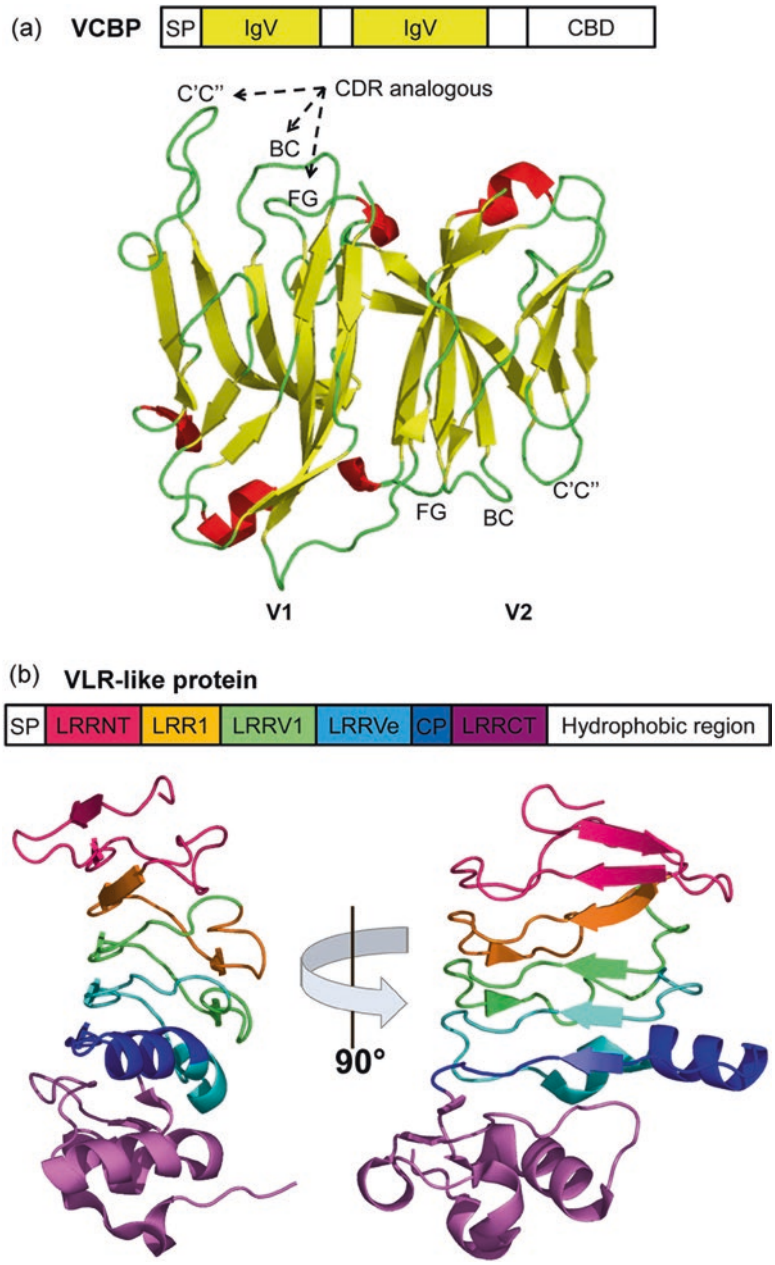

Fig. 11 Amphioxus V region-containing chitin binding protein (VCBP) and variable lymphocyte receptor (VLR). (**a**) Crystal structure of VCBP3 V1–V2. Secondary structure (β-strands, yellow; loop regions, green; helices, red). Loops corresponding to complementarity-determining regions (CDRs) in T cell receptor (TCR) and immunoglobulin: BC loop, CDR1; C′C″, CDR2; FG, CDR3. (**b**) Crystal structure of VLR LRRNT-LRRCT (leucine-rich repeat [LRR] N-terminal–LRR C-terminal)

Detailed studies of amphioxus immunity will certainly provide information on the reduction of innate immune complexity and the origin and evolution of AIS shaped by the conflict between microbiota and host during vertebrate evolution.

Concluding Remarks

In conclusion, the cephalochordate amphioxus has a rather complex immunity. On one hand, it has a vastly expanded innate receptor repertoire with 71 TLR models, 118 NLR models, 270 SR models, over 1200 C-type lectin models, over 1600 LRR-containing models, and hundreds of models containing complement-related domains, comparable with that of sea urchin. Amphioxus also has a sophisticated TNF system and a complicated complement system not previously seen in other invertebrates. It is estimated that amphioxus utilizes about 10% of its gene repertoires, and an ongoing domain reshuffling mechanism among these genes, for innate immunity, indicating an extraordinary innate complexity and diversity not observed in all other species. On the other hand, although an AIS mediated by lymphocytes bearing variable receptors in jawed vertebrates was not found in invertebrates, the identification of lymphocyte-like cells in the gill along with the genes related with lymphoid proliferation and differentiation suggests the presence of some basic components of an AIS in amphioxus. Moreover, the pro-MHC region, the RAG1/2 genomic locus, and some raw elements of AIS, including molecular structures, high levels of polymorphism, and signaling mechanisms, have been reported in amphioxus that are available for recruitment to AIS during chordate evolution. Detailed studies of amphioxus immunity in the future will certainly provide information on the reduction of innate immune complexity and the origin and evolution of the AIS shaped by the conflict between microbiota and host during vertebrate evolution.

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The Origin and Early Evolution of Adaptive Immune Systems

Masayuki Hirano

Introduction

To survive in a competitive environment, organisms have to protect themselves from pathogens, which seek their resources. Biologists have found that simple multicellular life forms such as sponges (phylum Porifera) have many of the components used by vertebrates for immune response and pathogen defense (Cooper 2006, 2010). These ancient defense strategies protect against infection by potential pathogens in a relatively non-specific manner, collectively known as innate immunity, which can be found in representative species at almost every level of the evolutionary tree of life (Beutler 2004; Hoffmann et al. 1999; Janeway 1989; Medzhitov 2007).

Lymphocytes with diverse anticipatory receptors are primarily responsible for adaptive immune responses, in particular the developmentally and functionally distinct lineages known as T and B cells in gnathostomes (jawed vertebrates). During their development in hematopoietic tissues and the thymus, B and T cells somatically generate diverse repertoires of immunoglobulin (Ig) domain–based antigen receptors that can be used to recognize a virtually unlimited range of antigens. The essential recognition elements of this type of adaptive immune system—the *Ig*, T-cell receptor (*TCR*), and major histocompatibility complex (*MHC*) genes—are present in all of the jawed vertebrates, whereas none of these essential components have been found in agnathans (jawless vertebrates). Instead, the two extant jawless vertebrates, lampreys and hagfish, use variable lymphocyte receptors (VLRs) composed of somatically assembled leucine-rich repeat (LRR) motifs to recognize antigens and induce specific immune responses.

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Adaptive immunity is mediated through various genetic and cellular processes that create appropriate somatic variants of antigen-binding receptors under evolutionary pressure by pathogens and other factors (Flajnik and Kasahara 2010). The core elements of adaptive immune systems are now mechanistically understood, such as compartmental differentiation of lymphocytes, the generation of diverse immune recognition, and the associated cellular complexity that develops various cell populations expressing suitable antigen-binding receptors and immunological memory. Immunologists and evolutionary biologists believed these general features of adaptive immunity were exclusive to the higher vertebrates. The discovery of a lymphoid cell–based system of adaptive immunity in jawless vertebrates that is strikingly similar to the system in jawed vertebrates was a total surprise (Pancer et al. 2004, 2005; Rogozin et al. 2007; Kasamatsu et al. 2010; Li et al. 2013; Guo et al. 2009; Hirano et al. 2013). We are within reach of significant advances in our understanding of how adaptive immune systems evolved in the context of "mature" innate immune systems and how these molecularly different systems are related to the evolutionary attainment of immunological complexity.

Immune Response Molecules in Invertebrates and Plants

Common components deployed for innate immune defense in invertebrates and plants may provide insight into how and when our complex adaptive immune systems evolved. Two protein families, which contain either the LRR motifs or the Ig superfamily (IgSF) domains, are widely involved in immune functions.

LRR-containing proteins consist of multiples of 20–30 amino acid units that form horseshoe-like solenoid structures in which parallel β sheets form the concave surface and an array of helices form the convex surface (Buchanan and Gay 1996). The Toll-like receptors (TLRs) are well-defined examples of LRR-containing proteins that function as pattern recognition receptors that constitute key components of innate immune systems throughout the animal kingdom (Hoffmann et al. 1999). Plants have a large number of Toll-like nucleotide binding site (NBS) LRR proteins (Monosi et al. 2004) that function in disease resistance (Fig. 1). Interestingly, sea urchins and amphioxus also have hundreds of *TLR* genes (Pancer and Cooper 2006; Rast et al. 2006).

IgSF members also have major immune defense functions in invertebrates, in addition to their main role as specific antigen receptors in the adaptive immune system of jawed vertebrates. Their roles in innate immunity include the Down syndrome cell adhesion molecule in insects (Watson et al. 2005), fibrinogen-related proteins in snails (Zhang et al. 2004), and the variable region-containing chitin-binding proteins in sea squirt (Azumi et al. 2003) and amphioxus (Cannon et al. 2002). These molecules can be diversified through alternative splicing (or even somatic mutation) to generate potential antigen recognition ability. These examples of invertebrate IgSF usage indicate the remarkable adaptability of Ig domains, although there are no distinctive structural and functional characteristics that can define these invertebrate immune components as direct ancestors of the vertebrate

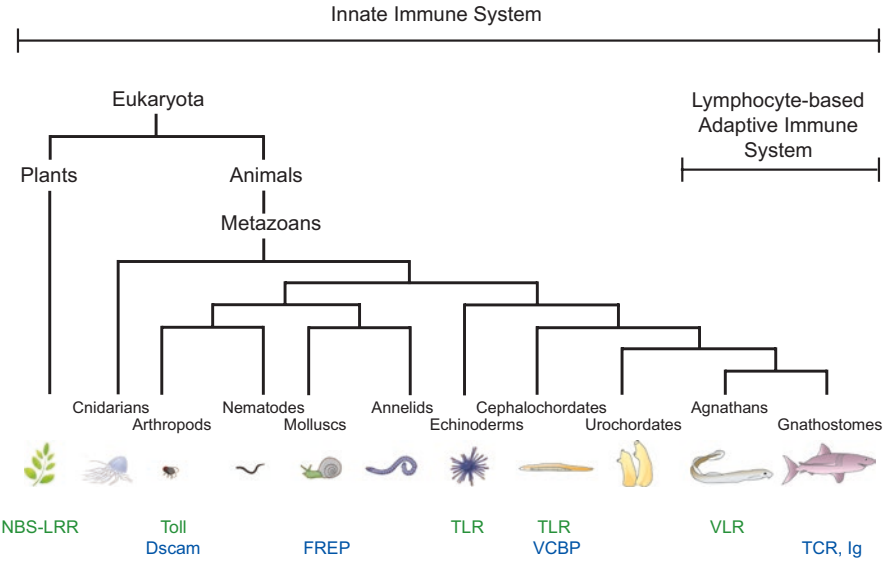
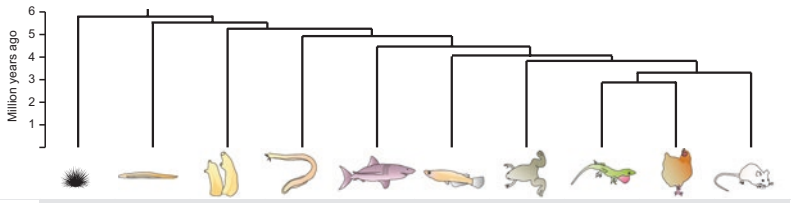


Fig. 1 Hypothetical evolutionary scheme of the emergence of adaptive immunity in conjunction with innate immunity. Families of leucine rich repeat (LRR)-based receptors used as immune molecules are indicated in green: nucleotide binding site–LRR (NBS-LRR), Toll-like receptors (TLRs), and variable lymphocyte receptors (VLRs). Immunoglobulin (Ig)-based receptors used in immune defense are indicated in blue: Down's syndrome cell adhesion molecule (Dscam), fibrinogen-related proteins (FREPs), Variable region-containing chitin-binding proteins (VCBP), T cell receptors (TCRs), and Igs. One representative for each lineage is named in parentheses. (Adapted from Hirano et al. 2011)

TCR and B cell receptor (*BCR*) gene family. An abundance of LRR motifs and IgSF domains were thus readily available for co-option to provide the basic molecular units for use in the somatic diversification of VLR in jawless vertebrates or of Ig/TCR antigen receptors in jawed vertebrates.

Evolution of Immune Cell Types: From Innate Immune Cells to Lymphocytes

As first reported by Metchnikoff in starfish embryos (Metchnikoff 1893), cells with monocyte or macrophage features, are present in protozoans and vertebrates. Unicellular protozoa can also take up pathogenic microbes by phagocytosis and release proteins to protect against pathogens (Korn and Weisman 1967; Takahashi et al. 1996). Protozoa are therefore considered to be a prototype of macrophages, because phagocytic activity is common to macrophages. Professional phagocytic cells that reside in tissues then developed during the evolution from unicellular to multicellular animals. Such cells, although given different names (e.g., archeocytes in Porifera and amoebocytes in Coelenterata), might be primitive macrophages



		Echinoderm	Cephalochordate	Urochordate	Agnathan	Cartilaginous fish	Teleost fish	Amphibian	Reptile	Bird	Mammal
Lymphoid tissues	Thymus	–	–	–	+*1	+	+	+	+	+	+
	Bone marrow	–	–	–	–	–	–	+	+	+	+
	Spleen	–	–	–	–	+	+	+	+	+	+
	Lymph node	–	–	–	–	–	–	–	?	?	+
	GALT	–	–	–	+*2	+	+	+	+	+	+
Cells	Monocyte/macrophage	+	+	+	+	+	+	+	+	+	+
	Dendritic cell	–	–	–	?	?	+	+	+	+	+
	NK cell	–	–	–	?	?	+	+	+	+	+
	αβT	–	–	–	VLRA	+	+	+	+	+	+
	γδT	–	–	–	VLRC	+	+	+	+	+	+
	B	–	–	–	VLRB	+	+	+	+	+	+
Genes	RAG1/RAG2	–	–	–	–	+	+	+	+	+	+
	AID (AICDA)	–	–	–	+*3	+	+	+	+	+	+
	Ig H-chain	–	–	–	–	M,D,NAR	M,D,Z/T	M,X,Y,D,F	M,Y,A,D	M,Y,A	M,G,A,D,E
	Ig L-chain	–	–	–	–	σ,κ,λ	σ,κ,λ	σ,κ,λ	κ,λ	λ	κ,λ
	VLR	–	–	–	A,B,C	–	–	–	–	–	–
	MHC-I/II	–	–	–	–	+	+	+	+	+	+
Functions	Gene rearrangement	–	–	–	–	+	+	+	+	+	+
	Gene conversion	–	–	–	+*4	–	–	–	?	+	+
	Affinity maturation	–	–	–	?	±	±	±	+	+	+
	Class switch	–	–	–	–	–	–	+	+	+	+
	Memory	–	–	–	?	+	+	+	+	+	+
Complement	Alloresponse	+	?	+	+*5	+	+	+	+	+	+
	Lectin pathway	+	+	+	+	+	+	+	+	+	+
	Classical pathway	–	–	–	+*6	+	+	+	+	+	+
	Alternative pathway	+	+	+	?	+	+	+	+	+	+

1: Thymoid, 2: Typhlosole, *3: CDA1/CDA2, *4: Copy choice/template switch assembly, *5: Skin rejection, Mixed lymphocyte reaction (MLR), *6: VLRB-mediated

Fig. 2 Evolution of immune systems, ranging from those in echinoderms to mammals. Key factors for the immune system are described on the left (vertically) and elements of each factor are listed. Existence of the different elements is indicated for each lineage. A phylogenetic tree of animals is shown on the top. (Adapted from Hirano 2015). GALT gut-associated lymphoid tissue

(Cheng and Streisfeld 1963; Bosch and David 1984). Amoeboid phagocytes develop from the mesoderm of tridermic invertebrates. In flatworms, planarian reticular cells with phagocytic functions are macrophage-like cells (Morita 1991). Bona fide monocytes are not found in invertebrate blood but are detected in the blood of lampreys and hagfish; macrophages are observed in their tissues, such as kidneys and gills (Tomonaga et al. 1986). Resident macrophages are observed in various tissues and found in species from cartilaginous fish to mammals. Cartilaginous and bony fish have tissue macrophages (Tizard 2001; Watanabe et al. 1995), and tissue macrophages in frogs are involved in phagocytosis of apoptotic muscle cells during tail involution (Watanabe et al. 1985; Mukaigasa et al. 2009). Macrophages have a ubiquitous distribution in reptile, avian and mammalian tissues (summarized in Fig. 2) (Terebey 1972).

Although the evolution of well-defined natural killer (NK) cells is still unclear, distant metazoans (marine sponges) have been shown to use cytotoxic cells to avoid fusion with the other cells (Paust and von Andrian 2011). In addition, alloresponses have been observed without adaptive immunity in many invertebrates, including cnidarians, echinoderms, and tunicates (McKitrick and De Tomaso 2010). Mononuclear cells with NK-like cell morphology have been observed in earthworms (Cooper et al. 1999). NK cell-like cells in bony fish express Ig-like receptors encoded by a multigene family of novel immune-type receptors (NITRs), which

may regulate cell-mediated cytotoxicity. Thus, NITRs may be functional orthologs of mammalian NK receptors (Cannon et al. 2008). NK-like cells in the Xenopus spleen show spontaneous killing of MHC-negative tumor targets (Yoder 2009), and natural cytotoxic activity has also been found in snakes (Sherif and Ridi 1992). The thymus-independent development of NK-like cells has been observed in chickens (Bucy et al. 1989) and their NK activities are found in the intestinal epithelium (Rogers et al. 2008).

Phagocytic cells form mobile cellular arms for innate immune defenses in almost all of the metazoan species as already described. However, lymphocytes bearing somatically diversified antigen receptors have been found only in the vertebrates, wherein they play fundamental roles in adaptive immunity. The thymus-derived T lymphocytes and bone marrow-derived B lymphocytes are the cellular pillars of adaptive immunity in jawed vertebrates. T and B lymphocytes are primarily responsible for cell-mediated immunity and humoral immunity, respectively, and they collaborate with phagocytic cells and other cells to mediate adaptive immunity efficiently. Migratory long-lived lymphocytes expressing antigen receptors and possessing the potential for self-renewal and selective clonal expansion hence represent an evolutionarily innovative type of specialized immunocompetent cells.

Lymphocyte-like cells have not been recognized in invertebrates, but cells with lymphocyte-like morphology, which express lymphocyte-related genes and respond to pathogenic bacteria with size increase, have been observed in amphioxus (Huang et al. 2007). Lymphocyte-like cells that express much of the molecular machinery used by lymphocytes in jawed vertebrates have been characterized in lampreys and hagfish, the most basal vertebrate representatives (Mayer et al. 2002; Nagata et al. 2002; Najakshin et al. 1999; Uinuk-Ool et al. 2002). The latter findings coupled with earlier observations that lampreys and hagfish produce specific agglutinins following immunization with bacteria and foreign red blood cells suggested that jawless vertebrates could have a lymphocyte-based adaptive immune system (Finstad and Good 1964; Fujii et al. 1979a, b; Linthicum and Hildemann 1970).

Evolution of Lymphoid Tissues

Specialized tissues used for vertebrate adaptive immunity are classified into primary and secondary lymphoid tissues. Primary lymphoid tissues (the bone marrow and thymus) are defined by their ability to differentiate from progenitor cells to lymphocytes. Secondary lymphoid tissues are structured for the necessary interaction of immune effector cells to perform immune responses.

The B lymphocytes are generated in hematopoietic tissues, such as fetal liver, bone marrow and kidney, which are spatially separated from the niches where erythroid and myeloid cells develop. The development of T lymphocytes from their progenitors occurs only in the thymus or thymus-equivalent lympho-epithelial tissue. Lamprey T-like cells, which express VLRA or VLRC, develop in a region at the tips of gill folds called the thymoid (Hirano et al. 2013; Bajoghli et al. 2011). The spleen or a spleen-like organ is most conserved among the secondary lymphoid

tissues, being present in all jawed vertebrates. Well-formed lymph nodes are more recently developed tissues, which is restricted to mammals. Comparison of locational and functional features of lymphoid tissues can reveal the development and evolution of adaptive immune responses (Fig. 2).

All jawed vertebrates possess a thymus for T cell maturation, but the precise anatomical position and organization of thymopoietic tissues varies greatly among vertebrate species. In sharks, thymic development is associated with specific pharyngeal pouches (Lloyd-Evans 1993); in teleosts, thymic tissue emanates primarily from the third pharyngeal pouch (Zapata et al. 2006). In birds, seven pairs of thymic portions are arranged in a string-like pattern along the major neck vessels (Kendall 1980). In mammals, the thymic lobes derived from the third and fourth pharyngeal pouches migrate and are placed in the thoracic region overlying the heart (Miller 1961), although ectopic loci of thymic tissue are occasionally found in the neck region (Terszowski et al. 2006).

Canonical bone marrow is found in amphibians, birds, and mammals (Fig. 2). Early experiments using larval frogs discovered that the lymph gland (LM1), which disappears at metamorphosis, is blood filtering and the center of humoral immunity until the bone marrow develops when frogs emerge onto land (Cooper 1971, 1976; Woodhams et al. 2016). LM1 is packed almost exclusively with lymphocytes separated by sinusoids that filter blood (Cooper 1971, 1976; Klempau and Cooper 1984). In bony fishes, the most cranial portion of kidney, the head kidney, is the bone marrow equivalent. It contains hematopoietic and lymphoid cells, and serves as the major hematopoietic tissue of bony fish throughout life (Zapata et al. 2006). The Leydig and epigonal organs are the bone marrow equivalents in cartilaginous fishes (Fange and Pulsford 1983). The Leydig organ, unlike the epigonal organ, is not found in all elasmobranch species.

Adaptive Immune System in Jawed Vertebrates

The mechanisms of the Ig-based adaptive immune system are well-defined in mammals. The two major lineages of clonally diverse lymphocytes that specifically recognize and respond to antigens are named T and B lymphocytes because they are generated in the thymus and the bone marrow (or the avian bursa of Fabricius), respectively (Cooper et al. 1965; Greaves et al. 1968). The T and B lymphocyte progenitors are derived from multipotent hematopoietic stem cells (Moore and Owen 1965; Owen et al. 1965). During their early developmental stages, the T and B lymphocyte progenitors rearrange different sets of variable (*V*), diversity (*D*), and/or joining (*J*) gene segments to generate the antigen binding regions of the TCRs and BCRs (Hedrick et al. 1984; Tonegawa 1983; Yanagi et al. 1984). The recombination-activating genes (*RAG1*/*RAG2*) encode enzymes that mediate *V*(*D*)*J* rearrangement (Schatz et al. 1989). The RAG1 and RAG2 proteins were found to recognize the recombination signal sequences (RSSs) flanking the *V*(*D*)*J* gene segments to initiate the double-stranded DNA breaks and the recruitment of other proteins required for recombination (Jung and Alt 2004; Oettinger et al. 1990). RAG1/RAG2 proteins form a transposase that can excise DNA containing the

recombination signal sequences and reinsert it elsewhere, thus supporting the theory that RAG1/RAG2 were once components of a transposable element (Agrawal et al. 1998; Hiom and Gellert 1997).

The antigen-binding domains of the different *V*(*D*)*J* combinations are diversified further through splicing variability and the enzymatic addition of nucleotides in the joints created during *V*(*D*)*J* segment assembly (Dudley et al. 2005). This random nature of diversification inevitably results in the generation of Ig/TCR repertoires that recognize self-antigens, requiring that T and B lymphocytes bearing potentially harmful receptors be eliminated or anergized in their thymic and bone marrow origins (Goodnow et al. 2005; Jameson et al. 1995; von Boehmer 2004). The selected populations of long-lived T and B cells then enter the circulation to begin the body guard via a migratory route that involves entry into the secondary lymphoid tissues, where they encounter invading pathogens, and their subsequent return to the stream (Gowans and Knight 1964).

The T cells can use their TCRs to recognize peptide presented by antigen presenting cells (APCs) within cell surface proteins encoded by the major histocompatibility complex class I and class II (*MHCI* and *MHCII*) genes (Bjorkman et al. 1987; Unanue 1980; Zinkernagel and Doherty 1974). T cells thus typically recognize antigens that are partially processed within specialized APCs, mainly dendritic cells, phagocytes, and B cells (Steinman et al. 1999; Storni and Bachmann 2003). In contrast, the secreted and membrane-bound antibodies made by B lymphocytes normally recognize exposed epitopes of intact molecules, including cell surface protein and carbohydrate moieties of invasive microorganisms. The Ig-based BCR and TCR antigen-binding proteins are associated with other transmembrane molecules that can trigger intracellular signaling to induce expression of genes necessary for immune responses. In most antigen-induced responses, the B cells receive help from T cells for their activation (Paul 2008).

Variable Lymphocyte Receptor (VLR)-Based Adaptive Immune System in Jawless Vertebrates

VLR Discovery in Jawless Vertebrates

Since a transcriptome analysis of lymphocyte-like cells of naïve lampreys was unsuccessful in revealing evidence of equivalent adaptive immune systems in jawed vertebrates, lamprey larvae were stimulated by an antigen and mitogen mixture to survey the transcriptome of activated lamprey lymphocytes. The purpose of stimulation was to catch the lymphocytes in the act of an immune reaction. Large activated lymphoblasts in blood were then sorted by their light scatter characteristics and used for the construction of a complementary DNA (cDNA) library (Pancer et al. 2004). Still no orthologs of Ig, TCR, and MHC genes were detected, but this experiment revealed a large number of transcripts for uniquely diverse LRR proteins, which were named VLRs because of their lymphocyte-restricted expression and sequence diversity. Each VLR transcript was found to encode a conserved

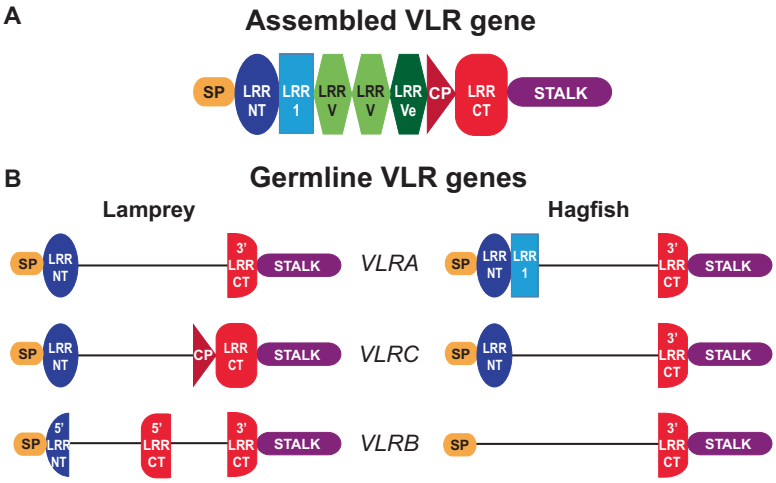


Fig. 3 Schematic depiction of the lamprey and hagfish variable lymphocyte receptor (VLR) genes. (**a**) Assembled 'mature' VLR: signal peptide (SP), N-terminal leucine rich repeat (LRR) (LRRNT), first LRR1, two variable VLR (LRRV), end LRRV (LRRVe), connecting peptide (CP), C-terminal LRR (LRRCT), stalk region. (**b**) Lamprey and hagfish germline *VLRA*, *VLRC*, and *VLRB* genes. Lamprey *VLRB* has two large non-coding intervening sequences separating the 5′LRRNT and 3′LRRCT, whereas all other VLR genes have a single, short intervening sequence (not drawn to scale). (Adapted from Herrin et al. 2015)

signal peptide (SP) followed by highly variable LRR modules: a 27–38 residue N-terminal LRR (LRRNT), the first 18-residue LRR (LRR1), variable numbers of 24-residue variable LRRs (LRRV), one 13-residue connecting peptide LRR (LRRCP), and a 48- to 65-residue C-terminal LRR (LRRCT) (Fig. 3).

After the discovery of the first lamprey VLR (now known as VLRB), two hagfish VLR genes, *VLRA* and *VLRB*, were identified in an expressed sequence tags (EST) database of hagfish leukocyte transcripts (Pancer et al. 2005). The homolog of *VLRA* in lampreys was identified by a subsequent search of the draft genome sequence database of the sea lamprey (Rogozin et al. 2007). A third VLR, designated VLRC, has been identified through an analysis of the sea lamprey EST database (Kasamatsu et al. 2010). The predicted VLRC structure is very close to that of lamprey VLRA and VLRB, except that VLRC lacks the thumb-like protrusion encoded in the LRRCT inserts of VLRA and VLRB that is critical for antigen recognition. We found the third VLR gene by searching the hagfish transcriptome data and by cDNA cloning (Li et al. 2013). The gene was phylogenetically close to the lamprey *VLRA* gene (Li et al. 2013).

Ancient T Cell-Like and B Cell-Like Lymphocyte Populations in Jawless Vertebrates

Two T cell-like populations of cells, VLRA and VLRC cells, and B cell-like lymphocytes, VLRB cells, have been characterized in lampreys (Fig. 4) (Pancer et al. 2004; Guo et al. 2009; Hirano et al. 2013; Alder et al. 2008). The VLRA$^+$

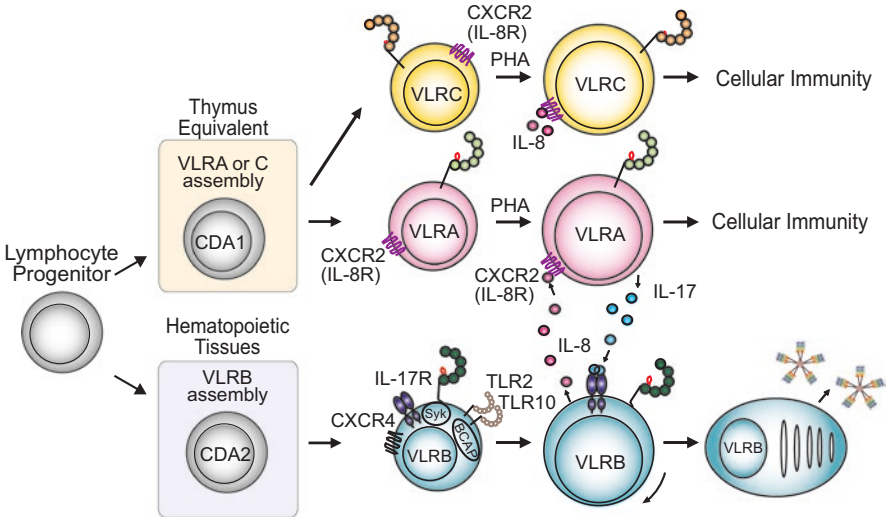


Fig. 4 Characteristics of variable lymphocyte receptor (VLR)-expressing lymphocyte lineages. In this simplified scheme, a hypothetical lymphoid progenitor gives rise to precursors that differentiate, after gene assembly in the thymus equivalent, presumably mediated by cytidine deaminase 1 (CDA1), into VLRC- and VLRA-expressing T-like cells. These cells express, in addition to their antigen receptors, other cell surface molecules, such as C-X-C chemokine receptor (CXCR) 2, that allow them to communicate with other immune cells, in this example those that secrete interleukin (IL)-8. After stimulation, they may themselves secrete effector molecules, such as IL-17, that can activate other cell types, in this example VLRB-expressing B-like cells. *VLRB* gene assembly in hematopoietic tissues is thought to be mediated by CDA2. The VLRB$^+$ cells can express chemokine receptors (for instance, CXCR4), cytokine receptors (such as IL-17 receptor [IL-17R]), and pattern recognition receptors, such as Toll-like receptors (TLRs). Antigenic stimulation of VLRB$^+$ lymphocytes may lead to limited clonal amplification and differentiation into plasma cells that secrete VLRB antibodies. For detailed references, see text. (Adapted from Boehm et al. 2018)

and VLRC$^+$ cells are generated in the thymus-equivalent (called thymoid) region at the tip of each gill fold (Bajoghli et al. 2011). Both of the cell types resemble the T lymphocytes in jawed vertebrates. They express their receptors only on the cell surface, respond preferentially to the classical T cell mitogen (phytohemaglutinin [PHA]) and express similar cytokine, chemokine, and transcription factor gene profiles (Guo et al. 2009; Hirano et al. 2013). On the other hand, the VLRB$^+$ cells seem to be generated in hematopoietic tissues, express their antigen-binding receptors on the cell surface and respond to antigenic stimulation by differentiating into plasma cells that secrete multimeric VLRB antibodies (Guo et al. 2009; Hirano et al. 2013; Alder et al. 2005, 2008; Herrin et al. 2008).

Hagfish also have three distinct lymphocyte populations which express VLRA, VLRB, or VLRC. These cells have not been well-characterized yet, but the currently existing data suggest that, like their lamprey counterparts, hagfish VLRB$^+$ lymphocytes are B-like and the VLRA$^+$ and VLRC$^+$ lymphocytes are T-like cells.

Gene Expression Profiles for T Cell-Like and B Cell-Like Lineages

Discriminating gene expression profiles in the three different lymphocyte populations include molecules for transcription factors, cytokine/chemokines and their receptors, integrins, TLRs, and various signaling proteins. VLRB$^+$ lymphocytes preferentially express gene orthologs of those that are expressed by B cells in jawed vertebrates (Guo et al. 2009; Hirano et al. 2013). These contain the hematopoietic progenitor homing receptor C-X-C chemokine receptor (CXCR) 4; the B cell transcription factors E2A, paired box protein 5 (Pax5), PR domain zinc finger protein 1 (also called BLIMP1), and B cell chronic lymphocytic leukemia (CLL)/lymphoma 6 (BCL6); the herpes virus entry mediator/tumor necrosis factor receptor superfamily member 14 (TNFRSF14) that binds to LIGHT on T cells; the interleukin (IL)-8 chemotactic inflammatory cytokine, IL-17 receptor; TLR2, TLR7 and TLR10; and two components of the BCR-mediated signaling cascades, the spleen tyrosine kinase (Syk) and B cell adaptor protein (BCAP) (Fig. 4). In contrast, the VLRA$^+$ lymphocytes preferentially express genes orthologous to those typically expressed by T cells in jawed vertebrates: several transcription factors that are used for T cell differentiation such as GATA binding protein 2/3 (GATA2/3), c-Rel, aryl hydrocarbon receptor (AHR), and BCL11b transcriptional factors used for T cell differentiation; the CCR9 chemokine receptor involved in thymus homing of lymphocyte progenitors; the T cell fate-determining molecule Notch1; the tyrosine phosphatase receptor CD45 that is essential for T cell development; and pro-inflammatory cytokines IL-17, macrophage migration inhibitory factor (MIF), and the IL-8 receptor CXCR2 (Guo et al. 2009). The transcriptional profile of VLRC$^+$ cells is similar to that of VLRA$^+$ cells, except that the VLRC$^+$ cells differ (Hirano et al. 2013) in their preferential expression of the SRY-box containing gene 13 (SOX13) encoding a fate-determining transcription factor, which is used for γδ T cell lineage commitment, an integrin αL (ITGAL), one component of the heterodimeric lymphocyte function-associated antigen 1 (LFA1), the very late antigen 4 (VLA4) components integrins α4 and β1 (ITGA4 and ITGB1), whose expression correlates with the adherence of human γδ T cells to epithelial cells, TLR3, and a modulator of T cell activation, IL-16. The transcriptional platforms for lamprey VLRA and VLRC cells are hence reminiscent of those of αβ and γδ T cells in jawed vertebrates, respectively.

Gene Assembly/Rearrangement Mechanisms for Somatic Diversification of Antibodies in Jawless/Jawed Vertebrates

Antigen receptor diversification of jawed vertebrates is mediated by two DNA alteration mechanisms: 1) RAG-mediated *V*(*D*)*J* recombination, and 2) activation-induced deaminase [AID]-mediated somatic hypermutation, class switch recombination and gene conversion (Flajnik 2002). Jawless vertebrates are instead thought to use AID/APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) family cytidine deaminases for antigen receptor assembly.

V(D)J Recombination for Immunoglobulin-Type Receptors in Jawed Vertebrates

Ig loci in mammals, birds, reptiles, and amphibians have a translocon configuration which features the location of multiple *V* segments upstream of multiple *D* and *J* segments (Fig. 5a) (Flajnik 2002). Chicken H and L chain genes also possess a translocon

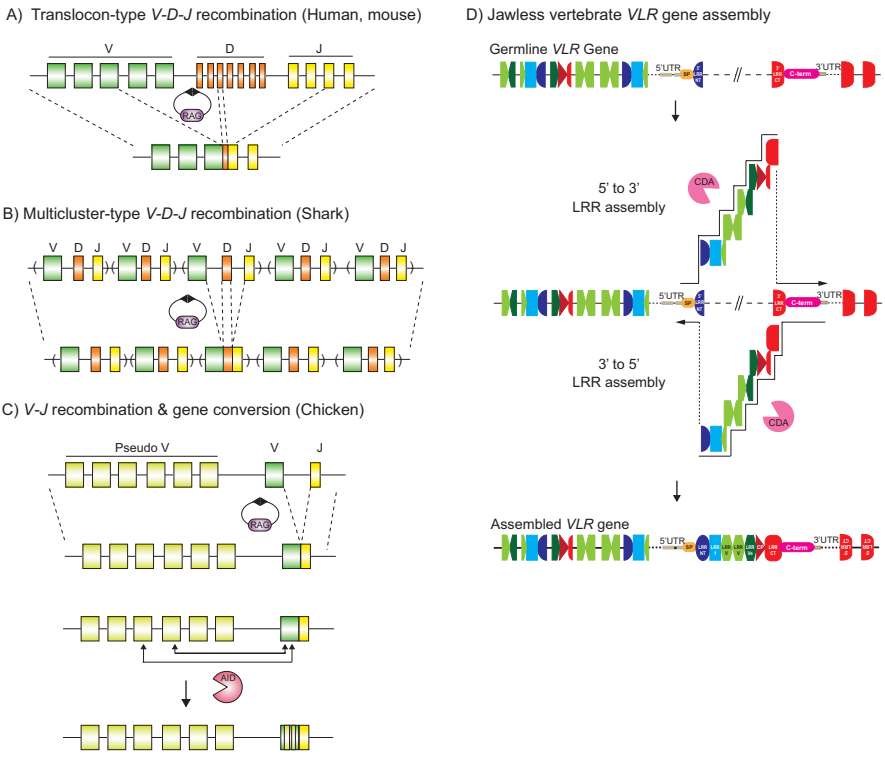


Fig. 5 Contrasting gene assembly mechanisms in jawed and jawless vertebrates. (**a**) Human and mouse immunoglobulin (Ig) loci form translocon configuration in which *V* (variable), *D* (diversity), or *J* (joining) gene segments are rearranged by recombination-activating gene (RAG) 1/RAG2 to make mature Igs. (**b**) Sharks (cartilaginous fish) also rearrange *V*, *D*, and *J* segments by RAG1/RAG2, but one set of *V(D)J* cluster is selected by an individual cell for rearrangement from the multiple sets of *V(D)J* units, multiclusters, in Ig loci. (**c**) Chicken H and L chain genes have a translocon configuration, but there are multiple pseudo *V* genes upstream of one functional V segment. Chickens use gene conversion to achieve Ig diversity and specificity after an initial RAG-mediated *V(D)J* gene rearrangement. The V region is extensively replaced using upstream pseudo *V* genes by AID-mediated gene conversion. (**d**) Variable lymphocyte receptor (VLR) gene assembly by a gene conversion-like mechanism. The germline VLR genes are flanked by hundreds of leucine rich repeat (LRR) cassettes. The non-coding intervening sequence between parts of the N-terminal LRR (LRRNT) and C-terminal LRR (LRRCT) is replaced by LRR fragments that are sequentially copied using the flanking donor LRR cassettes as templates. Mature VLR genes are assembled from either the LRRNT or LRRCT ends in a stepwise manner, which is directed by short sequence homology between the donor and acceptor LRR sequences for the completion of VLR gene assembly. (Adapted from Hirano 2015)

configuration, but multiple pseudo *V* genes are located upstream of the functional *V* segments. In chickens and rabbits, gene conversion is the primary mechanism used for Ig diversification. The initial *V*(*D*)*J* region is extensively modified using upstream *V* genes by gene conversion mechanism after *V*(*D*)*J* rearrangement (Fig. 5c). In cartilaginous fish, Ig loci form a multicluster configuration in which there are multiple clusters of single *V*(*D*)*J* units (Fig. 5b). Interestingly, the L chain segments form multiclusters in bony fish, whereas the H chain segments are located in a translocon configuration (Flajnik 2002), suggesting that the Ig genes emerged as multiclusters and evolved to a translocon configuration. AID-mediated Ig class switching has been observed in amphibians and cartilaginous fishes (Zhu et al. 2012; Chaudhuri et al. 2007; Basu et al. 2009). The TCRs are IgSF members and structurally similar to Igs. The TCR gene segments are assembled by somatic rearrangement from sets of *V*(*D*)*J* gene segments in the same way as Ig genes. Cartilaginous fishes have α, β, γ, and δ TCR genes, suggesting that all four TCR loci evolved early in jawed vertebrates and have not changed much throughout jawed vertebrate evolution (Rast et al. 1997).

Gene Assembly for Leucine Rich Repeat (LRR)-Type VLR Proteins in Jawless Vertebrates

Sequence diversity is observed primarily in the 3′-portion of LRRNT (3′LRRNT), LRR1, LRRV, terminal LRR variable modules (LRRVt), connecting peptide (CP), and the 5′-portion of LRRCT (5′LRRCT) are variable regions, and the 5′ portion of LRRNT (5′LRRNT) and the stalk region are constant regions of VLRs. In their germline configuration, all VLR genes are incomplete in that they normally have coding sequences only for the leader sequence and incomplete amino- and carboxyl-terminal LRR subunits (LRRNT and LRRCT) and stalk region (Fig. 5d) (Pancer et al. 2004, 2005; Rogozin et al. 2007; Kasamatsu et al. 2010; Li et al. 2013). Each germline VLR gene is flanked by hundreds of LRR-encoding sequences designated donor cassettes, because they can serve as randomly selected templates to add the LRR sequences required for production of a complete VLR gene (Fig. 5d).

A gene conversion-like mechanism has been proposed for the VLR gene assembly process (Alder et al. 2005; Nagawa et al. 2007) in which the intervening sequence is replaced in a stepwise way by random choice of donor LRR cassettes to serve as templates for copying the sequences necessary to complete a VLR gene (Fig. 5d). The assembly process can be initiated at either the 5′LRRNT or the 3′LRRCT ends using short stretches of nucleotide homology (6–30 base pairs) between donor and acceptor regions (Rogozin et al. 2007; Alder et al. 2005; Nagawa et al. 2007).

CRISPR/Cas and RNA Interference Systems

In terms of defense against genomic parasites such as phage, virus and plasmid, prokaryotes deployed the RNA-based immune defense mechanisms: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas systems (Jinek et al. 2012; Cooper and Overstreet 2014). After virus infection or plasmid conjugation, their CRISPR/Cas system recognizes and cleaves the exogenous DNA. The fragmented foreign DNA is incorporated into the leader end of the CRISPR locus as a novel repeat-spacer unit. On a second infection or transfer, the CRISPR repeat-spacer unit is transcribed to a pre-CRISPR RNA (crRNA). A Cas9 nuclease complex digests the pre-crRNA into crRNAs which guide the Cas9 complex to the target nucleic acid in the corresponding second invasion. This mechanism thus functions as a bacterial adaptive immune system (Deveau et al. 2010), albeit one that contrasts with the vertebrate adaptive immune systems. Plants have antiviral mechanism using RNA interference (RNAi), which provides nucleotide sequence-specific immune protection (Baulcombe 1996). The somatic RNAi molecules are acquired from virus and recognize specific sequences that are homologous to foreign RNA, resulting in inactivation of specific viruses (Voinnet 2001; Rimer et al. 2014). *Caenorhabditis elegans* and *Drosophila* also have the similar RNAi antiviral defense systems (Fire et al. 1998; Lu et al. 2005; Bronkhorst et al. 2012). In summary, bacteria, archaea, plants, nematodes, and arthropods have adaptive (acquired)-type immune systems in addition to innate-type immune defenses such as restriction-modification systems in prokaryotes (Wilson and Murray 1991).

Conclusions

Different species have their own adaptive (or acquired) immune systems that are genetically unrelated (one did not evolve from the other). Each type of system evolved independently but it keeps the same procedure for its function. Individuals learn from their own immune experiences and adjust their responses accordingly.

Both internal and external environments forced organisms to evolve their immune systems. Internally, organisms have evolved compartmentalized functions for nutrition intake (and oxygen uptake for multicellular organisms with mitochondria), reproduction, and transformation. Externally, organisms have evolved in response to multiple pathogens (Flajnik and Kasahara 2010) that escape the host immune defense (Bergstrom and Antia 2006; Schmid-Hempel 2008). Survived organisms also have to maintain a symbiosis with commensals and the microbiome requires balanced immune regulation (Kau et al. 2011). Simpler organisms less than vertebrates have fewer requirements for maintaining pathogen response and symbionts (Pancer and Cooper 2006; Litman et al. 2005), because they keep fewer cells and tissues. For prokaryotes, the relatively simple acquired immune system CRISPR/Cas9 is sufficient. The system targets genomic parasites and their DNAs or RNAs are incorporated into the host genome for the next threats and also transferred to future generations. Ancient multicellular invertebrates began to produce

professional innate-type immune cells, and also developed another basic acquired immune mechanism, RNAi. Both CRISPR/Cas9 and RNAi molecules are templated by specific polynucleotide sequences of the pathogens. They just maintain the polynucleotide sequences for the future immune responses. However, by using simple RNA strands these solutions are relatively limited in their effector mechanisms and are directed mainly against viruses or phages. More complex organisms require more and different immune strategies to take care of different defense tasks and to regulate different parasites and symbionts at different tissue types. Arthropods and molluscs have developed alternative splicing methods to generate immune response molecules (Down syndrome cell adhesion molecule in arthropods and fibrinogen-related proteins in molluscs). Those mechanisms could be sufficient for their internal complexity and for defense against bacterial pathogens, although they are more restricted than vertebrates in their combinatorial diversity (Rimer et al. 2014).

Vertebrates, much more complex multicellular organisms, have to handle microorganism pathogens and symbionts, and also to maintain more different tissues (Cohen 2007). Therefore, they evolved novel somatic strategies for generating combinatorial diversity. The strategies of these more complex organisms have been to prepare in advance, using somatically generated diversity mechanisms. A large number of recognizing molecules are randomly produced at low copy number. Upon demand (antigen encounter), the system moves to mass production (clonal expansion) and can advance to customized specificity (affinity maturation). Important patterns are preserved for future needs as immune memory. Adaptive immune cells, lymphocytes, have evolved on the basis of innate immune cells and express a variety of immune receptors (e.g., Igs, TCRs, VLRs).

Macfarlane Burnet has proposed that cells in the mammalian immune system could have evolved from the hemocytes in the invertebrates, with the view that such moving phagocytic cells are ancestral lymphocytes (Burnet 1968). Phagocytic cells are well-known to play a critical role in innate immune defense of the evolutionarily ancient starfish (Metchnikoff 1893; Cooper et al. 2002). In teleost fish and frogs, B lymphocytes have also been shown to have phagocytic activity (Li et al. 2006) in keeping with the idea that B cells are the evolutionary derivatives of phagocytes. It is interesting to note also that VLRB cells of jawless vertebrates express many of the TLRs that phagocytes and B cells of jawed vertebrates may express. Cells with cytotoxic competence could also have diverged from primitive phagocytic cells in that a protease for cellular cytotoxicity (Bilej et al. 1998) and allograft rejections have been reported in earthworms (Cooper et al. 1999) and the coelomocytes in sea urchin (phagocytic amoebocytes) show cytotoxic activity (Lin et al. 2001). Cytotoxic NK and T cells may have been derived from a common ancestor, given that they share many properties including cytotoxic granule production (Cooper 2006; Litman et al. 2010; Sun and Lanier 2009).

The demonstration of cells that resemble lymphocytes in amphioxus (Huang et al. 2007), which is considered to be the representative head of the chordate lineage (Putnam et al. 2008), and the presence of T- and B-like lymphocyte lineages in both jawless and jawed vertebrates may imply that bifurcation of the lymphocyte

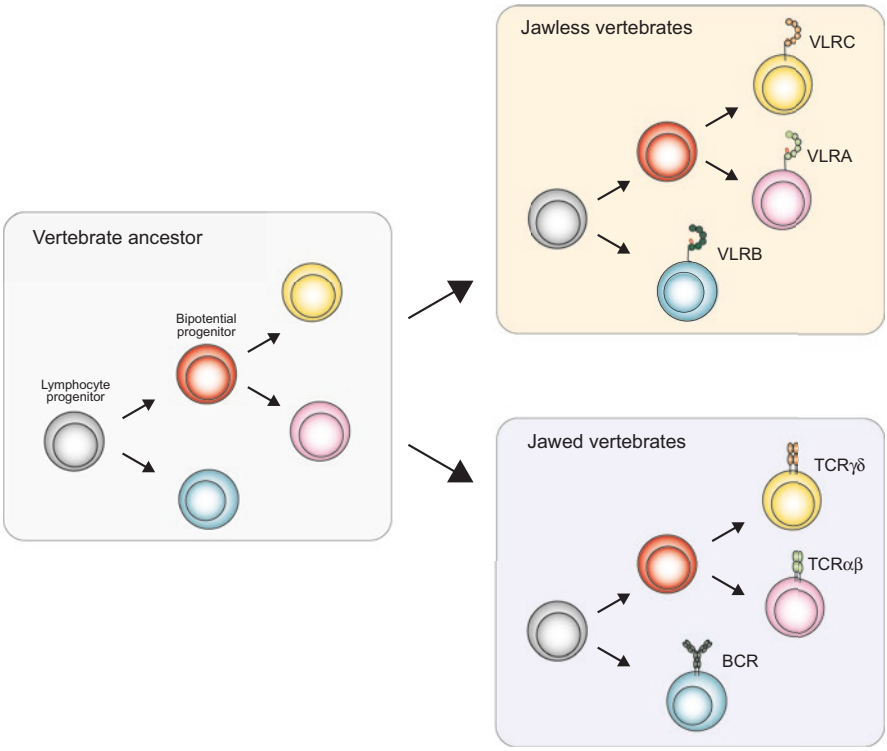


Fig. 6 Evolution of three major lymphocyte differentiation pathways in vertebrates. The two sister groups of vertebrates evolved different structural proteins and mechanisms to generate clonally diverse antigen receptors. Recent studies indicate that two T cell lineages and one B cell lineage emerged before the convergent evolution of different types of somatically diverse anticipatory receptors in cyclostomes and gnathostomes. It remains unknown which of T or B cells evolved first and which of the receptor genes came first: the *VLRA* or *VLRC* or *VLRB* gene in jawless vertebrates or the *TCRαβ* or *TCRγδ* or *BCR* genes in jawed vertebrates. The three primordial lymphocyte lineages might have served different functions in a primordial "non-adaptive" immune system of vertebrate ancestors, even before the development and deployment of somatically diversified antigen receptors. (Adapted from Hirano 2015). BCR B cell receptor, TCR T cell receptor, VLR variable lymphocyte receptor

lineage preceded the emergence of the diverse anticipatory receptors that characterize the alternative lymphocyte-based adaptive immune systems. This view would be consistent with observations suggesting that B and T cells differentiate from myeloid B progenitors and myeloid T progenitors, respectively (Bell and Bhandoola 2008; Wada et al. 2008).

This phylogenetic study of immunity indicates that all of the surviving vertebrates, both jawless and jawed, have a lymphocyte-based adaptive immune system (Fig. 6). This fact alone suggests a strong survival advantage for an adaptive immune system. The convergent evolution of two very different forms of clonally

diverse anticipatory receptors to achieve adaptive immunity in jawless and jawed vertebrates further attests to the survival value of an adaptive immune system, although we are unlikely ever to know whether or not other vertebrates fell victim to pathogen-mediated extinction because they failed to acquire a recombinatorial immune system.

Our analysis further indicates that the strategy of functionally interactive T and B lymphocyte arms is a fundamental feature of an adaptive immune system. The reason for this may be the inherent threat of autoimmunity, which is inevitable with the emergence of an adaptive immune system featuring a randomly generated receptor repertoire being expressed by lymphocytes with pro-inflammatory potential. The two functionally cooperating arms of an adaptive immune system could be essential to achieve balance and self-regulation. In keeping with this idea, one would anticipate that the repertoire of VLRA, VLRB, and VLRC lymphocytes is selected, beginning within the thymus equivalent for the VLRA and VLRC cells. Obviously, much remains to be learned about the biology of the agnathan T-like and B-like cells. At this point we can only conclude that the remarkable complexity of our integrated innate and adaptive immune systems is the result of strong and continuing selection, most probably to improve the possibility of pathogen protection.

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Chondrichthyes: The Immune System of Cartilaginous Fishes

Helen Dooley

Introduction

Cartilaginous fishes (Chondrichthyes) diverged from a common ancestor with other vertebrates approximately 450 million years ago (MYA) and comprise two extant subclasses, the Holocephali (chimaeras, such as the elephant shark, rat fishes, and rabbit fishes) and the much better known Elasmobranchii (sharks, skates, and rays), which split about 420 MYA (Inoue et al. 2010). Recovery of cartilaginous fishes following the Permian and Jurassic extinctions facilitated their diversification (Sorenson et al. 2014), giving rise to the >1000 cartilaginous fish species classified today, occupying almost every marine niche. Although they show huge diversity in their body forms and life histories, most grow slowly and sexually mature late; an extreme example is the Greenland shark (*Somniosus microcephalus*), whose females mature at ~150 years old and have a lifespan of over 350 years (Nielsen et al. 2016)—traits that put them at extreme risk of extinction through overfishing (Dulvy et al. 2014).

Because of their key phylogenetic position, cartilaginous fishes are an important research model to investigate the evolution of many fundamental vertebrate characteristics, such as hinged jaws, paired limbs, and an adaptive immune system. While lymphocyte-like cells secreting somatically recombining variable lymphocyte receptors (VLRs) (Pancer et al. 2004, 2005) have now been identified in the jawless fishes (hagfish and lamprey), the cartilaginous fishes are the most phylogenetically distant group relative to mammals to have an adaptive immune system that is also based on rearranging immunoglobulin (Ig) superfamily (IgSF) molecules, specifically Igs and T-cell receptors (TCRs), and polymorphic/polygenic major histocompatibility complex (MHC) molecules (Flajnik 2014).

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Our understanding of the cartilaginous fish immune system has increased considerably since the first functional studies were performed in the 1960s (Clem and Small 1967; Clem et al. 1967; Marchalonis and Edelman 1965; Day et al. 1970). The development of polymerase chain reaction and related molecular techniques enabled the sequencing of individual cartilaginous fish immune genes and/or transcripts, primarily by homology-based cloning. However, the large evolutionary distances involved, and the fast evolutionary rate observed for many immune genes, meant that this knowledge was hard won and, compounded by the use of many different study species, often fragmentary. Draft genomes have been published recently for the elephant shark (*Callorhinchus milii*) (Venkatesh et al. 2014a), little skate (*Leucoraja erinacea*) (Wyffels et al. 2014; Wang et al. 2012), and whale shark (*Rhincodon typus*) (Read et al. 2017) [with that of small-spotted catshark (*Scyliorhinus canicula*) well underway], as well as a rapidly increasing number of transcriptomes from different cartilaginous fish species [including the elephant shark (Venkatesh et al. 2014a), little skate (Wyffels et al. 2014; Wang et al. 2012), smooth hammerhead (*Sphyrna zygaena*) (Goshima et al. 2016), and small-spotted catshark (Mulley et al. 2014; Redmond et al., unpublished data)]. With increasing access to such sequence resources, we are beginning to fill the remaining gaps in our knowledge, allowing us to gain a more complete understanding of immunity in this key vertebrate group. This chapter summarizes our current knowledge regarding immune protection in these enigmatic animals and highlights some of the key questions that remain.

Innate Immunity in Cartilaginous Fishes

Pattern Recognition Receptors

Pattern recognition receptors (PRRs) are broadly specific, germline-encoded receptors that play a key role in pathogen recognition. These receptors identify so-called pathogen-associated molecular patterns (PAMPs), highly repetitive molecules such as lipopolysaccharides (LPSs), nucleic acids, peptidoglycans, lipoproteins, and glucans, as well as damage-associated molecular patterns (DAMPs), intracellular components released as a consequence of damage or death (Medzhitov and Janeway 1997).

Little is known about the repertoire of PRRs in cartilaginous fishes; however, homologs of various nucleotide oligomerization and binding domain (NOD)–like receptors (NLRs) have been annotated in the elephant shark genome, including genes for the intracellular PRRs *NOD1*, *NOD2*, and *NOD4*, and a substantial number (>50) of *NLRP3*-like genes (a key component of the inflammasome) (Venkatesh et al. 2014a). Along with cytoplasmic helicases such as *DHX58*, *IFIH1*, and *DDX58*, and the nucleic-acid sensing Toll-like receptors (TLRs; *TLR3*, *TLR7*, *TLR8*, and *TLR9*, all of which were clearly identifiable in the elephant shark genome), these PRRs monitor the intracellular environment for pathogen-derived PAMPs. In addition to the four intracellular TLRs, six more TLRs were found in the elephant shark

genome: two that showed homology to the mammalian TLRs 1, 6, and 10; two with homology to mammalian TLRs 2 and 5; and two that could not be assigned based on homology to mammalian TLRs and are likely to be "fish-type" TLRs (Kasamatsu et al. 2010; Roach et al. 2005). Surprisingly, no ortholog for *TLR4* was identified; upon closer inspection of the *TLR4* syntenic region, however, a pseudogene fragment was found (Venkatesh et al. 2014a), suggesting this gene has been secondarily lost. In mammals TLR4 does not bind LPS directly but requires various extracellular components to facilitate binding—namely LPS-binding protein (LBP), which binds LPS and transfers it to CD14, a Glycosylphosphatidylinositol (GPI)-anchored membrane protein that in turn transfers the LPS to the MD2–TLR4 complex, inducing TLR4 dimerization and subsequent downstream signaling (Ryu et al. 2017). The genes for all three of these components (*LBP*, *CD14*, and *MD2*) were also absent from the elephant shark genome, indicating loss of the entire TLR4 extracellular pathway (Venkatesh et al. 2014a) and likely explaining the previously observed lack of LPS responsiveness in elasmobranchs (Dooley and Flajnik, unpublished data).

The only cartilaginous fish PRR to be studied at a functional level to date is TLR2, the expression of which was analyzed in a range of bamboo shark (genus *Chiloscyllium*; species unknown) tissues before and after stimulation with TLR ligands; subcutaneous administration of peptidoglycan (a TLR2-agonist in mammals) upregulated *TLR2* transcription at the immunization site, whereas a more general upregulation in all of the tissues examined was induced 24 h after administration of a mix of agonists (peptidoglycan, LPS, poly I:C, and flagellin) into the peritoneal cavity (Anandhakumar et al. 2012). It is obvious that further functional work is required to catalog the range of agonists bound by cartilaginous fish TLRs and the effector pathways triggered after ligation.

Complement

The complement system is a cascade of soluble and cell-associated proteins responsible for the elimination of invading pathogens. Mammals have three pathways through which the complement system can be activated: the classical, alternative, and lectin pathways (Fig. 1a). During classical pathway activation antibody–antigen complexes are bound by the complement component C1q, which recruits the serine proteases C1r and C1s, forming the "C1 complex." C1r activates C1s, which in turn proteolytically cleaves C2 and C4, whose products (C4b + C2a) form C3 convertase. C3 is the lynchpin of the complement system and the point at which the three activation pathways converge. The activation of C3 produces the anaphylotoxin C3a and thioester-containing protein (TEP) C3b. The exposure of this reactive thioester bond allows C3b to bind to hydroxyl and amine groups on the surface of invading pathogens, producing a focal point for the cleavage of C5 and assembly of the membrane-attack complex (MAC) from complement components C6 through C9. The lectin pathway is similar in structure to the classical pathway; however, in this case the serine proteases mannose-binding lectin-associated serine protease (MASP)1, MASP2, and MASP3 (which are closely related to C1r and C1s) are

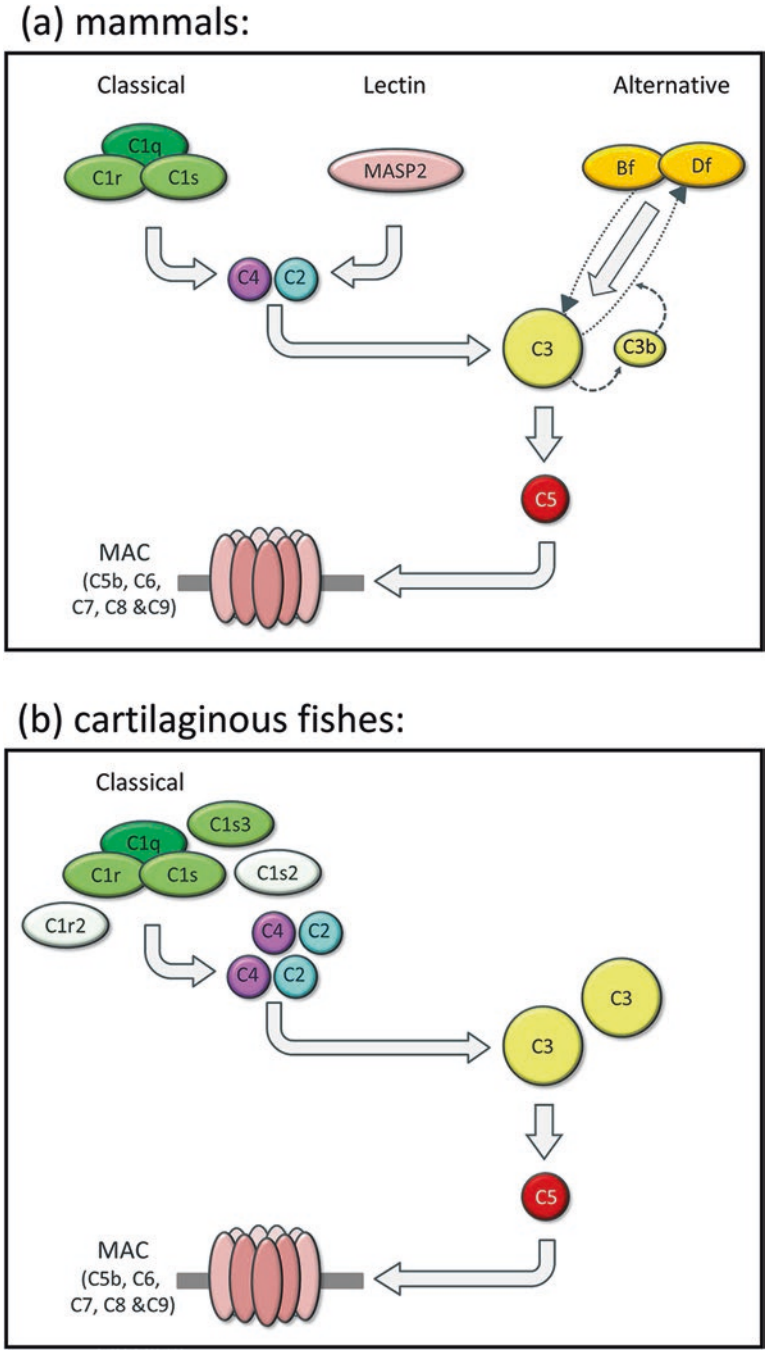

Fig. 1 (**a**) The mammalian complement system is triggered through three initiation pathways: the classical pathway, mediated through the binding of C1 complex to immune complexes; the lectin pathway, mediated through the recruitment of the mannose-binding lectin (MBL)-associated

recruited by multimers of mannose-binding lectin (MBL) or ficolin bound to sugars such as mannose, glucose, or N-acetylglucosamine present on the surface of microorganisms. Activated MASP2, like C1s, cleaves C2 and C4 to form the C3 convertase. In contrast to the other two pathways, the alternative pathway is initiated through the continuous, natural, low-level activation of C3. Under normal conditions the C3b generated is quickly inactivated by the inhibitors factor H and factor I; however, when a pathogen is present the C3b covalently binds to its surface and, now protected from inactivation, binds activated factor B (Bf) to form C3bBb, another C3 convertase that can cleave more C3 in an amplification loop, and C5 enabling MAC formation (Ricklin et al. 2010; Bubeck 2014).

Although shark serum was reported to have opsonin-like activity as early as 1907 (Ruediger and Davis 1907), the first hard evidence for a complement system in cartilaginous fish was provided by Jensen and co-workers who showed that fresh shark serum had bactericidal, virucidal, and hemolytic activity (Ross and Jensen 1973). That this activity was dependent on the presence of "natural" antibody (presumably pentameric IgM; see section "B Cells and Immunoglobulins") and could be inactivated by heating or freeze-thawing the serum (Ross and Jensen 1973) indicated that complement activation was likely occurring through the classical pathway. In a subsequent study, Jensen et al. (1981) provided functional evidence for the classical and terminal pathways through the isolation of six different components from nurse shark serum; these were named C1n, C2n, C3n, C4n, C8n, and C9n based on functional equivalence (i.e., C1n could substitute for C1 complex in depleted guinea pig serum). However, the exact composition and correspondence of these components to those of mammals remained unknown. While Jensen's studies did not find evidence for the alternative pathway in sharks, a later study by Culbreath et al. (1991) showed that shark complement could be activated in a Ca^{2+}-independent, Mg^{2+}-dependent manner, and that this activity was lost when serum was heated or treated with zymosan, inulin, or LPS, comparable to the alternative pathway in mammals. The subsequent cloning of shark orthologs for a number of complement genes—including a *MASP* gene with features intermediate between MASP1 and MASP2, two *C2/Bf-like* genes, and multiple MAC components (Table 1)—led researchers to conclude that the complement system in sharks was highly similar to that of mammals. However, these studies were rather piecemeal, with individual genes being cloned from different cartilaginous fish species, potentially confounding their subsequent analysis.

Fig. 1 (continued) serineprotease (MASP) serine proteases to MBL−/ficolin-bound sugars; and the alternative pathway, initiated through the low-level activation of C3. All three pathways culminate in the activation of C3 which provides a focal point on the pathogen for recruitment of further complement proteins and eventual formation of the membrane-attack complex (MAC), resulting in the death of the pathogen. (**b**) Current genomic and transcriptomic data suggests cartilaginous fishes have duplicated a number of their complement genes, including the classical pathway initiators C1r/C1s, and the complement system lynch-pin C3. Furthermore, key components of both the lectin and alternative pathways have been secondarily lost in this lineage. The functional significance of these changes has yet to be determined

Table 1 Components of the cartilaginous fish complement system described prior to the publication of the elephant shark genome, with the species the molecule was cloned from

Complement Component	Species	Reference
C2/Bf	Nurse shark (*Ginglymostoma cirratum*)	Shin et al. (2007)
	Banded houndshark (*Triakis scyllium*)	Terado et al. (2001)
C3	Nurse shark	Dodds et al. (1998)
C4	Nurse shark	Dodds et al. (1998)
	Banded houndshark	Terado et al. (2003)
C5	Nurse shark	Graham et al. (2009)
C6	Starspotted smooth-hound (*Mustelus manazo*)	Kimura and Nonaka (2009)
C8	Nurse shark (C8a)	Aybar et al. (2009)
	Whitespotted bamboo shark (*Chiloscyllium Plagiosum*) (C8a)	Wang et al. (2013a)
	Silver chimaera (*Chimaera phantasma*) (C8b)	Kimura and Nonaka (2009)
C9	Whitespotted bamboo shark	Wang et al. (2013b)
MASP	Banded houndshark	Endo et al. (2003)

MASP mannose-binding lectin-associated serine protease

More recent surveys of the elephant shark genome (Venkatesh et al. 2014a), a smooth hammerhead (*Sphyrna zygaena*) liver transcriptome (Goshima et al. 2016), and our recently generated normalized, multi-tissue small-spotted catshark transcriptome (Redmond et al., unpublished data) suggest that the complement system of cartilaginous fishes actually diverged from the vertebrate norm following their split from the common ancestor (Fig. 1b). Fitting with the functional work of Jensen et al. (1981), the initiators of the classical pathway were found; however, all three species carried multiple copies of *C1r* and *C1s* (elephant shark 2xC1r and 2xC1s; hammerhead 1xC1r and 2xC1s; catshark 3xC1r and 2xC1s), suggesting the duplications occurred before the chimera–elasmobranch split. Interestingly, some of the duplicates are missing one or more domains thought to be important for C1 complex formation in mammals (Goshima et al. 2016; Redmond et al., unpublished data). However, the retention of the extra C1r/s variants for over 400 million years certainly suggests they play some role and, as most have no serine protease activity (due to mutation of the active site residues in the serine protease domain, or loss of the entire domain), it currently seems most likely that they act as negative regulators of classical pathway initiation.

Moreover, duplications of *C3* and *C4* have also now been reported in multiple cartilaginous fish species; while only one *C3* gene was found in elephant shark (Venkatesh et al. 2014a) and hammerhead (Goshima et al. 2016), two *C3* variants have been described in nurse shark: one with a His residue at the thioester active site, the other where this His is replaced by Asp (Smith and Nonaka 2014). Similarly, two C3 molecules carrying either His or Asp active site residue were also found during our recent survey of small-spotted catshark complement molecules (Redmond et al., unpublished data). At least two *C4* genes have been found in the elephant shark and hammerhead, and again these group into His and Asp lineages (Venkatesh et al. 2014a; Goshima et al. 2016). While there is concordance between this finding

and the presence of two *C4* lineages in mammals, it appears that the *C4* duplication occurred in the common ancestor of all vertebrates, giving the His and Asp lineages. Mammals thereafter lost the Asp lineage (prior to speciation), then regenerated it via a recent gene duplication (Nonaka et al. 2017). Differential binding preferences have been shown by TEPs, dependent on the residue present at the active site; His TEPs bind to carbohydrates on target cells while Asp TEPs bind more efficiently to proteins (Law et al. 1984). It therefore seems that duplication of *C3* and *C4*, followed by diversification of the active site residue, is selectively advantageous by broadening the range of antigenic structures the complement system can target.

In contrast, functional evidence for complement activation through the lectin pathway has never been reported for cartilaginous fishes (Smith and Nonaka 2014), nor have MBL or ficolin (protein or transcript) been identified in any species yet examined (Venkatesh et al. 2014a; Goshima et al. 2016; Smith and Nonaka 2014). While MASP transcripts have been found in various species, a recent analysis performed by our group shows elephant shark, little skate, and catshark all lack MASP2, while it appears from transcriptome analysis that small-spotted catshark lacks all three MASP family members (Redmond et al., unpublished data). In the hammerhead, where MASP2 is present, the serine protease domain is missing (Goshima et al. 2016), making it impossible for this protein to function as a lectin pathway initiator. Moreover, recent phylogenetic analyses by Goshima et al. (2016) and our group (Redmond et al., unpublished data), both of which included more MASP family sequences from a broader collection of species, indicate that the original MASP1/2-like transcript found in the banded houndshark (Endo et al. 1998, 2003) is actually MASP3. Thus, hard evidence for a lectin pathway in cartilaginous fishes is currently lacking.

Confirming the presence or absence of the alternative pathway in cartilaginous fishes has been much more difficult, due in part to the shared origin of *Bf* and *C2* through gene duplication from a common ancestor (Smith 1998; Nonaka and Smith 2000). Thus, while two *Bf/C2-like* genes have been reported in the nurse shark, elephant shark, and hammerhead shark, the sequences are highly similar to one another and equally related to both mammalian *C2* and *Bf*, making it impossible to definitively assign them. However, the N-linked glycosylation patterns and intron/exon organization suggest that the two nurse shark genes are more *C2*-like than *Bf*-like (Shin et al. 2007; Smith and Nonaka 2014). It is also worth noting that activation of Bf is critically dependent on its cleavage by factor D (Df) and that, as yet, Df has not been found as a gene, transcript or protein in any of the cartilaginous fish species examined (Goshima et al. 2016; Smith and Nonaka 2014, Redmond et al., unpublished data). An absence of both Df and Bf would make activation of complement through the alternative pathway virtually impossible; however, this contradicts the functional data acquired by Smith and colleagues as detailed earlier (Culbreath et al. 1991; Smith and Nonaka 2014). Further work is required to resolve this discrepancy, and at this stage we certainly cannot rule out Df being present and simply missed, or the Bf/C2-like molecules being able to act in both the alternative and classical pathways. While further functional work is certainly required, it is intriguing to consider the immunological implications of cartilaginous fishes losing both

the alternative and lectin pathways for complement initiation (Fig. 1b). When combined with the possible downregulation of the surviving classical pathway, through the maintenance of proteolytically inert duplicates, it may provide some explanation of how sharks (unlike most other jawed vertebrates) can tolerate the presence of indigenous microflora in their blood and organs (Mylniczenko et al. 2007; Grimes et al. 1985; Tao et al. 2014).

Fitting with the observation of MAC lesions on sheep red blood cells by Jensen and colleagues (1981), all of the molecules of the terminal pathway (C5–C9) have now been identified in multiple cartilaginous fish species (Venkatesh et al. 2014a; Goshima et al. 2016; Kimura et al. 2009), confirming the lytic pathway evolved concomitant to the emergence of the jawed vertebrates. While there is currently little information on complement receptors in cartilaginous fishes, a few have been identified in the elephant shark genome, including a gene encoding a C3a/C5a receptor-like protein, tallying with observations of shark leukocyte chemotaxis in response to activated shark serum (Venkatesh et al. 2014a; Smith and Nonaka 2014 and references therein). Further, orthologs of the complement regulatory proteins C1-inhibitor *(SERPING1),* factor I (*CFI*), factor P (*CFP*), factor H (*CFH*), C1q-binding protein (*C1qBP*), and C4-binding protein (*C4BPa*) have all been found in the elephant shark genome (Venkatesh et al. 2014a).

Cytokines

Until recently only a handful of cytokines had been described in cartilaginous fishes, with even fewer studied at a functional level. The publication of the elephant shark genome enabled a more thorough survey to be conducted; however, even with such genomic resources, it remains difficult to find and assign cytokine gene homologs due to their rapid, pathogen-driven evolution (Secombes et al. 2014). In mammals, cytokines are classified into major groups as given in sections "Interleukins and Their Receptors, Transforming Growth Factor, Tumor Necrosis Factor Superfamily Ligands and Their Receptors, Interferons and Receptors, and Chemokines and Receptors".

Interleukins and Their Receptors

Interleukins (ILs) are the largest group of cytokines in mammals, with 37 members currently characterized in humans; these are grouped into families based on distinguishing structural features. ILs of cartilaginous fishes generally have low homology to those of mammals, and so are usually identified through a combination of structural characteristics and synteny analysis (Secombes et al. 2014). For this reason, only *IL-1β* (sharing < 30% homology with its human ortholog) had been unambiguously identified in cartilaginous fishes prior to publication of the elephant shark genome (Bird et al. 2002). From surveys of the genome, conducted by several groups (Venkatesh et al. 2014a; Secombes et al. 2014; Dijkstra, 2014), it has now been concluded that cartilaginous fishes have a "seemingly modern

complement of interleukin genes" (Venkatesh et al. 2014a). The main findings to date are summarized as follows:

- *IL-1 family*: So far, only *IL-1β* and *IL-18* have been identified in cartilaginous fishes (Venkatesh et al. 2014a; Bird et al. 2002), with *IL-1β* being the only cartilaginous fish IL to be studied at a functional level to date; IL-1β transcript levels increased in catshark splenocytes stimulated with LPS for 5 h in vivo (Bird et al. 2002), as did the transcript levels of pro-inflammatory genes following splenocyte stimulation with bacterially expressed recombinant IL-1β, although the exact details of the latter experiment remain unpublished (Secombes et al. 2014).
- *IL-2 family*: Orthologs for the IL-2 family members *IL-2, IL-4, IL-7, IL-15*, and *IL-21* have now been reported in cartilaginous fishes, however synteny analysis suggests that *IL9* is absent (Venkatesh et al. 2014a; Dijkstra 2014). IL-4 and IL-13 share functional similarities and probably were derived via a tandem duplication and divergence of a shared ancestral gene in tetrapods; it is therefore of note that three genes sharing homology to both IL-4 and IL-13 (named *IL-4/13A* through *C*) have been found in the same syntenic region in elephant sharks (Dijkstra 2014). In mammals, most members of the IL-2 family pair with the common γ-chain (IL2RG) to enable signaling; two linked *IL2RG* genes were found in the elephant shark genome (Venkatesh et al. 2014a). A similar finding has been made in several species of bony fishes, suggesting that two copies of *IL2RG* were present in the jawed vertebrate ancestor, with one copy being lost at some point prior to the divergence of mammals.
- *IL-3/IL-5 family*: Neither the IL-3/5 receptor common chain (*CSF2RB*) nor *IL-3* were found during the elephant shark genome survey. Two *IL-5*-like candidate genes (named *IL5A* and *IL5B*) were subsequently found by synteny analysis; however, work is still required to understand the precise relationship of these molecules to mammalian IL-3/5 family members (Venkatesh et al. 2014a; Dijkstra 2014).
- *IL-6 family*: Two *IL-6-like* genes were found clustered together in the elephant shark genome; however, neither *IL-11* nor *IL-31* were found (Venkatesh et al. 2014a). Our group has subsequently found orthologs of *IL-11* in the small-spotted catshark, little skate, and elephant shark (Redmond et al., unpublished data).
- *IL-10 family*: Orthologs of *IL-10* and *IL-22* were found in elephant shark, as were two genes with homology to *IL-19/20/24* that could not be confidently assigned (Venkatesh et al. 2014a; Secombes et al. 2014). No orthologs of *IL-26* or *IL-28* have yet been reported in cartilaginous fishes.
- *IL-12 family*: The IL-12 family is composed of four heterodimeric cytokines: IL-12 (composed of IL-12A + IL-12B chains), IL-23 (IL-23A + IL-12B), IL-27 (IL-27A + IL-27B), and IL-35 (IL12A + IL-27B). Thus far, genes encoding *IL-12A, IL-12B*, and *IL-27B* have been found in the elephant shark, as well as a potential *IL-28A* ortholog, suggesting IL-12, IL-27, and IL-35 can be formed in cartilaginous fishes (Venkatesh et al. 2014a; Secombes et al. 2014).
- *IL-17 family*: Several IL-17 family genes were found in the elephant shark, including two *IL-17A/F-like* and two *IL-17B/D-like* genes (Venkatesh et al.

2014a; Secombes et al. 2014) as well as a single gene for *IL-17C*. *IL-17E* (alternatively called IL-25) was not found.
- *Receptors*: Orthologs for most of the human IL receptors were found during the survey of the elephant shark genome [for a comprehensive list see supplemental table XI.7b in Venkatesh et al. (2014a)]; however, genes for *IL-2R2*, *IL-2RA*, *IL-6R*, *IL-23R*, *IL-17RE*, and *IL-31RA* are thought to be absent.

Transforming Growth Factor

Three isoforms of transforming growth factor (TGF) are present in mammals, called TGF-β1–3, with an additional fourth form, TGF-β6, in bony fishes. All of these isoforms appear to be present in cartilaginous fishes, with orthologs for each being found in the elephant shark (Venkatesh et al. 2014a; Secombes et al. 2014).

Tumor Necrosis Factor Superfamily Ligands and Their Receptors

Many orthologs of human tumor necrosis factor (TNF) superfamily (TNFSF) ligands were identified in the elephant shark genome, including those for CD40L (*TNFSF5*), FASL (*TNFSF6*), TRAIL (*TNFSF10*), RANKL ([*TNFSF11*]), and LIGHT (*TNFSF14*). Orthologs of many TNF receptors (TNFRs) were also easily identifiable, in addition to several TNFR superfamily (TNFRSF) genes with less clear orthology [for a complete list see supplemental table XI.8 in Venkatesh et al. (2014a)]. Notable differences between the mammalian TNFSF gene complement and that of cartilaginous fishes are as follows:

1. Orthologs of the key regulators of B cell development and survival in mammals, namely APRIL ([*TNFSF13*]) and BAFF ([*TNFSF13b*]), along with their receptors, BAFF-R (B-cell activating factor receptor [*TNFRSF13*]), TACI ([*TNFRSF13b*]), and BCMA ([*TNFRSF17*]), have now all been identified in cartilaginous fishes (Venkatesh et al. 2014a; Pettinello et al., unpublished data; Li et al. 2012), together with an additional TNFSF13 family ligand, BALM (BAFF- and APRIL-like molecule [*TNFSF13c*]) (Li et al. 2012; Redmond et al. 2017; Ren et al. 2011). Before its discovery in cartilaginous fishes, BALM was thought to be a bony fish-specific TNFSF ligand (Glenney and Wiens 2007). Subsequent analysis has, however, shown that BALM was present, along with APRIL and BAFF, in the gnathostome ancestor and has been secondarily lost in the tetrapod lineage (Redmond et al. 2017; Das et al. 2016). Expression data from bony fishes shows BALM is upregulated during infection (Granja et al. 2017); however, further work is required to uncover which receptor(s) BALM is capable of binding and how its function integrates with that of the other TNFSF13 family ligands. Further confounding this matter is the discovery that cartilaginous fish BAFF carries an extended loop between the a and a' β-strands, a region bordering the functionally important receptor binding groove, which may impact on its binding specificity (Li et al. 2012).
2. Conspicuously absent from the elephant shark genome were the genes for the lymphotoxin (LT) receptor (*LTR*) LTRβ (*TNFRSF3*) and its ligands LTα (*TNFSF1*) and LTβ (*TNFSF3*), as well as that for the closely related TNF-α

(*TNFSF2*) (Venkatesh et al. 2014a). A TNF-α-like transcript was, however, identified in nurse sharks by the same authors (Venkatesh et al. 2014a). We have subsequently found a single transcript in small-spotted catshark, along with multiple partial transcripts in little skate, that by phylogenetic analysis appear equally related to both LTα and TNF-α in other vertebrates (Pettinello et al., unpublished data). Our study supports that of Glenny and Wiens (2007), who found that mammalian TNF-α and LTα branch more closely to one other than to teleost TNF-α, suggesting that mammalian TNF-α and LTα share a recent, common evolutionary origin. This is significant as LTα and TNF-α are both required for the correct development of germinal centers (GCs) in mammals (Wang et al. 2001). Although the white pulp of the shark spleen has well-defined B and T cell areas (Rumfelt et al. 2002), "mammalian-like" GCs are not found in cartilaginous fishes (or any other cold-blooded vertebrate), and increases in antibody affinity during an immune response are magnitudes smaller than those observed in mammals (Dooley et al. 2006). Further work is required to understand if the duplication giving rise to functionally distinct LTα and TNF ligands permitted the evolution of more complex GC structures, better able to select high-affinity antibody variants [perhaps through the evolution of specialized follicular dendritic cells as suggested by Neely and Flajnik (2016)?].

3. Although orthologs of the TNFSF ligands CD70 (*TNFSF7*) and LIGHT (*TNFSF14*), both important for mammalian lymphocyte development and proliferation, were not identified in the elephant shark, we have subsequently found LIGHT in the small-spotted catshark (Pettinello et al., unpublished data). Additionally, no clear orthologs of the receptors *TNFRSF7*, *TNFRSF10A-D*, or *TNFRSF12A* could be identified in elephant shark (Venkatesh et al. 2014a) but *TNFRSF12A* and a single *TNFRSF10* gene appear to be present in catshark (Pettinello et al., unpublished data).

Interferons and Receptors

Approximately 20 distinct interferon (IFN) genes have been identified in humans, divided into three classes (type I, II, and III), and their production is key for the protection of cells from viral infection. Multiple type I genes and the lone type II IFN (*IFN-γ*) were identified in the elephant shark. To date, type III IFNs (also called IFN-λ) have only been found in mammals and birds, and in line with this were also not found in the elephant shark. However, potential orthologs of both type III IFN receptor chains (*IL-28RA* and *IL-10RB*) were found, indicating that type III IFN may be present but missed due to the quality of the genome in this syntenic area and/or their observed fast divergence. Genes for type I (*IFNAR1* and *IFNAR2*) and type II (*IFNγR1* and *IFNγR2*) IFN receptor chains were also present (Venkatesh et al. 2014a; Secombes and Zou 2017).

Chemokines and Receptors

Chemokines—small cytokines able to induce cellular chemotaxis—are generally classified into four main subfamilies: CXC, CC, CX3C, and XC. Chemokines exert their biological effects by interacting with G protein-linked membrane-bound

receptors found on the surface of their target cells. Human chemokines and chemokine receptors were used to interrogate the elephant shark genome for orthologs; of the 28 CC and 17 CXC chemokines in humans, 14 and 17 of each, respectively, were found in elephant shark. Only orthologs of *CCL 19, CCL20, CCL25*, *CXCL 8, CXCL12* (two copies), and *CXCL14* could be clearly identified, in addition to several genes with unclear orthology. Neither XC-like nor CX3C chemokines were found. Thirteen chemokine receptors were also found in the elephant shark genome, while orthologs for most of the human CXCR receptors are present [interestingly, *CXCR3* was found in the little skate but not the elephant shark (Zou et al. 2015)], only four (*CCR4, CCR6, CCR7*, and *CCR9*) of the ten human CCR receptors were found, reflecting the lower number of CC cytokines of this type [for a complete breakdown see supplemental table XI.5 and XI.6 in Venkatesh et al. (2014a)].

Adaptive Immunity in Cartilaginous Fishes

Major Histocompatibility Complex

In all other vertebrates examined so far, the peptides that are loaded onto class I and II MHC molecules for subsequent presentation to CD8+ cytotoxic T cells and CD4+ helper T cells, respectively, are derived via two major processing pathways. Endogenous proteins (as well as pathogen-derived proteins in the case of infected cells) are degraded by a multiprotein complex called the proteasome into peptides of 8–10 amino acids in length. These peptides are carried to the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) transporter proteins, where they bind to the antigen-binding cleft (ABC) of the class I molecules. Although the proteasome is composed of 14 different subunits, only three have proteolytic activity: PSMB5 (X), PSMB6 (Y), and PSMB7 (Z). Upon infection, these subunits are replaced by the IFN-γ-inducible subunits PSMB8 (LMP7), PSMB9 (LMP2), and PSMB10 (MECL-1), forming the "immunoproteasome" (Tanaka and Kasahara 1998). These changes in subunit composition are thought to increase chymotrypsin-like activity, generating peptides with hydrophobic C-terminal residues which better bind to the ABC of class I molecules. Once at the cell surface, the loaded class I molecules can be scanned by CD8+ cytotoxic T cells [reviewed in Neefjes et al. (2011)].

Class I molecules have now been described in multiple species of cartilaginous fish and, like other species, are both polygenic and polymorphic; the genes encode proteins of a similar structure to previously characterized class I molecules, with the ABC showing the highest levels of polymorphism (Bartl et al. 1997; Okamura et al. 1997). The class I partner β_2-microglobulin (*β2M*), immunoproteosome genes (*PSMB8*, *PSMB9*, and *PSMB10*), and TAP genes (*TAP1*, *TAP2*, and *TAPBP*), have also been characterized and are linked to those of class I (Venkatesh et al. 2014a; Ohta et al. 1999, 2002, 2011; Chen et al. 2010). Interestingly two PSMB8 paralogs have been found in cartilaginous fishes, one with an alanine at the active site (*PSMB8A*) and the other with a phenylalanine substitution at this residue (*PSMB8F*),

altering its cleavage specificity and the nature of the peptides generated (Ohta et al. 2002; Tsukamoto et al. 2012). Fitting with its role in immune surveillance, class I expression is highest in cartilaginous fish gill, intestine, PBL, and spleen with intermediate expression in kidney, liver, testis, and thymus (Ohta et al. 2002; Shen et al. 2014).

Unlike class I molecules which are constitutively expressed on all cells, class II MHC molecules are found predominantly on professional antigen-presenting cells (APCs). These cells sample antigens from the extracellular spaces for degradation by acid-dependent proteases, such as cathepsins, present in lysosomes. Following their synthesis in the ER, the class II molecules are bound by invariant chain (Ii), which allows them to fold correctly and subsequently transit to the Golgi. In the Golgi Ii is cleaved, leaving a small peptide, called CLIP (class II-associated invariant chain peptide), in the class II ABC until the non-classical class II chaperone DM binds, releasing CLIP and allowing antigen-derived peptides to load. The loaded class II molecules then move to the cell surface for sampling by $CD4^+$ helper T cells [reviewed in Neefjes et al. (2011)]. Again, polygenic and polymorphic class II genes have been identified in multiple cartilaginous fish species, along with Ii and multiple cathepsin genes. In contrast, DM appears to be a tetrapod invention and is missing from all cartilaginous (and bony) fishes surveyed to date (Venkatesh et al. 2014a; Kasahara et al. 1993; Bartl and Weissman 1994; Criscitiello et al. 2012; Dijkstra et al. 2013; Bartl and Nonaka 2014). Interestingly, the residues required for interaction with CD4 in other vertebrates (specifically β-chain residues S144 and E162) are not conserved in cartilaginous fish MHC class II molecules (Dijkstra et al. 2013), indicating an atypical mode of interaction. Indeed, while the molecules required for the synthesis of peptide-loaded class I and class II MHC molecules are possessed by cartilaginous fishes, the presence of a CD4-like molecule that can interact with class II is still a point of debate (see section "T Cells and T Cell Receptor"). Both class II and Ii expression is high in cartilaginous fish spleen, spiral valve, and gill, with slightly lower expression in kidney, intestine, PBL, and thymus. Expression levels were shown to increase in gill up to 8 h after in vivo challenge with bacteria, and over 24 h in liver and spleen (Criscitiello et al. 2012; Ma et al. 2013).

B Cells and Immunoglobulins

Cartilaginous fishes lack bone marrow, with B cells being generated instead in the epigonal organ, associated with the gonads, and the Leydig organ embedded within the walls of the esophagus. Recombination-activating genes (*RAG1* and *RAG2*) and terminal deoxynucleotidyl transferase (*TdT*) are expressed in these organs throughout the life of the animal, suggesting that B cells are continuously generated (Rumfelt et al. 2001, 2002; Miracle et al. 2001). Lacking lymph nodes (Zapata and Amemiya 2000), the cartilaginous fish secondary response occurs primarily in the spleen and, potentially, also the gut and gills (Rumfelt et al. 2002; Luer et al. 2014).

Three heavy (H) chain isotypes, IgM (μ), IgW (ω; previously called IgNARC, IgX, or IgR), and the lineage-specific isotype IgNAR, as well as four light (L) chain

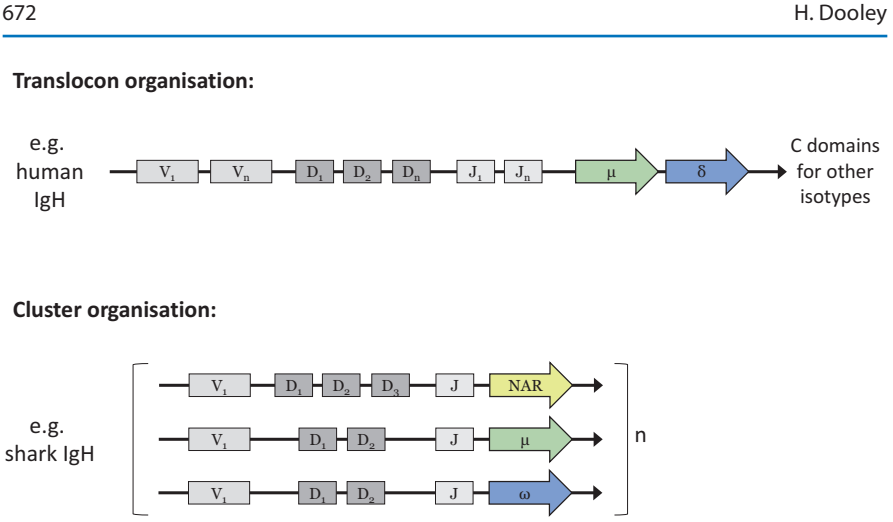

Fig. 2 The immunoglobulins (Igs) of most vertebrates are found in the translocon organization, typified here by human IgH; blocks containing multiple V (variable), D (diversity) (in the case of H chains but not L chains) and J (joining) segments are found 5′ to the C domains for all of the Ig isotypes expressed. The Igs of cartilaginous fishes, however, are present in a cluster organization, where a single V, 1–3 D, and a single J segment are 5′ to the C domains for only one isotype; rearrangement occurs almost exclusively within a cluster and not between different clusters. (Adapted from Pettinello and Dooley 2014)

isotypes; kappa (κ; NS4 or type III), lambda (λ; NS3 or type II), sigma (σ), and sigma-2 (σ-2; NS5, type I or σ-cart), have been found in cartilaginous fishes [reviewed in Pettinello and Dooley (2014)]. Unlike mammalian Igs that are present in a translocon configuration, shark H and L chain genes are arranged in discrete clusters (Fig. 2), with each cluster containing a variable (V) segment, several diversity (D) segments and a joining (J) segment as well as the constant (C) domain(s) required for completion of the Ig chain (Hinds and Litman 1986). The number of clusters for each isotype varies between species, ranging from two to three to many hundreds, and has also been shown to vary between individual members of the same species (Lee et al. 2008). Rearrangement occurs almost exclusively within a cluster and very rarely between clusters (Malecek et al. 2008). Further, and in stark contrast to mammals, there is no strict order to rearrangement of the segments within a cluster, implying all segments are simultaneously available for recombination (Malecek et al. 2008). Despite this, examination of individual IgM-expressing B cells by Hsu and colleagues showed a maximum of three IgM heavy chain clusters were rearranged out of the approximately ten clusters (= 20 alleles) present in the nurse shark, and the clusters/alleles appeared to be targeted at random (indicating each is recombinatorally autonomous) (Zhu et al. 2011). This led the authors to propose that in cartilaginous fish B cells a limiting nuclear factor(s) and/or time constraint allows only a few of the many clusters present to be activated and begin rearranging per cell. As it is highly unlikely that all clusters will begin this process at the same instant, rearrangement of the remaining clusters can be halted as soon as the first productive V region is produced (Zhu et al. 2011; Hsu 2014).

Unexpectedly, a small percentage of Ig clusters were found to be partly (VD-J) or fully (VDJ or VJ) pre-joined in the germline, likely due to the expression of RAG in germ cells (Kokubu et al. 1988; Lee et al. 2000). While the antibodies produced by these "germline-joined" clusters have limited CDR3 diversity, they have been shown to have an expression advantage early in development (Rumfelt et al. 2001; Diaz et al. 2002) and so may play a protective role in neonates and pups. Despite this, the presence of many clusters for each isotype, each requiring multiple rearrangement events to produce a functional V region, ensures that diversity in the primary repertoire is high. The repertoire is subsequently diversified further through the introduction of somatic mutations in an antigen-driven manner (Diaz et al. 1999). The levels of mutation observed in some isotypes far exceeds the upper levels reported for mammalian Igs and, uniquely to cartilaginous fishes, mutations are often found in tandem runs, suggesting they are introduced through a novel, non-templated mutation process (Diaz et al. 1999; Lee et al. 2002). As mentioned in section "Tumor Necrosis Factor Superfamily Ligands and Their Receptors", conventional GCs have not been found in cartilaginous fishes; despite this, replacement-to-silent mutation (R/S) ratios and structural analysis are consistent with the selection of mutant clones (Dooley et al. 2006; Diaz et al. 2002).

The cluster organization of cartilaginous fish Ig genes, as well as a lack of switch regions, prevents the type of isotype switching seen in other vertebrates. However, recent studies in nurse sharks have shown that the V region from one cluster can be expressed with the C domains from a different cluster, despite the fact that they are separated by large (>120 kb) distances (Zhu et al. 2012; Zhang et al. 2013). Unlike mammals, this unconventional form of "switching" is not unidirectional so, for example, IgW V regions have been found to be associated with IgM C domains and vice versa. Switching is heightened following immunization and occurs concomitantly with somatic hypermutation (SHM), so is likely mediated by activation-induced cytosine deaminase (AID), whereby introduced lesions lead to DNA strand breakage and subsequent re-joining (Zhu et al. 2012). How (or even if) this process is directed, as well as its biological relevance, remains to be clarified.

Of the three heavy chain isotypes, IgM is most abundant in cartilaginous fish serum, making up roughly half of the total protein (Marchalonis and Edelman 1966), and is present as both a monomer (mIgM or 7S) and a pentamer (pIgM or 19S) (Clem et al. 1967). The two forms of IgM are produced independently, likely by different lineages of B cells (Dooley and Flajnik 2005), with the production of pIgM being dependent on co-expression of the J chain (Castro et al. 2013). In 2001 a third form, called $IgM1_{gj}$, was found to be the predominant form of IgM in the serum of neonatal nurse sharks; the $IgM1_{gj}$ H chain lacks the second constant domain (Cμ2) making it convergent in structure to mammalian IgG, forms both monomers and dimers in serum, and carries a V region that is completely (VDJ) germline-joined (Rumfelt et al. 2001). This heavy chain preferentially partners with a germline-joined L chain, giving this antibody a "pre-determined" binding site (Flajnik and Hsu, unpublished data). Interestingly, the heavy chain gene does not encode a transmembrane region and so $IgM1_{gj}$ is only produced as secreted protein (Rumfelt et al. 2001; Hsu et al. 2006); the presumed lack of B cell receptor (BCR)

on the surface of $IgM1_{gj}$-expressing cells suggests they either do not proliferate, or proliferate in response to a signal other than BCR engagement, and that $IgM1_{gj}$, having forfeited its role as a BCR, has taken on some other (immune?) function in young animals (Hsu 2014). As shark pups age the levels of pIgM and mIgM gradually increase in the serum until they overwhelm the $IgM1_{gj}$ present (Rumfelt et al. 2001).

IgNAR (Ig new antigen receptor [NAR]) is a novel heavy chain homodimer that naturally does not associate with light chains (Greenberg et al. 1995; Roux et al. 1998) and whose V region is structurally more closely related to that of Ig L chains or TCRs than that of Igs (Stanfield et al. 2004). The membrane-bound form of IgNAR has three or five C domains, whereas the secreted form has five C domains, the last four being homologous to those of IgW; thus, it seems that IgNAR arose through the invasion of the V domain of NAR-TCR (a cartilaginous fish-specific, doubly rearranging TCR; see section "T Cells and T Cell Receptors") into an IgW cluster (Criscitiello et al. 2006). In all cartilaginous fish species studied so far there are fewer (2–20) IgNAR clusters than IgM; however, IgNAR sequence diversity is bolstered by the presence of three D segments in each cluster, necessitating four rearrangement events (with associated trimming and N/P additions) to generate a fully functional V region (Greenberg et al. 1995); following exposure to antigen, the V regions are then mutated to an exceptionally high level (Diaz et al. 1998). Although IgNAR is found as a monomer in the blood of nurse shark, in the small-spotted catshark and spiny dogfish it is present as both a monomer and a multimer (Smith et al. 2012; Crouch et al. 2013).

The third cartilaginous fish isotype, IgW, has been difficult to identify at the protein level [due to its low serum levels and apparent sensitivity to proteolysis (Dooley and Flajnik, unpublished data)]; however, transcript levels are high in spleen, epigonal, pancreas, thymus, and gill of unimmunized animals (Zhang et al. 2013; Smith et al. 2012; Rumfelt et al. 2004), suggesting a potential role in mucosal protection. From analysis of transcripts two cell-bound forms (containing 2 and 4Cδ) and at least seven secreted forms (containing 2, 4, 6, or 8Cδ as well as a 6Cδ form that lacks a V region) of IgW have been found thus far, all of which are generated by alternate splicing. Interestingly, the short (2Cδ) secreted form of IgW has a long, cysteine-rich tail that is unlike that of any other Ig (in cartilaginous fish or other species) (Rumfelt et al. 2004), strongly suggesting it has a different effector function. At least some IgW-expressing cells also express J chain (Castro et al. 2013), suggesting IgW can form multimers; however, the biological relevance of this requires further investigation. Although IgW is the shark ortholog of IgD (Ohta and Flajnik 2006), it seems that, unlike other vertebrates, the cluster organization of cartilaginous fish Ig genes prevents IgM and IgW from being expressed on the surface of the same B cell (Eason et al. 2004). Interestingly, IgW (along with the genes for IgNAR and σ light chains) appears to have been lost from the genome of the elephant shark (Venkatesh et al. 2014a).

Immunization studies performed in nurse sharks indicate that pIgM provides the "first line" of defense. Due to its large size, pIgM is restricted to the intravascular space where it binds invading pathogens with low affinity but high avidity (Dooley

and Flajnik 2005; Small et al. 1970). The functional affinity of pIgM does not increase significantly over the course of a response (Dooley and Flajnik 2005); this, and its presence in the serum of neonates, suggests the production of pIgM is independent of T cell help. In contrast, mIgM and IgNAR are absent from neonatal serum and exhibit a lag period before an antigen-specific response is observed, suggesting their production requires T cell help (Rumfelt et al. 2002; Dooley and Flajnik 2005; Fidler et al. 1969). Like other exothermic vertebrates, there are indicators that humoral immunity is impacted by environmental factors such as temperature, the response being slightly faster in summer than in winter in nurse sharks (Dooley and Flajnik, unpublished data). Antigen-specific IgM and IgNAR titers remain high for prolonged periods (1–3 years) post-immunization before dropping back to pre-bleed levels. However, if the animal was subsequently boosted with the same antigen (without adjuvant) then a response was observed in a much shorter time period (4–6 weeks), indicating that cartilaginous fish are capable of a memory response (Dooley and Flajnik 2005). Due to a lack of specific antibodies it has not been possible to study the role of secreted IgW during an immune response.

T Cells and T Cell Receptors

As with other jawed vertebrates, T cell maturation occurs in the thymus, a multilobed, bilateral organ located dorsomedial to the gills, in cartilaginous fish (Miracle et al. 2001; Luer et al. 1995). In some species of shark the thymus has been shown to involute with age, whereas in other species it remains large throughout the life of the animal (Rumfelt 2014). Nurse shark thymus shows symmetrical petal-shaped lobules of cortex around a central medulla; RAG and TdT show highest expression in the subcapsular region of the cortex, the area where mammalian TCRs also rearrange their variable regions (Criscitiello et al. 2010). Orthologs of all four mammalian TCR chains (α, β, δ, and γ) have been identified in cartilaginous fishes, with single genes for all except *TCR-β* which has multiple (two or three) copies in the species examined so far (Venkatesh et al. 2014a; Criscitiello et al. 2010; Pettinello et al. 2017; Rast et al. 1995, 1997); no major differences in sequence or transcript level was observed between the two *TCR-β* genes in catshark, suggesting both contribute to the TCR repertoire. Unlike their Igs, the TCR genes of cartilaginous fish are thought to be present in the typical translocon configuration (Criscitiello et al. 2010; Rast et al. 1997; Chen et al. 2009), possibly with the exception of *TCR-β* (Pettinello et al. 2017), although this requires confirmation at the genomic level.

Cartilaginous fish TCR genes are rearranged by RAG-mediated recombination, during which TdT introduces non-templated additions, generating a diverse V region repertoire for all four TCR chains (Criscitiello et al. 2010). Moreover, recent studies have shown that cartilaginous fish use SHM to further increase the diversity of their TCR V regions. Evidence of SHM was first observed in TCR-γ of the sandbar shark by Chen et al. (2012); as observed in shark Ig V domains, changes were due to either point mutations or tandem mutations, indicating a common means of generation (Diaz et al. 1999; Lee et al. 2002; Chen et al. 2012). Retrospective

analysis of sequences isolated from nurse shark by Criscitiello and colleagues (2010) uncovered evidence of SHM in TCR-γ and TCR-α V domains that had been missed in the initial study, but not in TCR-β or TCR-δ V domains (Criscitiello et al. 2010, 2014; Chen et al. 2012). No appreciable differences were found in the R/S ratios between the CDRs and framework regions, indicating little or no selection pressure on the mutated TCR chains; it also suggests that SHM is being used for repertoire diversification, or perhaps the rescue of autoreactive clones, rather than affinity maturation (Chen et al. 2012; Criscitiello 2014).

In addition to their four canonical TCR chains, cartilaginous fish also possess a novel TCR chain, composed of a TCR-δ C domain carrying two independently rearranging V domains. As the N-terminal V domain is most closely related to that of the shark Ig isotype IgNAR, this novel TCR chain was christened NAR-TCR and in nurse sharks, where NAR-TCR was first discovered, approximately 20% of TCR-δ C-containing transcripts have this two V form (Criscitiello et al. 2006). As IgNAR V domains do not require a partner domain to bind its target (Roux et al. 1998; Stanfield et al. 2004), it is assumed (but not yet proven) that NAR-TCR pairs with a conventional TCR-γ chain leaving the NARV domain free to bind its target as a soluble single domain. Interestingly, the CDR3 regions of the "supporting" TCR-δ V domains are as diverse in length and sequence as those of the NARV, suggesting that the binding site of this domain may not be fully occluded by the NARV domain on top (Criscitiello et al. 2006). The short transmembrane region and conservation of hallmark residues in the TCR-δ C domain predict that NAR-TCR is able to form a complex with CD3 allowing cell surface expression and signal transduction. In line with this, transcript levels of NAR-TCR are highest in thymus followed by spleen, spiral valve, and peripheral blood cells, reflecting the pattern observed for conventional TCR-δ transcripts (Criscitiello et al. 2006). While the role of NAR-TCR has yet to be determined, it seems to expand the TCR repertoire by enabling the binding of soluble antigens. The identification of NAR-TCR in the genome of the elephant shark (Venkatesh et al. 2014a) and its continued maintenance in many cartilaginous fish species (Criscitiello et al. 2006) suggests that whatever the role NAR-TCR plays, it is an important and/or advantageous one. Cartilaginous fish also possess other, divergent, TCR chains that are generated as a product of trans-rearrangement between a TCR V region and that of a (presumably) closely linked Ig (Venkatesh et al. 2014a; Criscitiello et al. 2010). All the transcripts found so far involve trans-rearrangement of the V segment from an IgM or IgW cluster to the D-J and C of TCR-δ. The CDR3 of these clones are almost always in-frame, suggesting selection of those that produce a functional protein (Criscitiello et al. 2010; Pettinello et al. 2017). Quantitative polymerase chain reaction shows trans-rearranged TCRs are transcribed in the same tissues as conventional TCRs and NAR-TCRs, albeit at a lower level (Criscitiello 2014). It therefore seems that cartilaginous fishes use an assortment of strategies, including gene duplication, the "appropriation" of Ig V regions, and SHM, to expand their TCR repertoire, thereby increasing its binding potential.

In mammals, T cells carrying a TCR composed of γ/δ chains do not appear to be MHC restricted, rather binding to soluble antigens. Those composed of α/β chains

are sub-classified into "helper" (Th) and "cytotoxic" (Tc) T cells based on the presence of the co-receptors CD4 and CD8, respectively. The presence of CD4 on the surface of a T cell enables interaction with MHC class II molecules, whereas CD8 enables interaction with MHC class I molecules. Mammalian Th cells are further divided into subsets (Th1, Th2, Th9, Th17, Th22, Tfh, and Treg [regulatory T cells]) based predominantly on the cytokines they express following stimulation. Initial analysis of the elephant shark genome by Venkatesh and colleagues (2014a) found genes for all the hallmark molecules of the cytotoxic T cell lineage, principally the cell surface glycoprotein CD8 (*CD8a*), the effector molecules IL-7 (*IL-7*), IL-15 (*IL-15*), perforin (*PRF1*), granzyme (*GZM*), IFN-γ (*IFNG*), and TNF-α (*TNF*), as well as *RUNX3*, a transcription factor critical for CD8 T cell lineage commitment (Kohu et al. 2005). However, despite the long-proven presence of cartilaginous fish MHC class II and Ii (as detailed earlier), and the identification of the gene encoding ThPOK (*ZBTB7B* [the transcription factor that controls CD4 expression and Th lineage commitment (He et al. 2005)] in the elephant shark genome, no canonical gene for CD4 was identified. While a "classical" *CD4* gene was not found in elephant shark (or a nurse shark transcriptome searched by the same authors) a "CD4/LAG-like" candidate gene was found (CD4/LAG3-related is a single protein with similarity to both *CD4* and the closely related *LAG3*). The protein encoded by this gene carries all the structural features of CD4 except the CXC motif in its cytoplasmic tail that, in mammals, mediates interaction with the tyrosine kinase Lck and is thought to be critical to its role as co-receptor (Venkatesh et al. 2014a). As mentioned earlier, cartilaginous fish class II MHC molecules lack key residues that mediate CD4 interaction in other vertebrates (Dijkstra et al. 2013). Thus, should the CD4/LAG3-related protein indeed be used as a co-receptor on T cells, its interaction with both MHC and intracellular signaling molecules (whether Lck or another tyrosine kinase) is likely to be unique amongst jawed vertebrates (Dijkstra 2014; Venkatesh et al. 2014b).

During their initial analysis, Venkatesh and colleagues failed to find a number of Th subset-specific transcription factors and key effector cytokines in the elephant shark. This led to the suggestion that cartilaginous fishes possess a basic Th system based on Th1-like activity alone (Venkatesh et al. 2014a). Subsequent interrogation of the genome by Dijkstra identified candidate orthologs for some of the "missing" genes (specifically *IL-2*, *IL-4*, *IL-5*, and *IL-13*), which is highly indicative of a Th2 lineage also being present (Dijkstra 2014). The current data regarding the presence of Treg and Th17 lineages in cartilaginous fishes are more ambiguous. In mammals, the transcription factor FOXP3 (forkhead box P3) acts as a master regulator of Treg differentiation in concert with IL-2. While both an *IL-2* candidate and *FOXP3* are present in the elephant shark genome, the *FOXP3* gene was reported to lack critical amino acids in its forkhead (FKD) domain that facilitate DNA binding and nuclear factor of activated T cells (NFAT) interaction in its mammalian orthologs (Venkatesh et al. 2014a). However, recent studies have shown that these residues vary naturally between the different FOXP subfamily members in mammals (all of which bind DNA) (Bandukwala et al. 2011). Additionally, *FOXP3* completely lacking a FKD retains the ability to regulate numerous genes by acting as a bridge between other

transcription factors and downstream effector molecules (Xie et al. 2015). Thus, the lack of conservation found in the FKD of elephant shark *FOXP3* does not necessarily equate to a lack of functionality or the absence of Treg cells (Venkatesh et al. 2014a; Dijkstra 2014). Further, while the gene encoding RORγT, the master regulator of Th17 cells, has yet to be found in cartilaginous fishes, all the cytokines required to induce Th17 cell development (IL-6, TGF-β, and IL-21) are present (Venkatesh et al. 2014a; Secombes et al. 2014). Although IL-23 and the IL-23 receptor both seem to be absent (Secombes et al. 2014; Redmond et al., unpublished data), it is possible that Th17 maintenance and expansion is driven by IL1β, as previously observed in humans (Miyahara et al. 2008).

In a recent study of the CD3 complex, responsible for converting TCR engagement into an intracellular signal, Pettinello and colleagues (2017) showed that the genes of the complex were highly conserved across gnathostomes except for a duplication of the *CD3γδ* gene apparent in all cartilaginous fish species studied. Closer scrutiny of the duplicates showed that one variant, CD3γδ-B, is missing some of the expected CD3 characteristics (notably, the negatively charged residue in the transmembrane region required for non-covalent bonding with TCR chains) and is expressed at a much lower level than the other in catsharks, suggesting functional silencing following duplication (Pettinello et al. 2017).

Conclusion and Future Perspective

Due to their key position in phylogeny, cartilaginous fishes remain an important experimental model to explore the evolution of immune systems in general, and "mammalian-like" adaptive immunity in particular. As our knowledge regarding the immune molecules and pathways in this group improves, it is increasingly apparent that the immune system of cartilaginous fishes is not merely a rudimentary version of that found in mammals. Indeed, exploration of new genomic and transcriptomic datasets indicates that cartilaginous fish, like mammals, use a highly sophisticated repertoire of immune cells, recognition molecules, regulators, and effectors to defend themselves against pathogen invasion. Further, functional work shows that these pathways culminate in the generation of a highly specific, multi-layered humoral response, involving multiple Ig isotypes and exhibiting immunological memory (Dooley and Flajnik 2005; Crouch et al. 2013).

However, major questions regarding the degree of T cell help and the selection of antigen-specific B cells remain. The claim that cartilaginous fishes have only a basic Th cell repertoire (Venkatesh et al. 2014a) is rapidly losing ground as new databases are interrogated for T cell-associated molecules, or different/more advanced bioinformatic techniques are used to re-examine previously published ones (Dijkstra 2014; Redmond et al., unpublished data; Venkatesh et al. 2014b). Thus, despite the extended period required to observe antigen-specific Ig in immunized sharks, it seems unlikely that this is due to inadequate T cell help. However, it is also apparent that much more work is required to catalogue the T cell subsets present in cartilaginous fishes, as well as their biological roles. Further, as mentioned earlier,

mammalian-like GCs have not been identified in cartilaginous fishes (Neely and Flajnik 2016); despite this, selection of higher affinity variants is clearly occurring (Diaz et al. 2002; Lee et al. 2002; Stanfield et al. 2007). While affinity maturation in the absence of GCs is not unprecedented (Matsumoto et al. 1996), the lower magnitude of improvements in shark Igs [and those of other cold-blooded vertebrates, which also lack GCs (Zapata and Amemiya 2000)] than in mammals (Dooley et al. 2006; Dooley and Flajnik 2005) suggests that selection of clones outside of GCs is suboptimal. The discovery of AID in sharks (Conticello et al. 2005) can now be used to locate actively hypermutating B cells and, when combined with new T cell markers, should enable the "GC equivalent" areas in cartilaginous fishes to finally be identified and characterized.

The secondary loss of two of the three complement initiation pathways, with potential downregulation of the third (classical) pathway (as detailed earlier), is also intriguing, particularly when combined with the reports of bacteria being present in the blood and tissues of apparently healthy sharks (Mylniczenko et al. 2007; Grimes et al. 1985; Tao et al. 2014). It is currently unknown whether these bacteria are opportunistic pathogens, benign commensals, or resident symbionts that serve some role in cartilaginous fish physiology [e.g., the regulation of tissue urea concentrations as hypothesized by Rita Colwell and colleagues (Knight et al. 1988)]; however, their very presence suggests the cartilaginous fish immune system functions in a fundamentally different manner to that of mammals. Future studies to understand the residual function of the cartilaginous fish complement system, and to profile the PAMPs recognized by their PRR repertoire, should shed some light upon this apparent incongruity.

As we move forward, simply proving the presence (or probable absence) of a gene in a single cartilaginous fish species will not be sufficient to make judgements about the ancestral jawed vertebrate state or immune characteristics of the lineage. Differences in the possession of immune molecules have already been noted between elephant sharks and other species (Venkatesh et al. 2014a; Pettinello et al. 2017). Additionally, it is now apparent that significant redundancy is built into the immune system (Nish and Medzhitov 2011; Ozaki and Leonard 2002), meaning individual molecules or, potentially, whole pathways can be functionally substituted by others. New genomic and transcriptomic resources will help with the first problem; however, functional studies are required to address the second. To facilitate such studies, we need to develop new tools for use in vivo and in vitro, as well as embracing innovative, enabling technologies. Undoubtedly, to answer the questions that remain regarding the immune system of cartilaginous fishes, we (comparative immunologists) will need to show the same level of adaptability and resilience as the amazing animals we study.

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Osteichthyes: Immune Systems of Teleosts (Actinopterygii)

Teruyuki Nakanishi, Jun-ichi Hikima, and Takashi Yada

Characteristics of Teleost Fish Immune System

Elasmobranchs and teleosts are the most primitive groups that have adaptive immune systems akin to mammals possessing immunoglobulins (Igs), T-cell antigen receptors (TCRs), and the major histocompatibility complex (MHC) class I and II molecules as well as B and T lymphocytes. However, the immune system of teleosts is different from that of mammals. First, it is rather simple and undifferentiated compared to that of mammals: they lack bone marrow, lymph nodes, and germinal centers as immune organs/tissues (Sunyer 2013). Teleost fish possess a smaller number of immune molecules than mammals. For instance, only three Ig classes, IgM, IgD, and IgT/Z, have been identified so far, and no IgG, IgA, and IgE are present. As for cytokines, only the IL-1β, but not IL-1α or IL-1 receptor, antagonist is present. Similarly, tumor necrosis factor (TNF) α has been identified but not LTα or LTβ.

Second, the teleost immune system is very diverse from species to species. For instance, Atlantic cod lacks the genes for CD4, MHC class II, and an invariant chain

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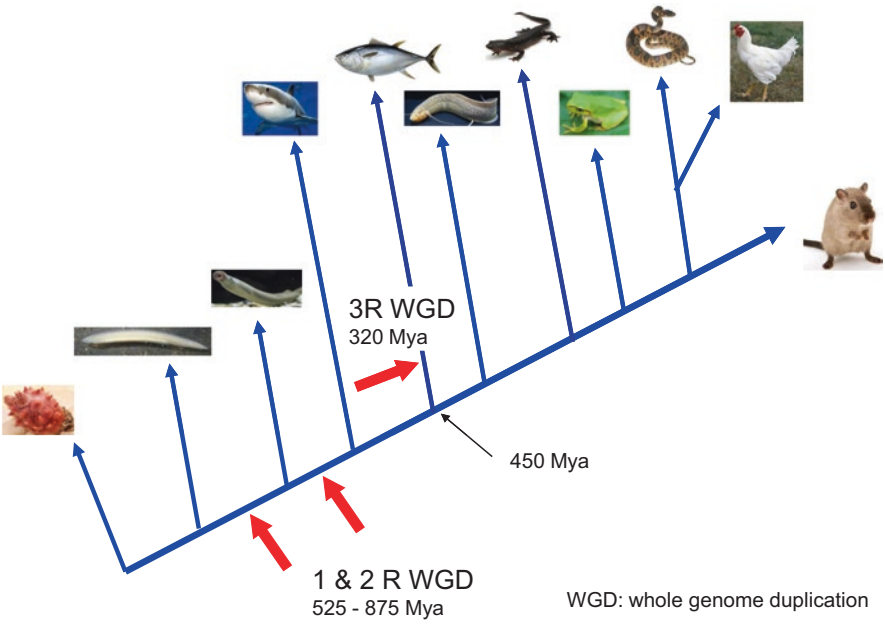

Fig. 1 Whole genome duplication (WGD) is the driving force in the presence of multiple isoforms in teleost immune genes

involved in making and transporting MHC class II genes (Star et al. 2011). However, Atlantic cod is not exceptionally susceptible to disease under natural conditions (Pilstrom et al. 2005). Instead, Atlantic cod has a highly expanded number of MHC class I genes and unique and markedly expanded Toll-like receptor (TLR) genes, resulting in the highest number of TLRs found in a teleost.

Third, teleost fish develop defense strategies different from that of mammals by producing diversified isotypes to compensate for the their rather simple and undifferentiated immune system. A number of reports have demonstrated the presence of multiple genes, for example, cytokines: TNFα, IL-1β; lymphocyte cell surface markers: CD4, CD8; complement components: C2, C3, and so forth. There is conclusive evidence that fish-specific whole genome duplication took place in ray-finned fish around 320 million years ago, in addition to two rounds of the whole genome duplication events early in vertebrate evolution (Fig. 1). Furthermore, cyprinid and salmonid fishes appear to be tetraploid because of their chromosome number and high DNA content. The additional number of genes resulting from genome or chromosomal duplication might have had creative roles in evolution such as speciation, adaptation, diversification, and promotion of new functions, although differential roles of the isoforms have yet to be clarified in most cases. This is evidenced by the fact that teleosts are the largest group in the class Actinopterygii, comprising 96% of all extant species of fish, and they are the dominant fishes in both marine and freshwater habitats from the deep sea to the high mountains (Fig. 2).

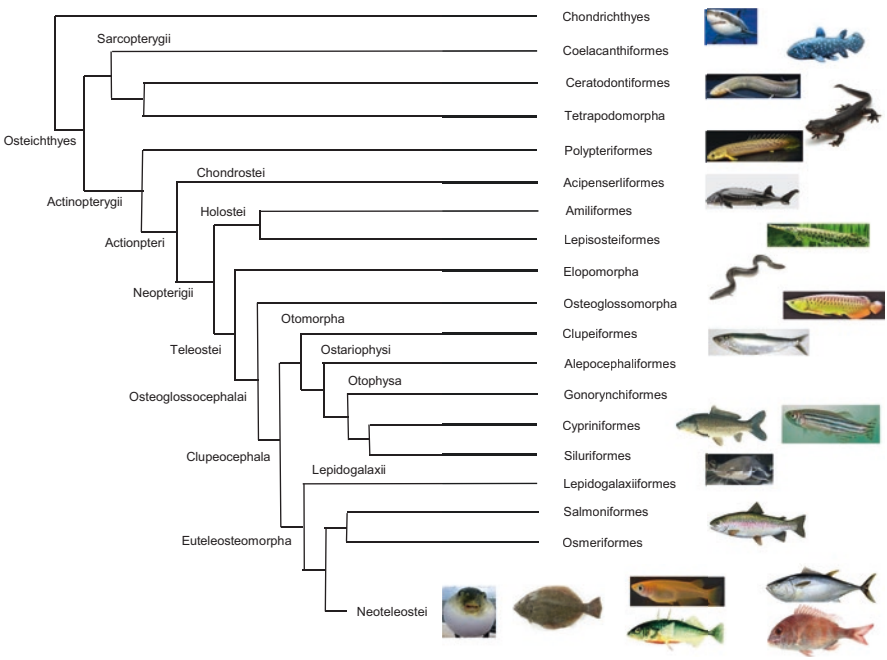

Fig. 2 Phylogeny of fish

Immune Tissues and Organs

The thymus is an essential organ for the development of T lymphocytes from early thymocyte progenitors to functionally competent T cells. It has been well documented that early steps in T-cell (thymocyte) development and thymic organogenesis in teleosts are similar to those in higher vertebrates, as documented by histology and gene expression patterns in zebrafish (reviewed in Langenau and Zon 2005) and other fish species (reviewed in Tatner 1996; Rombout et al. 2005; Nakanishi et al. 2015).

In teleosts, hematopoiesis occurs in the kidney, where all lineages of hematopoiesis are observed, including erythropoiesis, myelopoiesis, and lymphopoiesis. Thus, the teleost kidney is regarded as a comparable organ to bone marrow in mammals (Zapata et al. 1996). The teleost kidney is composed of two parts, a head kidney (HK) and a trunk kidney (TK). The HK is considered to be a more important hematopoietic organ than the TK since intertubular lymphoid tissues are more developed in the HK, while the TK contains abundant urinary tissues.

Kobayashi et al. (2008) developed in vivo transplantation systems in the zebrafish and isogeneic ginbuna crucian carp (*Carassius auratus langsdorfii*). They demonstrated that hematopoietic stem cells (HSCs) were present in teleost kidney and HSCs adhere to the epithelial cells of renal tubules, which are key components of HSC niches in teleosts. Interestingly enough, more HSCs are present in the TK than the HK and side population (SP) cells in which HSCs are enriched were detected

only from TK in ginbuna (Kobayashi et al. 2006, 2007). These results suggest that the TK but not the HK is the major source of HSCs since the TK contains abundant renal tubules where HSC niches reside.

The intestine of teleost is also considered to be lymphoid tissue as in higher vertebrates. Although there have been no reports showing the presence of Peyer's patches, M cells, IgA, or J-chains in the intestines of teleost fish, the presence of T cells, including intraepithelial lymphocytes (IELs), has been demonstrated in the intestinal tissue of several fish species using monoclonal antibodies that recognize T-cell subpopulations and by the expression analysis of T-cell marker genes (reviewed in Rombout et al. 2010; Salinas 2015).

The gill is another mucosal tissue exposed to the environment. The presence of considerable numbers of T cells in gills has been reported in several fish species, for example, carp (Rombout et al. 1998), Atlantic salmon (Koppang et al. 2010), rainbow trout (Takizawa et al. 2011), and European sea bass (Ortiz et al. 2014). In Atlantic salmon, Haugarvoll et al. (2008) reported interbranchial lymphoid tissue (ILT) at the base of the gill filaments, where mRNA expression of MHC class II and TCRα was detected.

Innate Immune System

Defense at Body Surface (First Barrier, Mucosal Environments)

The main form of fish mucus is a mucopolysaccharide that is secreted from mucus cells distributed in the epithelium. The primary role of mucus is to reduce the resistance of water, flush foreign substances that have adhered to the body surface, and minimize the physical contact injury, but the latter two roles themselves act as a defense against invaders. Aside from the mucus secreted on the body surface, various bioreactive substances that are useful for defense are also secreted in the mucus. These bioreactive substances include complement factors, lectins, hydrolytic enzymes (e.g., lysozyme, cathepsin B, proteases), transferrin, C-reactive protein (CRP), interferon (IFN), and antibody (immunoglobulin, IgM, and IgT) (section "Immunoglobulins") in fish skin mucus (Ellis 2001; Molle et al. 2008; Rakers et al. 2013). Fish skin generates a large variety of antimicrobial peptides (AMPs) such as hepcidin (Shike et al. 2002; Hirono et al. 2005; Cuesta et al. 2008a, b), defensinlike peptides (Zou et al. 2007a, b; van der Marel et al. 2012), cathelicidins (Chang et al. 2005; Maier et al. 2008), certain apolipoproteins (Villarroel et al. 2007), piscidin (or moronecidin) (Silphaduang and Noga 2001; Lauth et al. 2002), and pleurocidin (Cole et al. 1997), often with selective properties against pathogenic bacteria, fungi, algae, viruses, or parasites (Rakers et al. 2013). Particularly after wounding, fish skin is susceptible to proteolytic attack via such a bioreactive compound and needs to have some structural and immunological properties to prevent infections by pathogens (Rakers et al. 2013).

On the other hand, it is considered that bacterial flora in the intestinal tract of fish enhances the immune defense. Fish intestinal flora has been investigated in

numerous species including rainbow trout. The protective effect for fish pathogenic bacteria has been reported using a useful bacterial species isolated from the intestinal flora of mammals by fixing this in the gut of the target fish (Nayak 2010). This technique is referred to as probiotics.

Cellular Factors

Fish leukocytes are basically classified into lymphocytes, granulocytes, monocytes, and thrombocytes (cells involved in blood coagulation corresponding to platelets in mammals), like the mammalian system. Lymphocytes are divided into T and B cells, which are directly involved in specific immunity (see the section on *adaptive immunity*) (Secombes 1996) and are further divided into nonspecific cytotoxic cells (NCCs). Granulocytes are divided into neutrophils, eosinophils, and basophils according to the staining of cytoplasmic granules. It is generally rare to find both eosinophils and basophils in fish. Monocytes differentiate into macrophages. Neutrophils, monocytes (macrophages), and B cells have phagocytic activity among the fish leukocytes (Secombes 1996; Li et al. 2006). Eosinophils and thrombocytes also engulf foreign substances in some fish species. Neutrophils, monocytes/macrophages, and NCCs play an especially important role in nonspecific host defense.

Neutrophils

Neutrophils play a pivotal role in the innate immune response, are the most abundant cells among granulocytes and monocytes in the blood, show active migration and phagocytic activity, and sterilize/digest phagocytosed foreign substances. Neutrophils in mammals have multinucleated, lobulated spherical nuclei, while neutrophils in fish are polynuclear in salmonid fish, but in many fish species, at best they are horseshoe shaped. In zebrafish, neutrophils express two CXCL8 genes (also known as IL-8, chemokine) for inducing neutrophil recruitment through Cxcr2 (de Oliveira et al. 2013). Neutrophils also possess nonspecific cytotoxic activity in carp and ginbuna as one NCC (Kurata et al. 1995).

For phagocytic cells to engulf a foreign substance, the foreign substance needs to attach to the phagocytic cell surface with opsonic activity. Opsonin is a general term for a biological substance that binds to the surface of foreign substances and efficiently promotes phagocytosis by neutrophils. Complement component fragments (C3 origin) derived from antibodies (Fc) and lectin are important opsonins (Sunyer and Lambris 1998; Tosi 2005). Many reports show that opsonin exists in fish. Furthermore, it has already been reported that C3b receptors that recognize opsonins on phagocytes are present in carp neutrophil cell surfaces (Matsuyama et al. 1992). Fc receptors have been identified from neutrophils of peripheral blood in catfish (Stafford et al. 2006). Opsonic activity is conspicuous in the phagocytosis by neutrophils, while opsonins are not always necessary in macrophages, as is the case in fish (Iida et al. 2001).

Monocytes/Macrophages

Monocytes/macrophages slowly come together in an inflamed site after neutrophils. They migrate actively, phagocytose, and sterilize/digest as well as neutrophils. Macrophages that have infiltrated into an inflamed site phagocytose debris (dead cells) of neutrophils and the foreign substances that cannot be treated in neutrophils. It is considered that the life of neutrophils that leaches into inflamed parts is short and they normally die in the inflammation section. On the other hand, the life of macrophages is longer and some go back to the kidney from the inflamed part after phagocytosis of the foreign substance. Macrophages are present in heart, gills, kidney, spleen, the peritoneal cavity, and bowels, even when inflammation does not occur (Nakamura and Shimozawa 1994; Zapata et al. 1996).

Teleost macrophages possess two functional phenotypes, including proinflammatory responses for antimicrobial host defense (classical, M1-type) and anti-inflammatory responses for regulatory functions (M2-type) (Hodgkinson et al. 2015). In M1-type macrophages, induced proinflammatory reactions through stimulation by microbial patterns, interferon-γ, TNFα, or colony stimulating factor (CSF)-1 lead to increased antimicrobial responses similar to a mammalian M1 phenotype. In M2-type-like macrophages, alternative activation of teleost macrophages can be achieved by cAMP stimulation. Lipopolysaccharides (LPSs), interleukin (IL)-10, and glucocorticoids can deactivate their macrophages (e.g., induction of IL-10 and reduction of IL12) (Hodgkinson et al. 2015). Teleost M1-type macrophages have high iNOS gene expression, while alternatively activated M2-type macrophages exhibit upregulated arginase transcript levels (Grayfer et al. 2014).

Dendritic Cells

It has been determined that dendritic cells (DCs) in mammals have phagocytic activity and are important in antigen-presenting cells, but many questions remain regarding fish, although DC-like cells have been reported (Pettersen et al. 2008; Wittamer et al. 2011; Zoccola et al. 2015). DCs in rainbow trout developed nonadherent cells from hematopoietic tissue that had irregular membrane processes and expressed surface MHC II. Trout DCs possess DC markers (i.e., CD83, B7), the ability to phagocytose small particles, the capacity to be activated by T receptor ligands, and the ability to migrate in vivo (Bassity and Clark 2012). Furthermore, $CD8\alpha^+$ MHC II^+ DC-like subpopulations in the skin have been identified, showing phenotypical and functional characteristics of semimature DCs, and DCs also express CD141 and CD103 genes (Granja et al. 2015).

NK Cells/NCCs, Innate Lymphoidlike Cells

It is well known that natural killer (NK) cells nonspecifically adhere to and attack virus-infected cells and cancer cells in mammals. It is considered that NCCs correspond to NK-like cells in fish and have been identified in rainbow trout, catfish, tilapia, and zebrafish (Evans and Jaso-Friedmann 1992; Ghoneum et al. 1988; Moss et al. 2009). However, NK-like cell lines that are distinct from NCCs (which are negative for markers defining neutrophils, monocytes, and NCCs) have been developed from catfish peripheral blood leukocytes (PBLs) (Shen et al. 2004). The

relationship between NK-like cells and NCCs in catfish remains unknown, except that the source of cells differs, that is, NK-like cells are isolated from PBLs while NCCs are organ-derived cells. NCCs possess nonspecific cell-mediated cytotoxicity (CMC) (Nakanishi et al. 2011) and express a novel type III membrane protein called NCC receptor protein 1 (NCCRP-1) (Evans et al. 1998).

Recently, B cells that possess innate immune functions have been reported in rainbow trout. IgM^+ and IgT^+ B cells of rainbow trout could express multiple AMP genes including four cathelicidin genes and one β-defensin gene (Zhang et al. 2017a, b). The cathelicidin peptides could significantly enhance the phagocytic, intracellular bactericidal, and reactive oxygen species activities of trout IgM^+ and IgT^+ B cells, a phenomenon previously reported only in macrophages, and these activities might also be mediated by the $P2X_7$ receptor (Zhang et al. 2017a, b).

Thrombocytes

In common carp, thrombocytes represent nearly half of the phagocyte population on the total PBLs, and phagocytosis efficiency is further enhanced by serum opsonization. Particle internalization led to phagolysosome fusion and killing of internalized bacteria, pointing to a robust ability for microbe elimination. This potent phagocytic activity is shared across other teleosts such as Japanese flounder (*Paralichthys olivaceus*) and amphibian (*Xenopus laevis*), implying its conservation throughout the lower vertebrate lineage (Nagasawa et al. 2014, 2015). Thrombocytes express MHC class II genes, suggesting antigen presentation.

Phagocytic B Cells (B-1 Cells)

As described in section "Neutrophils", fish B cells show phagocytic activity and antigen presentation. The phagocytic and intracellular bactericidal capacities of B cells were first demonstrated in rainbow trout (Li et al. 2006), and the percentage of phagocytic IgT^+ B cells was similar to that of IgM^+ B cells (Zhang et al. 2010). Phagocytic capacity was also found in amphibians (Li et al. 2006) and reptiles (Zimmerman et al. 2010). The existence of subsets of B cells with such capacities has been reported in mouse liver (Nakashima et al. 2012) and mouse peritoneal cavity B-1 cells (Gao et al. 2012). Moreover, it has been reported that B-1 B cells in mouse peritoneal cavity have phagocytic and microbicidal capacities and present phagocytosed antigen to $CD4^+$ T cells (Parra et al. 2012). Currently, the phagocytic activity of IgM+ B cells in several fish species, such as zebrafish, lumpfish, and tongue sole, have also been confirmed (Zhu et al. 2014; Ronneseth et al. 2015; Yang et al. 2017).

Humoral Factors

Complement System

Complements play an important role in host defense to activate the function of the antigen–antibody complex and to react nonspecifically to bacterial cell wall components. The complement system involves more than 30 protein molecules, including

the 9 main components of C1 to C9, factor B, factor D, factors involved in the inhibition of activation (i.e., C4b binding protein, factor I, factor H), and complement-related factors (i.e., CR1, CR3, which is on the phagocytic cell surface) (Nonaka and Smith 2000). In teleosts, the main components C1 to C9, factors B and D (Nonaka and Kimura 2006), the membrane-attack complex (MAC), which consists of C5 and C9 (Yano 1995), and factors I and H (Anastasiou et al. 2011; Xiang et al. 2015) were observed. A complement may have three activation pathways: classical (first route), alternative (second route), and the lectin pathway (third route), which was recently revealed (Nonaka and Smith 2000; Nakao et al. 2011). The mechanism of the lectin pathway has been clarified; the complement is activated by the recognition and binding of mannose-binding lectin (MBL) to mannose on the target cell. MBL-associated serine protease (MASP)-1 and -2 are bound to this MBL, and this complex plays the same role as C1 in the classical pathway, and the subsequent activation is the same as in the classical pathway. For the lectin pathway in fish, MBL (Gercken and Renwrantz 1994) and MASP (Endo et al. 1998) are found, and it is believed that lectin pathways also exist. However, since potential C2 and factor B are the same molecule in fish as described earlier, the lectin pathway of fish could be the same as the alternative route (Nonaka and Smith 2000; Nakao et al. 2011).

Some activated fragments of complement components bind to a target cell of foreign substances and react as opsonins. Opsonin is a general term for serum factors that induce phagocytosis by phagocytic cells by binding to the surface of the phagocytic particles of bacteria and foreign substances. Phagocytic cells have a receptor on the cell surface for opsonins. C4b, C3b, iC3b (inactivated C3b on the cells of foreign substances by a C3b inactivator), and C3d (a fragment that can be C3b is decomposed further) have opsonic activity in complement component fragments. The opsonic activity of C4b is not as strong and the main opsonization of complement is by C3. Many studies have reported that normal serum (complement) of fish shows opsonization (Moritomo et al. 1988; Matsuyama et al. 1992; Jenkins and Ourth 1993). Further, it has also been reported that the phagocytic cells of fish express opsonic receptors (Matsuyama et al. 1992).

Lysozyme

Lysozyme is an enzyme that hydrolyzes $\beta 1 \rightarrow 4$ binding between the N-acetylmuramic acid and N-acetyl glucosamine present in bacterial cell walls and prevents bacterial infection in many organs (Jollès and Jollès 1984; Callewaert and Michiels 2010). In general, lysozyme has a direct effect against the peptidoglycan layer of Gram-positive bacteria, and it is effective against Gram-negative bacteria only when such bacteria are damaged by complement. In fish, it has been reported that lysozyme shows bactericidal effects not only against Gram-positive but also Gram-negative bacteria, although its lytic activity is not perfect (Yousif et al. 1994a). As mentioned earlier, fish are constantly exposed to the risk of many bacteria invading their body through the mucus and skin. In this situation, it is considered that fish lysozyme plays an important role in nonspecific host defense.

There are two types of lysozyme in fish, chicken type (C-type) and goose type (G-type) (Hikima et al. 2002; Callewaert and Michiels 2010). The C-type lysozyme

has been identified in numerous fish species, such as rainbow trout, Japanese flounder (*Paralichthys olivaceus*), common carp, brill (*Scophthalmus rhombus*), Senegalese sole (*Solea senegalensis*), grass carp (*Ctenopharyngodon idellus*), and others (Dautigny et al. 1991; Hikima et al. 1997, 2000; Savan et al. 2003; Jiménez-Cantizano et al. 2008; Fernández-Trujillo et al. 2008; Ye et al. 2010). The G-type lysozyme was previously only detected in avian (Périn and Jollés et al. 1976; Nakano and Graf 1991) before the fish G-type lysozyme gene was identified from Japanese flounder (Hikima et al. 2001). Since this discovery, the G-type lysozyme gene has been found in many fish species (Yin et al. 2003; Zheng et al. 2007; Kyomuhendo et al. 2007; Larsen et al. 2009; Whang et al. 2011) and mammals (Irwin and Gong 2003).

The lytic activity of fish lysozyme has been detected generally in the skin mucus, serum, kidney (HK and TK), liver, gills, and eggs (Yano 1996; Saurabh and Sahoo 2008). Tissue expression showed the presence of the lysozyme gene in these tissues (Hikima et al. 2002; Callewaert and Michiels 2010). In addition, the gene expressions of C- and G-type lysozymes increase in the HK and spleen after pathogenic bacterial infection (Hikima et al. 1997; Jiménez-Cantizano et al. 2008; Ye et al. 2010). In turbot (*Scophthalmus maximus*), two G-type lysozymes were identified, and one of the genes showed a significant upregulation in intestine following *Vibrio anguillarum* and *Streptococcus iniae* challenge (Gao et al. 2016).

In experiments with Japanese flounder recombinant lysozymes (i.e., C-type and G-type lysozymes), which were produced in insect cells, only slight lytic activity was shown against *Edwardsiella tarda*, a pathogen of Japanese flounder. However, stronger lytic activity was revealed against *V. anguillarum* and *Pasteurella piscicida* (currently *Photobacterium damselae* subsp. *piscicida*), which are not pathogens. The results suggested that there was some relationship between the host specificity and antibacterial activity of the lysozyme (Hikima et al. 2001; Minagawa et al. 2001). The activity of other fish G-type lysozymes against pathogenic Gram-negative bacteria has been reported in grass carp against *V. parahaemolyticus*, *E. tarda*, and *Aeromonas sobria* (Ye et al. 2010) and in yellow croaker against *A. sobria, V. parahaemolyticus*, and *V. vulnificus* (Zheng et al. 2007). In addition, since the C-type lysozyme has a lytic activity against fish bacterial pathogens (such as *E. tarda*) (Hikima et al. 2001; Minagawa et al. 2001), it has been revealed that lysozyme is actually important for infection by an experimental system using the chicken lysozyme gene transgenic zebrafish (Yazawa et al. 2006).

Lectin

Lectin is present in most living organisms and causes agglutination by binding to sugar on the cell surface. Lectin has at least two sugar-binding sites, and its binding specificity is high. In fish, lectin activity is observed in body surface mucus, blood, tissue, and eggs (Yano 1996). It is suggested that lectin in eggs may contribute to biological defense since it helps normal fertilization and the development of eggs (Krajhanzl 1990) and it aggregates specific bacteria (Yousif et al. 1994b). Lectin in the body surface (skin) also aggregates bacteria (Kamiya et al. 1988). In addition, it is considered that skin lectin plays some role against bacterial infection because

lectin shows higher activity in bacterial infection. Lectin plays an important role for the complement activation pathway (lectin pathway) since MBL is present in fish blood (Gercken and Renwrantz 1994). Further, it is also known that human MBL shows opsonic activity (Matsushita and Fujita 2001). It is suggested that fish lectin functions for the lectin pathway and plays an important role as a typical host defense factor since MBL genes have been identified from carp, goldfish, zebrafish, rainbow trout, and lamprey, and those show the ability to bind to foreign substances (Vitved et al. 2000; Nikolakopoulou and Zarkadis 2006; Takahashi et al. 2006). In two pufferfish, *Takifugu rubripes* and *T. niphobles*, pufflectin possessing two conserved mannose-binding domains was found and expressed in the skin, gills, brain, and muscle. The recombinant pufflectin protein exhibited binding activity specific for d-mannose, and both pufflectins were resistant and susceptible to the monogenean parasite *Heterobothrium okamotoi* in gill (Tasumi et al. 2016).

Galectins are also well known as another lectin and belong to the S-type lectin family that binds to β-galactoside and are involved in cell adhesion and regulation of growth and differentiation. Fish galectin (gene or protein) has been isolated and identified from conger eel, rainbow trout, and zebrafish and is present in many tissues such as body surface, gills, kidney, and spleen (Muramoto and Kamiya 1992; Inagawa et al. 2001; Tasumi et al. 2004; Vasta et al. 2004). Galectin is widely involved in the body's defense such as the differentiation of B and T cells and macrophage activation (Vasta et al. 2004).

Transferrin

Transferrin is an iron-binding protein present in serum that chelates two irons into one molecule. Transferrin is involved in the capture of absorbed iron and transporting it to hematopoietic tissue to construct hemoglobin. This is why free iron is present only in small amounts in the body. Iron is also essential for bacteria to live. Since free iron in the blood is very low because of transferrin, normal bacteria eventually die because they can't absorb iron. Thus, transferrin does not kill bacteria directly but by inhibiting bacterial proliferation. This is also referred to as bacteriostatic action.

Transferrin also exists ubiquitously in fish (Jamieson 1990). The apparent toxicity of *E. tarda* and *V. anguillarum* increases when iron is preinjected into eel (Iida and Wakabayashi 1990; Nakai et al. 1987). It is considered that the amount of free iron in a body is increased beyond the iron-chelating ability of transferrin. Thus, transferrin plays a role in nonspecific host defense. Transferrin is a multiple phenotype and the relationship between the expression type and disease resistance mainly in salmonid fish has been reported (Suzumoto et al. 1977; Winter et al. 1980; Withler and Evelyn 1990).

The structures of various fish transferrin genes have been revealed (Hirono et al. 1995; Lee et al. 1998). It has been clarified that a transferrin molecule is composed of two regions having a structure similar to that in mammalian transferrin. However, the expression type described earlier, that is, the relationship between genotype and disease resistance, is not clear. It has been shown that goldfish transferrin is involved in the activation of phagocytic cells by molecular and biological analysis (Stafford and Belosevic 2003). Furthermore, it has also been reported that recombinant

transferrin induces nitric oxide production of macrophages in goldfish and mouse (Stafford et al. 2004). In goldfish, macrophages contributed to transferrin cleavage produced during pathogen-induced acute inflammation, and the appearance of transferrin cleavage products correlated with the influx of leukocytes but did not necessarily correlate with the induction of robust respiratory burst and nitric oxide responses (Trites and Barreda 2017).

Pattern Recognition Receptors

Pattern recognition receptors (PRRs) play key roles in the innate immune system of animals, including teleost fish, in the recognition of pathogen-associated molecular patterns (PAMPs) derived from invading pathogenic microorganisms (Kawai and Akira 2011). Whereas PRR-recognizing PAMPs are very diverse, no such varied molecules are recognized by T-cell receptors and immunoglobulin in acquired immunity. PAMPs include bacterial components (e.g., lipoprotein, lipopolysaccharide, peptidoglycan, flagellin), viral nuclei (dsDNA, ssRNA, and dsRNA), and other components. The recognition of PAMPs by PRRs activates the innate immune system. PRRs include five receptor/sensor categories: TLRs (section "Toll-Like Receptors"), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (section "Retinoic Acid-Inducible Gene I (RIG-I)-Like Receptors"), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (section "Nucleotide-Binding Oligomerization Domain (NOD)-Like Receptors"), C-type lectin receptors (CLRs) (section "C-Type Lectin Receptors"), and cytosolic/cytoplasmic DNA sensors (CDSs) (section "Cytosolic/Cytoplasmic DNA Sensors") (Fig. 3).

Toll-Like Receptors

Of these, TLRs are the most-researched and best-known microbial recognition molecules of vertebrates including fish; their identification followed the discovery of the homolog gene of *Drosophila* Toll receptor. Ten TLR genes (i.e., TLR1–TLR10) have been found in humans and mice, and TLRs 11–13 have also been detected. In fish, TLR genes in many species have been found using in silico genomic databases such as those for Japanese pufferfish and zebrafish, and 8–15 TLR genes have been identified in teleosts, depending on their species (Roach et al. 2005; Takano et al. 2010; Aoki et al. 2013; Liao et al. 2017). The secretion type TLR5 (TLR5S) , TLR14 (identical to the TLR18 in zebrafish), TLR19, TLR20, TLR21, TLR22, and TLR23 appear to be TLR molecules that are specifically present in fish; these TLRs have been identified in many teleosts (Hwang et al. 2011a, b; Takano et al. 2010; Aoki et al. 2013). However, TLR6, TR10, TLR11, and TLR12 are present in mammals but not in fish. TLR1 and TLR6 genes are present in tandem in the human genome. However, it has been revealed that the TLR1 gene is found in the Japanese pufferfish genome but TLR6 gene has not been found in the vicinity using synteny analysis (Oshiumi et al. 2003).

It was revealed that TLR6 is evolutionarily close to TLR1 since the amino acid sequence is similar. The TLR1 found in fish is considered to be an ancestral gene of

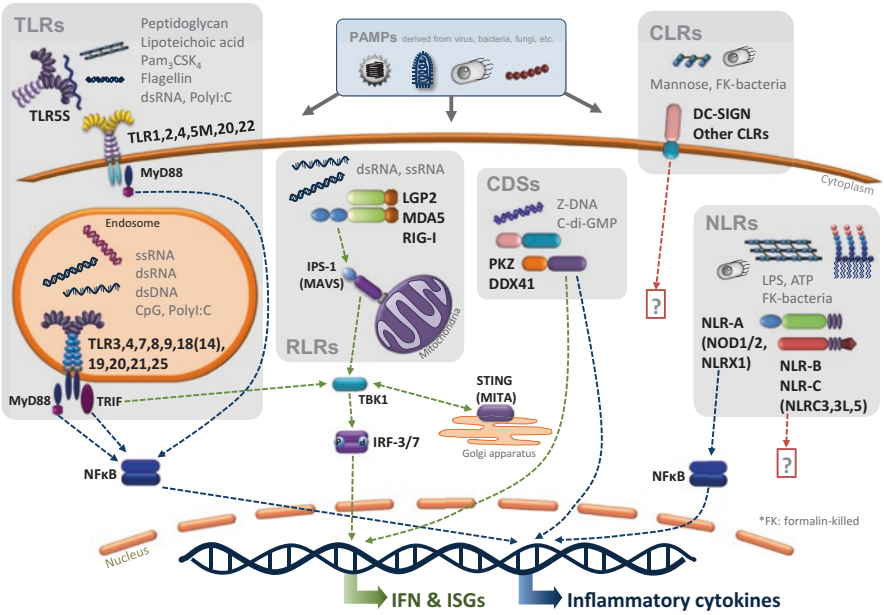

Fig. 3 PPR repertoires and their PAMPs in teleosts

TLR1 and TLR6 in mammals. The region of the Japanese flounder TLR2 gene matches the locus involved in resistance against lymphocystis disease and has been found by quantitative trait locus analysis searching in the vicinity of the Japanese flounder genome (Hwang et al. 2011a). TLR5S, which was cloned from rainbow trout, recognizes and binds to bacterial flagellin and activates the signaling into a TLR cascade in the same manner as the membrane-type TLR5 (TLR5M) in mammals (Tsujita et al. 2004). The presence of TLR5S and TLR5M has also been confirmed in Japanese flounder and Japanese pufferfish (Hwang et al. 2010; Oshiumi et al. 2003). The TLR4 gene has been identified in cyprinids such as zebrafish, but it is known that it does not show NF-κB-transcriptional activation by LPS stimulation, unlike TLR4 in mammals (Sepulcre et al. 2009). Furthermore, TLR4 has been found only in the cyprinid fish genome, suggesting that the TLR4 gene in fish is different. Therefore, this suggests that the genomic diversity of the PAMP-recognition mechanism is present even within teleosts (Roach et al. 2005). The expression of the teleost TLR9 gene is upregulated by GpG-ODNs (Skjaeveland et al. 2008; Cuesta et al. 2008a, b). Interestingly, the rainbow trout TLR9 gene is also upregulated by *E. coli*-produced rIFN-γ, and it shows a higher induction than CpG-ODN stimulation (Skjaeveland et al. 2008). In Japanese flounder, TLR9 promotes the expression of inflammatory cytokines in the presence of CpG-ODN (Takano et al. 2007). Atlantic salmon and zebrafish TLR9s bind CpG-ODN and induce ISRE-promoter activity (Iliev et al. 2013; Yeh et al. 2013). It is known that there is a difference in TLR responses depending on the type of CpG motif. In zebrafish, TLR9 broadly recognizes CpG-ODN with different CpG motifs, but

CpG-ODN with "GACGTT" or "AACGTT" better showed the NF-κB-activity. In contrast, TLR21 responded preferentially to CpG-ODN with "GTCGTT" motifs (Yeh et al. 2013).

Retinoic Acid-Inducible Gene I-Like Receptors

In mammals, the expression of the type-I IFN gene is dramatically induced by viral nucleic acids, for example, single-stranded (ss) RNA or double-stranded (ds) RNA, triggered by their recognition through RLRs (Takeuchi and Akira 2010), except for the aforementioned TLRs. Cytosolic viral PAMPs are recognized by RLRs, including RIG-I, MDA5 (melanoma differentiation associated gene 5), and LGP2 (Laboratory of Genetics and Physiology 2), and the signaling enhances the production of type I IFN through RLR adaptors, IPS-1 (IFN-β promoter stimulator-1, alternatively called MAVS) (Loo and Gale 2011). MDA5 and LGP2 counterparts were identified in many fish species, although RIG-I was found only in Acanthopterygian fish, including fish species in Salmonidae, Cyprinidae, and Gadinae, suggesting that MDA5 may have evolutionarily emerged before RIG-I (Aoki et al. 2013). Teleost RIG-I and MDA5 recognize cytosolic viral RNA, leading to IFN antiviral response (Biacchesi et al. 2009; Simora et al. 2010). In contrast, teleost LGP2 exhibits controversial functions, in other words, contradictory phenomena as positive and negative antiviral responses appear in fish, and the same happens to mammalian LGP2 (Ohtani et al. 2010; Han et al. 2016; Yu et al. 2016; Liu et al. 2017; Zhang et al. 2017a, b). In Japanese flounder, LGP2 and MDA5 encourage an antiviral state by inducing strong expression of type-I IFN and IFN-inducible genes such as ISG15 and Mx in HINAE cells (i.e., Japanese flounder embryo cells) infected with VHSV (Ohtani et al. 2010, 2011). In zebrafish, a full-length LGP2 and two splicing variants, LGP2v1 and LGP2v2, play distinct roles during IFN antiviral response. The full-length LGP2 not only potentiates IFN response in the absence or presence of poly(I:C) at limited concentrations but also inhibits IFN response by relatively high concentrations of poly(I:C) in the RLR pathway; however, LGP2v1 and LGP2v2 only retain an inhibitory role (Zhang et al. 2017a, b).

Nucleotide-Binding Oligomerization Domain-Like Receptors

NLRs contain leucine rich repeats and are a family of intracellular sentinels due to a lack of signal peptides and transmembrane domains that are involved in both defense against pathogenic microorganisms and cellular damage (Wilmanski et al. 2008; Shiau et al. 2013). In teleosts, NLRs include three distinct subfamilies: NLRA, NLRB, and NLRC (alternatively called NLR-A, NLR-B, and NLR-C), and of those, NLRC as a large subfamily is unique to bony fish (Laing et al. 2008; Sha et al. 2009; Biswas et al. 2016; Li et al. 2016). In the zebrafish genome, a family of nearly 400 NLR proteins are encoded and contain several functional domains such as NACHT, CARD, Pyrin, B30.2, and so on. NLRs have experienced massive species-specific expansions and domain shuffling, all of which fits the common phenomenon of parallel evolution (Howe et al. 2016). The B30.2 (PRY-SPRY) domain as a characteristic domain of the NLRC subfamily plays an important role in immune recognition, and this domain is maintained as a component of immune

defense (Rhodes et al. 2005). The expression of NLRC genes (e.g., NLRC, NLRC3, NLRC5) in fish was induced by viral and bacterial pathogens (Unajak et al. 2011; Biswas et al. 2016; Wu et al. 2017) and environmental particulates such as silica (Morimoto et al. 2016). NLRA, such as NLRX1, NOD1, and NOD2, also plays an important role in response to fish pathogens (Sha et al. 2009; Park et al. 2012; Li et al. 2015). Furthermore, goldfish NLRC3-like (NLRC3L) molecules interact with apoptosis-associated spec-like protein (ASC), indicating that NLRC3L may participate in the regulation of inflammasome responses (Xie and Belosevic 2018).

C-Type Lectin Receptors

C-type lectin receptors (CLRs) containing carbohydrate-recognition domains (CRDs) or C-type lectin-like domains (CTLDs) comprise a large family of extracellular receptors that bind to carbohydrates in a calcium-dependent manner (Zelensky and Gready 2004). Teleost MBL (section "Lectin") also belongs in this group as a soluble CLR. In teleosts, many types of CLRs have already been identified and are characterized by ligand affinity (Uribe et al. 2013) and their function against pathogenic microbes (Ao et al. 2015; Ma et al. 2016). In zebrafish and miiuy croaker, DC-SIGN (alternatively known as CD209) as a typical transmembrane CLR possesses CRD for ligand recognition and associates with various antigen-presenting cell (APCs), including macrophages, B cells, and a possible DC-like $CD80^+/DC83^+$ population (Lin et al. 2009; Shu et al. 2015). However, Dectin-1/2 and Mincle as typical mammalian CLRs for recognizing fungi-derived PAMPs have not been found in teleosts.

Cytosolic/Cytoplasmic DNA Sensors

In mammals, CDSs are comprised of various members and possess the potential to recognize dsDNA derived from viruses and intracellular bacteria (Gasser et al. 2017), whereas only two CDSs, including PKZ (protein kinase containing Z-DNA binding domains) and DDX41 (DExD/H-box 41), have been identified in teleosts (Rothenburg et al. 2005; Quynh et al. 2015). In zebrafish, PKR binds Z-DNA (dsDNA of left-handed Z conformation) by two Zalpha domains and plays a role in host response to viruses (Rothenburg et al. 2005; de Rosa et al. 2013). In Japanese flounder, DDX41 induces antiviral and inflammatory cytokine gene expression through cytoplasmic C-di-GMP treatment (Quynh et al. 2015). It has been proposed that these sensors are involved in the enhancement of antiviral and antibacterial immune responses after infection with pathogens in the cytoplasmic area.

Adaptive Immune System

Cells Involved in Adaptive Immunity

Adaptive immunity is mediated by two lymphocyte populations classified as T cells and B cells. Conventional T cells possess a TCR and CD3 together with costimulatory and co-inhibitory surface molecules. T cells are divided into two functional

groups of cytotoxic T cells expressing CD8α/β molecules and helper T cells with CD4 molecules. In teleosts, both $CD4^+$ and $CD8^+$ T cells have been identified, and their functions have been characterized, although the studies at the T-cell subset level are limited in several species, such as ginbuna crucian carp, rainbow trout, and sea bass, due to the availability of antibodies against CD4 and CD8. As for B cells, three major B-cell lineages have been described, those expressing either IgT or IgD and the most common lineage, which coexpresses IgD and IgM. Recently, B-cell subsets with phagocytic and intracellular bactericidal activities have been reported (Li et al. 2006) (section "Phagocytic B Cells (B-1 Cells)"). This finding led to the identification of B cells with phagocytic and microbicidal abilities even in mammals (Sunyer 2012).

Toda et al. (2011a, b) demonstrated in vitro proliferation of $CD4^+$ T cells by allogeneic combination of mixed leukocyte culture (MLC) and antigen-specific proliferation of $CD4^+$ T cells after in vitro sensitization with OVA, suggesting the primordial functions of helper T cells in fish. Recently, a culture system of $CD4^+$ αβ T cells was established in carp, and $CD4^+$ αβ T cell clones sharing some features with mammalian Th2 cells were obtained by selecting single cells from the bulk culture of helper T cells (Yamaguchi et al. 2013). In channel catfish, five groups of clones, including alloantigen-specific TCR $\alpha\beta^+$ cytotoxic clones (presumably CTLs), NK-like cells, were identified employing MLC followed by limiting dilution (Stuge et al. 2000). Effector cells in CMC against allogeneic cells or virus-infected syngeneic cells were first characterized as surface Ig (sIg) negative cells and, later on, as cells expressing CD8α or TCR α or β mRNA. Only $CD8\alpha^+$ CTLs among $CD8\alpha^+$, $CD4^+$, $sIgM^+$, and $CD8\alpha^-CD4^-sIgM^-$ cells showed specific cytotoxicity against allogeneic cells, while $sIgM^+$ cells, including NK-like cells, exhibited nonspecific killing (Toda et al. 2009). This is the first demonstration of the presence of CTLs in a defined T-cell subset in fish.

The presence of several forms of $CD4^+$ cells, CD4-1^+, and CD4-2^+ (CD4-related or CD4-rel) cells has been reported in several fish species (Fig. 4). Recently, Takizawa et al. (2016) reported the presence of three types of $CD4^+$ cells in rainbow trout, that is, CD4-1 single positive, CD4-2 single positive, and CD4-1/CD4-2 double positive cells. They reported that CD4-1/CD4-2 double positive cells were the dominant population accounting for 83–91% of the total $CD4^+$ lymphocytes in all tissues examined, while CD4-2 single positive cells were around 10%, and CD4-1 single positive cells turned out to be monocytes/macrophages. However, it has been reported that CD4-1 and CD4-2 are expressed in different T-cell lines in channel catfish (Edholm et al. 2007), common carp (Yamaguchi et al. 2013), and Japanese flounder (Kato et al. 2013). Furthermore, Somamoto et al. (2014) reported that CD4-1 and CD4-2 transcripts were expressed in different cell populations, and CD4-1^+ cells did not contain $CD4^+$ monocytes. Therefore, the tissue distribution of three types of $CD4^+$ cells and their expression of CD4-1 and CD4-2 may be different among fish species, although the presence of CD4-1/CD4-2 double positive cells is suggested by the expression of both genes in MACS sorted CD4-1+ cells (Somamoto et al. 2014), as has been reported in zebrafish (Yoon et al. 2015; Dee et al. 2016).

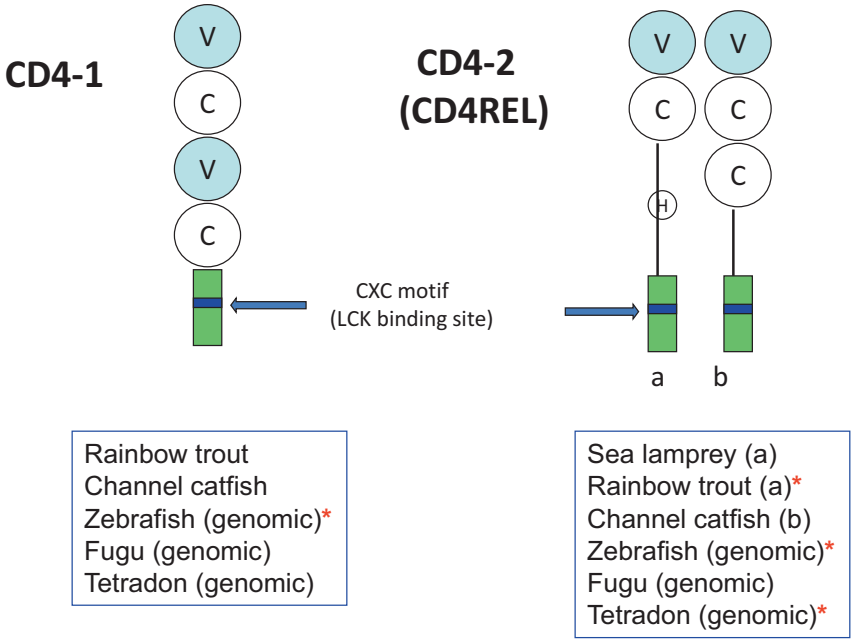

Fig. 4 Presence of two CD4 molecules in teleost fish

Regulatory T-cell (T_{reg})-like cells with the phenotype CD4–2$^+$, CD25-like$^+$, and Foxp3-like$^+$ have been reported from a pufferfish, which showed a suppressive effect on MLR and nonspecific cytotoxic cell (NCC) activity in vitro (Wen et al. 2011). Recently, APCs resembling mammalian DCs have been identified in zebrafish. Zebrafish DCs possess the classical morphological features of DCs and exhibit the expression of genes associated with DC function and activate T lymphocytes in an antigen-dependent manner (Lugo-Villarino et al. 2010).

Molecules Involved in Adaptive Immunity

Immunoglobulins

It was generally thought that IgM was the only functional immunoglobulin class in teleosts until a new Ig H chain gene, named *ighτ* in rainbow trout (Hansen et al. 2005) or *ighζ* in zebrafish (Danilova et al. 2005), was discovered. The assembled immunoglobulins containing the *ighτ* and *ighζ* product were named IgT and IgZ, respectively. Since then, genes encoding IgT or IgZ have been cloned and characterized in a number of teleost species (reviewed in Zhang et al. 2011). Teleost IgH genes possess a translocon configuration, VH-DH-JH-C*τ*/*ζ*-(VH)-DH-JH-Cμ-Cδ, although there are differences in C*τ*/*ζ* gene locations between two teleost groups: (1) in zebrafish, fugu, and three-spined stickleback, the C*τ*/*ζ* genes are inserted

between VH and DJ genes (Danilova et al. 2005; Gambón-Deza et al. 2010; Savan et al. 2005a, b); (2) in rainbow trout, this gene is located within the VH gene region (Hansen et al. 2005). Interestingly, in channel catfish, Cτ/ζ genes are not found either in the 3′ region of the VH gene cluster or within the 55 VH genes (>100 kb) (Bengtén et al. 2006).

In this review, we will use IgT ("T" for teleost) since the structure is similar for the two immunoglobulins, although the genomic organization and domain numbers of IgT are variable among different species. Thus, three immunoglobulin classes have been identified in teleosts: IgM, IgD, and IgT. IgM is the primary antibody present in teleost serum and mucus and is expressed as a tetramer. In contrast, IgT is expressed as a monomer in rainbow trout serum and a tetramer in gut mucus (Zhang et al. 2010). Teleost IgM possesses varying levels of intermonomeric disulfide polymerization, yielding tetramers, trimers, dimers, and monomers. A direct association of affinity with disulfide polymerization has been reported in IgM. Polymerization of IgM is suggested to contribute affinity maturation in teleosts that lack class switching (Ye et al. 2011).

In mammals, peripheral B cells are present as $mIgM^+/mIgD^+$ or $mIgM^-/mIgD^+$ B cells. $mIgM^-/mIgD^+$ B cells have been reported in catfish (Edholm et al. 2010) and rainbow trout (Ramirez-Gomez et al. 2012), although $mIgM^+/mIgD^+$ B cells are the most common lineage. In catfish, circulating granular leukocytes are armed with surface IgD, suggesting that the binding of B-cell-derived IgD to granulocytes may be part of an evolutionarily conserved immune pathway that is potentially important in the activation of the antimicrobial, opsonizing, inflammatory, and B cell-stimulating factors through binding to basophils via a calcium-mobilizing receptor in humans (Chen et al. 2009). In contrast, $mIgT^+/mIgM^+$ B cells have not been identified yet, suggesting that teleost B cells express either IgT or IgM and the two are distinct B-cell lineages.

The teleost IgD gene in all teleosts is reported to be expressed as a chimeric transcript with Cμ1 as the first C domain (Hikima et al. 2011). No class-switching sequences are found in the intergenic region of Cμ to Cδ, where the IgH enhancer Eμ is located (Magor et al. 1994), suggesting that Ig transcripts are produced by alternative mRNA splicing rather than class switching involving chromosomal recombination (Hansen et al. 2005; Saha et al. 2004a; Srisapoome et al. 2004; Stenvik and Jørgensen 2000; Wilson 2014). Furthermore, the teleost primordial Eμ3′ enhancer differs in important respects in terms of its structure and functions (i.e., transcriptional regulations depending on different transcription factors such as E-proteins and Oct factors) from the mammalian Eμ enhancer (Magor et al. 1994; Cioffi et al. 2001; Hikima et al. 2004, 2006a, b; Lennerd et al. 2007).

As in higher vertebrates, teleost immunoglobulins produced by B cells are present either as a secretory form (antibody) or as a membrane form (B cell receptor) for all three immunoglobulins including IgD (catfish: Bengten et al. 2002; trout: Ramirez-Gomez et al. 2012) and IgT (Zhang et al. 2011). Teleost IgM has not been found to possess a J chain, suggesting that the H chain may possess the binding motif for pIgR (Zhang et al. 2010). The C-domain structure of IgT varies among species, and rainbow trout and zebrafish have four constant Ig domains, while

stickleback (Gambon-Deza et al. 2009) and fugu (Savan et al. 2005a, b) possess three and two, respectively. Interestingly enough, the genomic organization of IgT and IgM is similar to that of the mouse TCRδ and TCRα. The genomic structure of the locus-encoding IgT and IgM heavy chains exclude the possible class-switch recombination between the genes encoding IgT and IgM (Zhang et al. 2010).

T-Cell Receptors

The initial description of the TCRα and TCRβ was reported in rainbow trout (Partula et al. 1995, 1996), and all four TCR chains, including TCRγ and TCRδ, were identified in Japanese flounder (Nam et al. 2003). Since then, orthologs for all four TCR chains have been reported in many other teleosts (reviewed in Castro et al. 2017; Laing and Hansen 2011). The basic TCR structure is well conserved in teleosts. The organization of the teleost TCRα and TCRδ locus is similar to that in mammals, and TCRα and TCRδ genes are encoded in the same locus. The TCRβ locus is also generally organized as in humans and mice. For instance, 11 TCRβJ genes followed by a TCRβC were found downstream of a TCRβD gene, all in the same transcriptional orientation (De Guerra et al. 1997). In mammals, the TCRδ locus is nestled into the TCRα locus, and this also happens in teleosts (Nam et al. 2003), although the second TCRδ isotype including two genes was found close to the TCRγ locus. However, teleost fish TCRs display novel characteristics not observed for mammals. For instance, the teleost TCRβ chain locus contains two highly divergent constant domain regions, and salmonids express five distinct constant region genes for TCRγ. Furthermore, the connecting region of the teleost TCRβC is shorter than in mammals, as seen in sharks, amphibians, and chickens (Partula et al. 1995).

The great diversity of T-cell repertoires is attributed to mutations at the V-(D)-J junctions in mammals. This also happens in teleosts, and the length diversity of the complementarity-determining region (CDR) was much higher than that of human and mouse (see review by Castro et al. 2017).

In mammals, αβ-T cells are the more abundant in lymphoid organs and blood, whereas γδ-T cells are distributed in mucosal tissues.

MHC Class I/II

MHC genes including classes IA, B2m, IIA, and IIB have been reported from a number of fish species. In teleosts, MHC class I and II genes are separately located on different chromosomes, although the MHC I and II linkage is observed in sharks and in mammals (Stet et al. 2003). The extensive polymorphism of classical MHC class I (Ia) genes has been observed in rainbow trout. Trans-species polymorphism is a common feature throughout vertebrates, for example, the amino acid sequence of the α2 domain of MHC class Ia gene is more closely related to that of the carp and zebrafish than that of other salmonids. Ubiquitous expression of MHC Ia genes has been reported in many species of fish. Currently, five different MHC class I lineages, including U, Z, S, L, and P, and three MHC class II lineages, including A, B, and E, have been identified in teleosts based on phylogenetic clustering (Grimholt 2016). The polymorphic and classical MHC class I and class II gene sequences

belong to the U and A lineages, respectively (Grimholt 2016). Nonclassical class I and II genes defined as low (limited) polymorphism, more restricted expression pattern in tissues, and lower conservation of amino acid residues involved in peptide binding are also present in teleosts, although several of those are only present in some teleost species. Huge MHC class I expansion in Atlantic cod has been suggested to compensate for the lack of MHC class II. However, similar MHC class I expansion is seen in tilapia and stickleback, although the species possesses a functional MHC class II. Thus, the driving force of MHC class I expansion remains unknown.

Enhanced expression of MHC class II has been noted in lymphoid tissues of Atlantic salmon following vaccination (Fischer et al. 2013). The important role of the MHC class II linkage group in tissue rejection has been reported in Gila topminnow. The MHC class I linkage group was found to be the major determinant for in vivo allograft rejection. Correlation between polymorphism in MHC class Ia genes with behavioral traits such as aggression has been reported in rainbow trout (see review by Nakanishi et al. 2011).

Function of T and B Cells

The teleost immune system is rather simple and undifferentiated at both the cellular and molecular levels when compared to that of mammals as mentioned earlier in this chapter as characteristics of teleost immune system. These features may evoke poor reactivity against foreign antigens. However, teleost fish exert immune responses against a variety of antigens with specificity and memory.

T-Cell Function

It is well known that in mammals T cells play a central role in adaptive immune response, and the several subsets of T cells have a distinct function involved in both humoral and cell-mediated immune responses. In fish, functions of T cells similar to those known in mammals have been reported in in vivo and in vitro experiments, for example, Th cells assist other cells such as B cells and macrophages, and CTLs kill virus-infected cells and transplanted allogeneic cells and tissues.

In Vivo Studies

Skin or scale allograft rejection is a representative phenomenon of specific cell-mediated immunity. Cellular reactions that occur at the grafting site are essentially the same as those in mammals, as characterized by specificity and memory (reviewed in Manning and Nakanishi 1996). Agnathans and elasmobranchs reject first-set grafts in a chronic manner, while teleosts can evoke allograft rejection in an acute fashion. Accelerated response on second-set grafts is commonly observed in all groups of fish. The involvement of T cells in allograft rejection has been suggested in sea bass (Abelli et al. 1999). Recently, Shibasaki et al. (2015) reported that $CD4^+$ and $CD8\alpha^+$ T cells play crucial roles and work together with

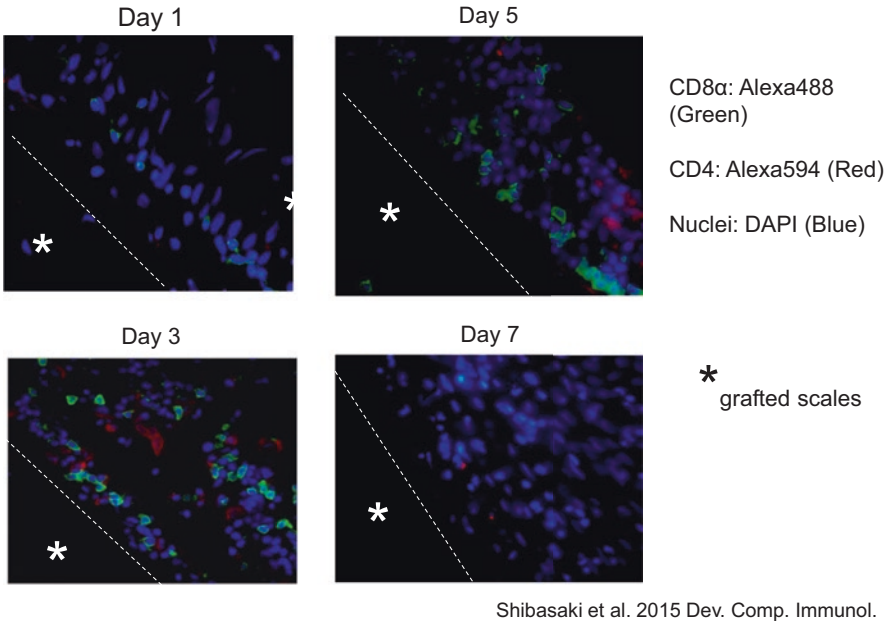

Fig. 5 Accumulation of CD4- and CD8α-positive T cells to grafted scales

other cell types including sIgM$^+$ cells and macrophages/granulocytes for the completion of allograft rejection (Fig. 5). They also showed that four IFNg isoforms differentially contributed to allograft rejection in ginbuna crucian carp (Shibasaki et al. 2016).

The graft-versus-host reaction (GVHR) is a phenomenon of cell-mediated immunity in which CTLs play the major role. The presence of GVHR in a teleost fish has been demonstrated employing a model system of clonal triploid ginbuna and tetraploid ginbuna–goldfish (*Carassius auratus*) hybrids (Nakanishi and Ototake 1999). In this model system tetraploid recipients cannot recognize the cells from triploid donors, while triploid donor cells recognize antigens originating from goldfish in tetraploid recipients (Fig. 6). When triploid cells sensitized by scale grafts from tetraploid donors were injected into tetraploid recipients, a typical GVHR was induced, leading to the death of the recipients within 1 month (Fig. 7). The induction of GVHR was also reported using clonal diploid and triploid amago salmon (*Oncorhynchus rhodurus*) (Qin et al. 2002). Most features of acute graft-versus-host disease (GVHD) in fish are quite similar to those reported for mammals, suggesting the existence of similar mechanisms. More recently, essential roles of donor-derived CD8α$^+$ T cells together with CD4$^+$ T cells in the induction of acute GVHR/D in teleosts have been reported (Shibasaki et al. 2010).

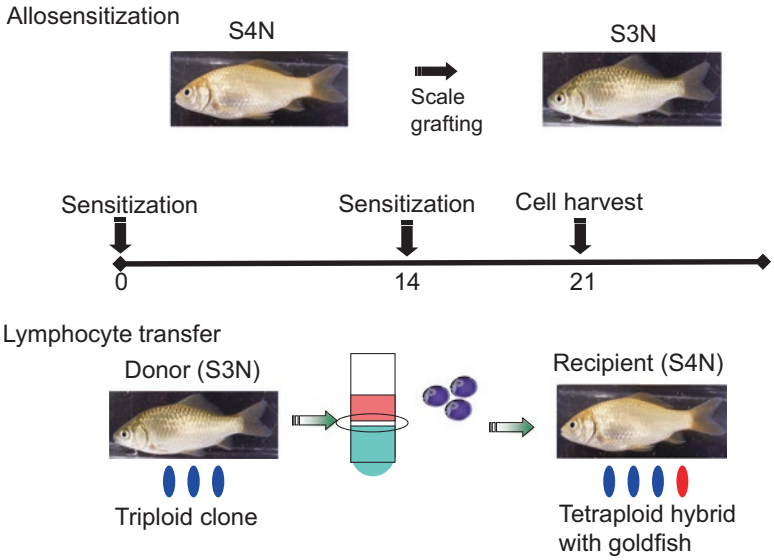

Fig. 6 Protocol for induction of GVHR

In Vitro Studies

Helper Function of CD4⁺ T Cells

Conservation of CD4+ helper T cell functions among teleost fish has been suggested in a number of studies employing MLC and the hapten/carrier effect. MLC has been reported in several fish species upon in vitro incubation of allogeneic leukocytes (Meloni et al. 2006). In channel catfish it has been reported that surface Ig-negative (sIg−) lymphocytes were the responding cells in MLC (Miller et al. 1986), and they cooperated with B cells (sIg+) and macrophages for in vitro antibody responses (Miller et al. 1985). Specific proliferation of CD4+ T cells after stimulation with alloantigen or thymus-dependent antigen such as ovalbumin (OVA) has been reported (Toda et al. 2011a, b). The requirement of CD4+ cell help in the secondary antibody response and the induction of secondary cell-mediated immunity in the presence of either CD4+ cells or leukocytes other than CD4+ cells has been reported (Somamoto et al. 2014).

Specific Cytotoxicity of CD8⁺ T Cells Against Allogeneic Cells and Tissues

In an earlier study, TCR αβ + alloantigen-specific cytotoxic cells were reported in channel catfish, although CD8α expression was not examined due to a lack of genetic information on CD8 in that species. Cells involved in alloantigen-specific cytotoxicity have been identified as CD8α⁺ T lymphocytes in ginbuna employing mAbs against CD8α (Figs. 8 and 9) (Toda et al. 2009).

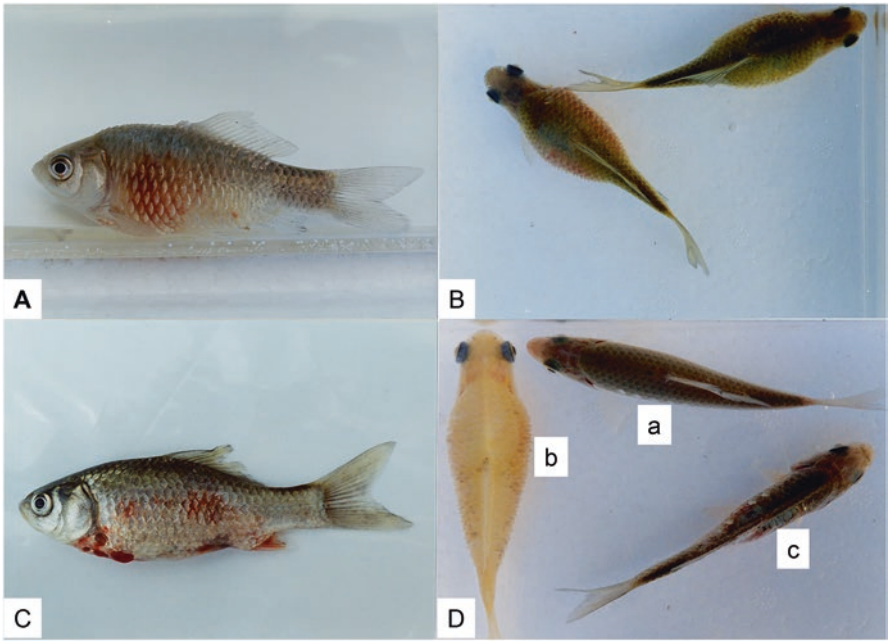

Fig. 7 Graft-versus-host disease (GVHD) in the triploid ginbuna crucian carp and tetraploid ginbuna–goldfish system. (**a**) Side view of fish suffering from GVHD to show scale protrusion and hemorrhage in ventral region. (**b**) View from top to show scale protrusion. (**c**) Side view to show severe hemorrhage and local destruction of ventral skin. (**d**) a: control fish receiving cells from nonsensitized donors, b: GVHD-suffering fish with scale protrusion, c: fish becoming very thin and feeble due to loss of appetite and constipation. Photographs were taken 2 weeks after donor cell injection for (a) and (b) and 3 weeks after for (c) and (d)

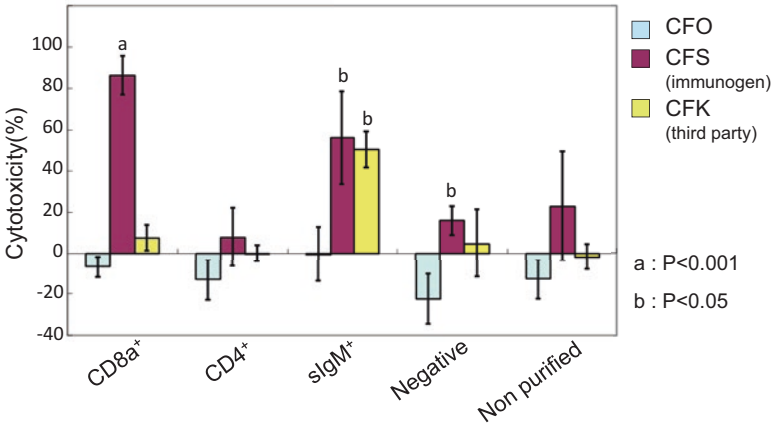

Fig. 8 Cytotoxicity of lymphocyte populations against allogeneic cells

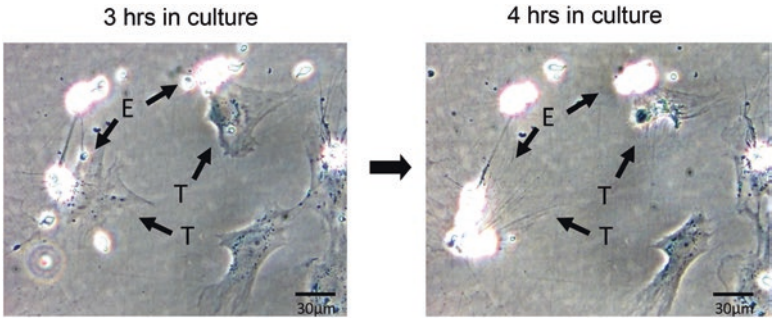

Fig. 9 Killing of allogeneic target cells by CD8+ T cells in ginbuna

Specific Cytotoxicity of CD8⁺ T Cells Against Virus-Infected Cells

CTL-mediated virus-specific cytotoxicity in fish was first described by Somamoto et al. (2000), although a few earlier papers had described the lysis of virus-infected cells by NK-like cells in fish (see review by Nakanishi et al. 2002). Convincing data showing the essential roles of CTLs against viral infection were reported by Somamoto et al. (2002). They confirmed the transferability of antiviral activities by the adoptive transfer of immune leukocytes from syngeneic donors. Recently, Utke et al. (2007) reported that PBL from low-dose viral hemorrhagic septicemia virus (VHSV)-infected rainbow trout killed MHC class I-matched VHSV-infected cells using a system of MHC class I-matched effector and target cells where the allele of classical MHC class I locus Onmy-UBA in rainbow trout clone C25 and in the cell line RTG-2 is identical. More recently, presentation of viral antigen-derived peptides by MHC Ia and its regulation by IFN has been reported in grass carp (Chen et al. 2010).

Specific Cytotoxicity of CD8⁺ T Cells Against Cell-Associated Bacteria

Edwardsiella tarda is an intracellular bacterial pathogen that causes edwardsiellosis in fish. Yamasaki et al. (2013) reported on the important role of cell-mediated immunity rather than humoral immunity against intracellular bacterial infection in ginbuna crucian carp. Bacterial clearance in kidney and spleen was also observed following elevated cytotoxic activity of CTLs and increased numbers of CD8α + cells, suggesting that CTLs might contribute to the elimination of *E. tarda*-infected cells with specific cytotoxicity. In contrast, *E. tarda*-specific antibody titers did not increase until after bacterial clearance, suggesting the primary role of cell-mediated immunity in protecting against intracellular bacterial infection, as in mammals. Furthermore, Yamasaki et al. (2014) showed the important role of CD4⁺ and CD8α⁺ cells in protection against *E. tarda* infection by the adoptive transfer of sensitized lymphocytes.

Killing Mechanisms

CTLs kill their cellular targets via either of two mechanisms whereby each requires direct contact between effector and target cells, that is, the secretory and

nonsecretory pathways mediated by perforin/granzymes and Fas/FasL, respectively. In fish, the presence of FasL has been reported at both the protein and gene levels in several fishes (Toda et al. 2011a, b). Recombinant FasL protein induced apoptosis in a Japanese flounder cell line indicating that fish possess a Fas ligand system (Kurobe et al. 2007). A major role for the perforin/granzyme pathway in the killing mechanism of alloantigen-specific CTLs has been reported in channel catfish, carp, and ginbuna (Toda et al. 2011a, b; Zhou et al. 2001). These studies strongly suggest that pathways of killing similar to those of mammals are operative in fish.

Very recently, a granzyme (Gzms) was identified and characterized in ginbuna crucian carp (Matsuura et al. 2014, 2016). The gcGzm was predominantly expressed in $CD8^+$ T cells and greatly enhanced by allosensitization and infection with an intracellular pathogen. However, its enzymatic activity was different from that of mammalian Gzms, suggesting that the gcGzm is a novel secretory serine protease involved in cell-mediated immunity in fish, with a structure similar to that of human GzmB but with a different substrate specificity.

B-Cell Function

Teleost fish exert specific antibody responses to a variety of antigens with memory (reviewed in Ye et al. 2013), although they have a limited number of immunoglobulin classes. As ectothermic vertebrates, both their primary and secondary responses are dependent on environmental temperature (Manning and Nakanishi 1996). In teleosts IgM is a major isotype in sera and has a wide variety of immune characteristics, playing many effector functions, such as complement fixation, agglutination, binding of mannose binding lectin, and mediating ADCC (reviewed in Ye et al. 2013). A specific response with memory has been demonstrated in most studies, and these findings represented a major contribution to the development of vaccines in fish.

Role of IgT in Mucosal Immunity

Cyprinid fish such as carp and zebrafish have two subclasses of IgT with differential expression patterns. Expression of IgT1 has been reported at the early developmental stage of zebrafish, although this tendency has not been observed in other teleost species (reviewed in Zhang et al. 2011). In adult zebrafish IgT1 was localized in primary lymphoid organs such as head kidney and thymus, while IgM was detected in both primary and secondary lymphoid tissues. In carp expression, the level of IgT2 was higher than that of IgT1 in intestine and gills, suggesting the important roles of IgT2 in mucosal tissues.

IgM^+ B cells are major populations among B cells in mucosal tissues as well as in main systemic lymphoid organs in fish, although a few reports exist on IgM responses in teleost gut, and the results are conflicting (Ye et al. 2013). Thus, IgM has been regarded as the only functional antibody in both systemic and mucosal areas of teleost fish. However, the IgT/IgM ratio was much higher in gut mucus when compared to serum (Zhang et al. 2010). Moreover, IgT responses to an intestinal protozoan parasite *Ceratomyxa shasta* was only detected in the gut of rainbow trout, and most trout intestinal bacteria were coated with IgT. These findings

suggest that IgT$^+$ may play a specific role in gut mucosal immunity acting like IgA in higher vertebrates. Teleost B cells share many similarities with mammalian B cells, including immunoglobulin (Ig) gene rearrangements, allelic exclusion, production of membrane Ig, and secreted Ig forms (reviewed in Edholm et al. 2011).

Affinity Maturation of Fish IgM

It has been believed that affinity maturation of fish antibodies does not occur due to a lack of germinal center. However, the existence of Ig somatic hypermutation was reported in xenopus (Wilson et al. 1992) and nurse shark (Diaz et al. 1998), suggesting the presence of affinity maturation in teleost antibodies. Finally, Kaattari et al. (2002) demonstrated the affinity maturation of antibodies in rainbow trout employing sensitive assays together with TNP-KLH as antigen. They found higher affinity antibodies relatively late in the antibody response, although the antibody affinity increase observed was not as high as those reported in mammalian Igs. The researchers also found that the increase of high-affinity antibody paralleled that of the tetrameric forms of IgM, which exist as monomers, dimers, trimers, or tetramers in serum. It is noteworthy that increased polymerization and affinity maturation contribute to the increase of binding avidity of antibodies. Furthermore, fish homolog of activation-induced cytidine deaminase (AID) involved in both Ig somatic hypermutation and class-switch recombination was reported in channel catfish (Saunders and Magor 2004). Since then a number of other fish AID homologs along with well-conserved functions have been reported (reviewed by Magor 2015).

Immunomodulation/Immunoregulation

Cytokines/Chemokines and Their Receptors

Interferons

Interferon (IFN) was discovered as a factor that inhibits nonspecific proliferation of a virus, and it was classified into types I, II, and III in mammals. The type-I IFN includes IFN-α, IFN-β, IFN-ω, IFN-ε, IFN-κ, IFN-ζ (only in mouse), IFN-τ (only in cattle), and IFN-δ (only in pig); type II type includes IFN-γ, and type III shows IFN-λ (Pestka et al. 2004; Ank et al. 2006). It was previously reported that virus-infected fish cells produce type-I IFN (Sano and Nagakura 1982) and IFN-γ (type II) (Graham and Secombes 1990). Type-I IFN genes have been identified from many fish species since the discovery of zebrafish type-I IFN gene by in silico data mining in fish genomes (Altmann et al. 2003), and type-II IFN (IFN-γ) gene was also discovered in fugu genome (Zou et al. 2004a) followed by its identification in many fish species (Robertsen 2006). However, type-III IFN was reported in mammals and amphibians (Qi et al. 2010), but not in fish. As a structural feature of the type-I IFN gene, there is no intron in the mammalian type-I IFN gene, whereas the fish type-I IFN gene is separated by four introns (Zou et al. 2007a, b).

In general, IFNs are produced by bacterial and viral infection or by stimulation by pathogen components. Type-I IFN is mainly secreted from fibroblasts and

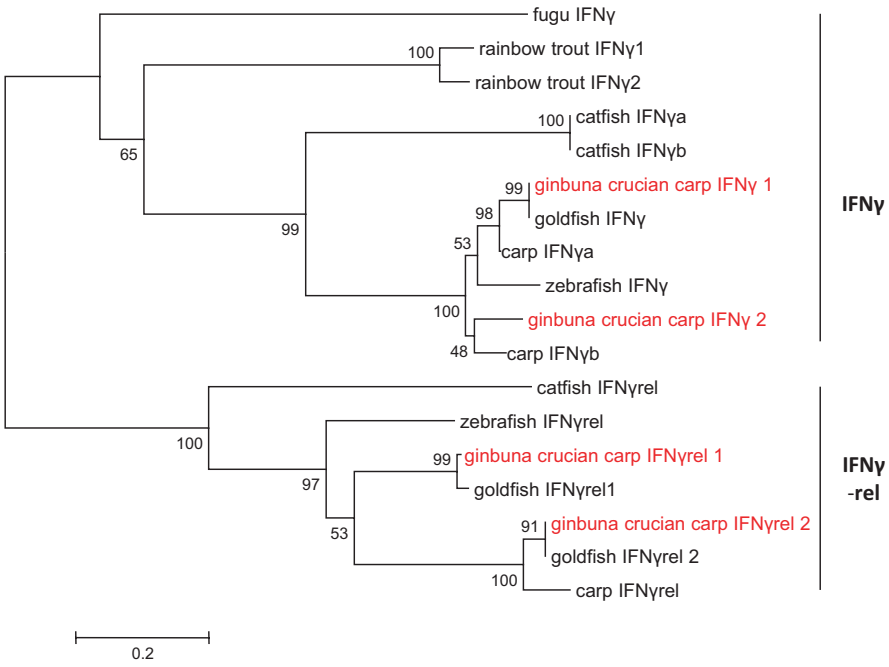

Fig. 10 Presence of four IFNγ isoforms in ginbuna

leukocytes, while IFN-γ is produced in NK cells and T cells. The secreted type-I IFN activates the JAK-STAT signaling pathway through the IFN receptor, which then leads to the induction of expression of IFN-inducible genes such as ISG15 and Mx to promote antiviral activity (Pestka et al. 2004; Robertsen 2006). On the other hand, type-II IFN, also through the JAK-STAT pathway, activates macrophages, increases NO production, and promotes antigen presentation (Robertsen 2006). As in mammals, fish type-I IFN also shows antiviral activity by enhancing gene expression of ISG15 and Mx (Verrier et al. 2011). It has been reported that recombinant type-II IFN enhances the expression of inflammatory cytokine genes in phagocytes and induces NO production in carp (Arts et al. 2010). In fish, expression of the type-I IFN gene is immediately induced by viral nucleic acids, for example, dsDNA, ssRNA, or dsRNA, and its expression is triggered by their recognition through many PPRs, including TLRs, RLRs, NLRs, and CDSs (section "Pattern Recognition Receptors" in the section "Innate Immunity").

Unlike mammals, some teleost fish species, including zebrafish, catfish, goldfish, and ginbuna crucian carp, possess two IFNγ subtypes, IFNγ and fish-specific isoform IFNγ-related (IFNγrel) proteins. Both subtypes are structurally different in the C-terminus region, and IFNγrel lacks a putative nuclear localization signal (NLS). Furthermore, the presence of two IFNγrels (named IFNγrel 1 and IFNγrel 2) have been reported in ginbuna (Fig. 10) (Shibasaki et al. 2014). IFNγrel 1 contains not a NLS but a NLS-like sequence that induces the translocation of a GFP-tagged

NLS-like sequence from the cytoplasm to the nucleus. IFNγrel 2 lacks this sequence, although both IFNγrel 1 and IFNγrel 2 showed high antiviral activities. Interestingly, both recombinant IFNγrels exhibit biological activity as monomers like mammalian type-I and III IFNs, while the functional conformation of IFNγ is a homodimer (Shibasaki et al. 2014). Shibasaki et al. (2016) have reported differential roles and their interaction of four IFNγ isoforms in allograft rejection in ginbuna.

Interleukins

In mammals, the IL-1 family includes 11 members, that is, IL-1α (IL-1F1), IL-1β (IL-1F2), IL-1 receptor antagonist (IL-1ra/IL1F3), IL-18 (IL-1F4), IL-1F5–10, and IL-33 (IL-1F11). Of these, only homologs of IL-1β and IL-18 have been discovered in teleosts (Secombes et al. 2011). The presence of IL-1β has been reported in many fish species, and more than one IL-1β gene is present due to allelic variation. Although fish IL-1β has many similarities to its mammalian counterparts, there are differences in exon/intron structure in some species, and no clear ICE cut site was found. IL-18 has been reported in fugu and rainbow trout (Huising et al. 2004; Zou et al. 2004b) and shows high level of conservation in terms of its gene organization.

The IL-2 subfamily includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, all of which contain a common gamma chain (γc) involved in signaling of T-cell proliferation. All of IL-2 subfamily genes except IL-9 have been identified in fish (Secombes et al. 2011). IL-2 was first reported in fugu together with IL-21 (Bird et al. 2005). Enhanced expression of IL-2 has been reported in leukocytes stimulated in vitro by B7 family protein in the presence of PHA in fugu (Sugamata et al. 2009). IL-2 is composed of three chains, α, β, and γ. However, α chain (CD25) has not been identified in fish so far. To date, two IL-4 homologs have been identified in fish within the IL-4 locus in mammals. The first gene was described in tetradon (Li et al. 2007) and the second in zebrafish (registered in GenBank by Bird and Secombes 2006). Both genes are considered to be ancestral genes of IL-4 and IL-13 and now referred to as IL-4/13A and IL-4/13B (Ohtani et al. 2008). The IL-7 gene was discovered in fugu by synteny analysis, and constitutive expression in lymphoid tissues and enhanced expression with mitogens in vitro were reported (Kono et al. 2008).

Two IL-15 genes have been discovered in fish to date and have been termed IL-15 and IL-15 L, in which two alternative splice variants exist (IL-15La and IL-15Lb, Gunimaladevi et al. 2007). Expression of the IL-15 gene was upregulated by rIFNγ in cell lines of rainbow trout and rIL-15 enhanced IFNγ expression in spleen leukocytes. The IL-21 gene has been identified as neighboring IL-2 in the fugu genome (Bird et al. 2005), followed by tetrodon (Wang et al. 2006) and rainbow trout (Wang et al. 2011). Enhanced expression of IL-21 was reported in leukocytes stimulated with PHA and in several tissues including gill and gut of fish injected with LPS or poly I:C in fugu and tetrodon, although the expression is low in tissues of naïve fish.

IL-10 is an anti-inflammatory cytokine belonging to the class II cytokine family that includes IL-19, IL-20, IL-22, IL-24, and IL-26 and the IFNs (Lutfalla et al.

2003). IL-22 and IL-26 genes have been identified in fugu (Zou et al. 2004a) and in zebrafish (Igawa et al. 2006) using a gene synteny approach. IL-22 was constitutively expressed in intestine and gills, and the expression was upregulated in tissues of fish injected with poly I:C or LPS.

The IL-17 subfamily is composed of six members, IL-17A to IL-17F in mammals. This gene family is considered to be ancient because the homologous gene has been reported in invertebrates such as Pacific oyster *Crassostrea gigas* and ascidian *Ciona intestinalis* (reviewed in Secombes et al. 2011). Several genes homologous to IL-17A and F (e.g., IL-17A/F1–3), IL-17C and IL-17D, have been reported in zebrafish, medaka, fugu, and Atlantic salmon (Kono et al. 2011; Secombes et al. 2011). In rainbow trout and carp, the recombinant IL-17A/F1, rIL-17A/F2, and rIL-17D induce the expression of β-defensin and the proinflammatory cytokines such as IL-1β, IL-6, and IL-8 (Monte et al. 2013; Du et al. 2014, 2015). Moreover, IL-17N is uniquely found in teleosts such as medaka, rainbow trout, and muiiy croacker (Kono et al. 2010; Wang et al. 2015; Yang et al. 2016), and its expression is upregulated by Poly I:C stimulation. However, the function of IL-17N remains unknown. The IL-17 receptor (IL17R) subfamily members, such as IL-17RA to IL-17E, have also been found in lamprey and other teleosts (Han et al. 2015; Ding et al. 2016). IL-17D binds to rIL-17RA and to the surface of IL-17RA$^+$ B-like cells and monocytes in lamprey (Han et al. 2015).

In mammals, IL-12 is present as heterodimers like IL-23, IL-27, and IL-35. IL-12 is composed of p35 and p40, IL-23 of p19 and p40, IL-27 of p28 and EB13, and IL-35 of p35 and EB13. P35 and p40 were first discovered in fugu (Yoshiura et al. 2003). The p35 gene is constitutively expressed in several tissues including brain and is also found in mammals, and the expression is upregulated in lymphoid tissues after in vivo stimulation with poly I:C. In both p35 and p40 genes the presence of multiple isoforms has been reported (Huising et al. 2006; Nascimento et al. 2007). At least three p40 isoforms (p40a, p40b, p40c) are present in carp, zebrafish, and pufferfish, and the expression pattern in tissues is different between isoforms.

Inflammatory Cytokines

Inflammatory cytokines include IL-1, IL-6, IL-12, IL-18, TNF, IFNγ, and granulocyte-macrophage colony stimulating factor (MCSF). Since IL-1, IL-12, and IFNγ are described in other sections, members belonging to TNF and IL-6 families will be outlined here.

TNF superfamily (TNFSF) and the TNF receptor superfamily (TNFRSF) are involved in inflammation, apoptosis, lymphocyte homeostasis, and tissue development (Bodmer et al. 2002). In teleosts, orthologs of the human genes encoding TNFSF9, TNFSF11, TNFSF12, TNFSF13, TNFSF13B, TNFSF14, TNFSF15, EDA, FASLG, and CD40LG as well as TNFα have been identified (Wiens and Glenney 2011). Although LTα and LTβ are not present in fish, a novel TNF gene (TNF-N) was described next to TNF in both fugu and zebrafish (Savan et al. 2005a, b). Fish species vary in the number of TNF genes presumably due to fish-specific gene/genome duplication events. Actually, multiple copies of TNFα genes have been reported in carp, goldfish, rainbow trout, and Atlantic salmon (Fig. 11)

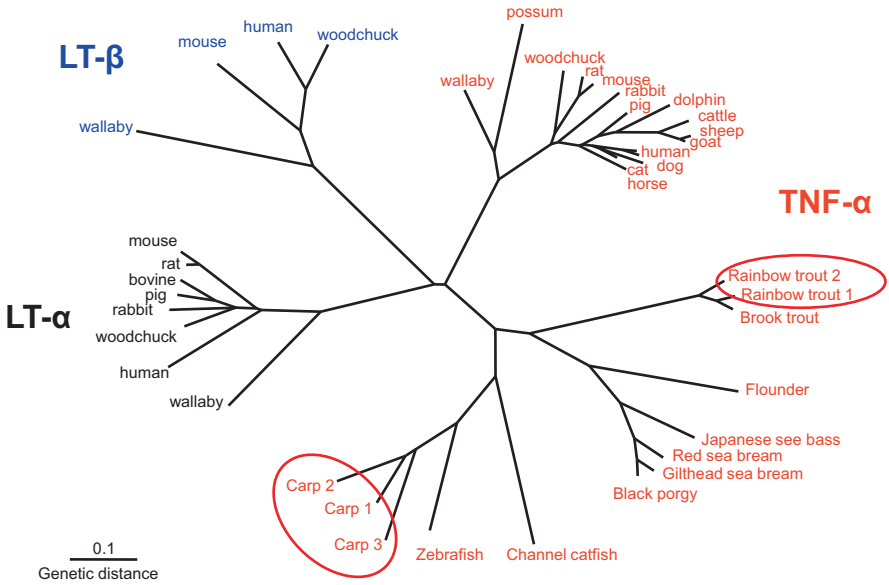

Fig. 11 Presence of multiple isoforms in teleost TNFα

(reviewed by Zou and Secombes 2016). Genes putatively homologous to human TNSF are located on five chromosomes in zebrafish (Wiens and Glenney 2011), while human TNFSF ligand members are found clustered on four MHC-paralogous chromosomes. As mentioned in the previous section, fish MHC does not form a complex, and MHC class I and II genes are located on different chromosomes in teleosts. Thus, the relationship between MHC and TNF in terms of the localization on chromosomes seems complicated. In zebrafish, a gene encoding TNFR1 with 5 CRD domains and an intracellular death domain have been identified, and the recombinant zTNF1 specifically binds to zTNFR1 (Eimon et al. 2006). Extensive in vivo functional analyses of TNF have been made in zebrafish. The injection of an expression construct for the precursor form of zebrafish TNF induced recruitment of neutrophils (Roca et al. 2008). Important roles of TNF against mycobacterial infection and a number of roles in homeostasis in addition to inflammation have also been reported (reviewed by Wiens and Glenney 2011). TNF-related apoptosis inducing ligand (TRAIL) orthologs whose numbers are much greater in fish than in mammals have been reported in a number of teleost species (Wiens and Glenney 2011). Recently putative homologs of human GITRL and GITR have been reported in zebrafish (Poulton et al. 2010), although the possibility of GITRL as CD70 or TNFSF9 has been proposed.

The IL-6 family of cytokines includes IL-6, IL-11, and IL-31, along with CNTF, LIF, OSM, CT-1, and CT-2 in mammals. Of these, IL-6 and IL-11 are present in fish (Secombes et al. 2011). IL-6 was first identified in fugu, followed by other fish species. The expression level of IL-6 in naïve tissues varied among fish species, while

mRNA expression was upregulated in in vitro and in vivo studies after stimulation with mitogens or infection with bacteria (reviewed in Secombes et al. 2011).

The IL-11 gene has been discovered in rainbow trout, carp, and flounder and two genes, IL-11a and IL-11b, exist in fish (Huising et al. 2005). Enhanced expression of IL-11 has been reported in vitro and in vivo stimulated with mitogens and bacterial and viral infections.

Chemokines

Chemokines are defined by the presence of four conserved cysteine residues and are divided into four subfamilies: CXC (α), CC (β), C, and CX_3C classes (Bacon et al. 2002). Since chemokines or their receptors have not been identified in invertebrates so far, the chemokine system is considered to have appeared at the emergence of vertebrates (DeVries et al. 2006).

In mammals, CXC chemokines contain an ELR (Glu-Leu-Arg) motif at the N-terminus of their sequence, which is involved in the receptor binding and activation of neutrophils. In fish, however, the ELR motif is replaced by a DLR motif (Asp-Leu-Arg), and this DLR motif is not essential for the attraction of neutrophils in fish (Cai et al. 2009). Phylogenetic analyses revealed that teleost CXC chemokines are divided into six clades: CXCa, CXCb, CXCc, CXCd, CXCL12, and CXCL14, although chemokines from each clade have not been identified in every species (reviewed in Alejo and Tafalla 2011; Bird and Tafalla 2015).

As for CC chemokines, the number of identified genes greatly varies between species, with 81 having been reported in zebrafish and 18 and 26 in rainbow trout and channel catfish, respectively. This may be partly attributed to species-specific intrachromosomal duplications (reviewed in Alejo and Tafalla 2011). C chemokines have only been identified in zebrafish, and CX_3C chemokines have not been reported yet, whereas a new family of chemokines, called CX, has been described in zebrafish (Nomiyama et al. 2008). IL-8 is one of the fish chemokines for which more functional assays have been conducted. Roles of CXCL12 and its receptor CXCR4 in development have been reported in zebrafish (reviewed in Alejo and Tafalla 2011).

Interaction Between Immune and Endocrine Systems

It has become apparent that immune–endocrine interactions also occur in nonmammalian vertebrates, particularly in fish. There have been several reviews on interactions between endocrine and immune systems in fish (Balm 1997; Weyts et al. 1999; Harris and Bird 2000a; Yada and Nakanishi 2002; Engelsma et al. 2002; Verburg-van Kemenede et al. 2009). In this section, we describe the interactions between the immune and endocrine systems and update current knowledge with an emphasis on the roles of mediators in interactions.

Endocrine Control of Immune Functions

Peptide hormones secreted from the pituitary are important factors modulating fish immune functions. There was a tendency to a decrease in immune responses

following hypophysectomy of several species of teleosts, suggesting the importance of hypophyseal hormones for the maintenance of the fish immune system (Rasquin 1951; Slicher 1961; Pickford et al. 1971; Ball and Hawkins 1976; Yada et al. 1999, 2002). Immunomodulation by growth hormone (GH) secreted from pituitary has been reported repeatedly in fish (Kajita et al. 1992; Sakai et al. 1995, 1996a, b, c; Calduch-Giner et al. 1997; Narnaware et al. 1997; Muñoz et al. 1998; Yada et al. 1999, 2001, 2002, 2004). The growth-promoting effect of GH is known to be mediated by other peripheral endocrine factors, such as insulin-like growth factors (IGFs), and the immune-stimulating effect of GH seems to be followed by IGF in fish (Yada 2009; Yada et al. 2012). Prolactin (PRL) is thought to be derived from an ancestral gene in common with GH, and the effects of PRL on fish immune functions seem to closely resemble that of GH (Sakai et al. 1995, 1996a, b; Yada et al. 2004; Olavarría et al. 2010). Other hypophyseal hormones, stimulatory effects of adrenocorticotrophic hormone (ACTH), and pro-opiomelanocortin (POMC)-derived peptides are also known in the fish immune system (Bayne and Levy 1991a, b; Harris and Bird 1997, 1998, 2000b; Harris et al. 1998; Takahashi et al. 1990, 2000; Sakai et al. 2001; Castillo et al. 2009).

Several neuropeptides show immunomodulatory effects on fish immune functions, directly or through the regulation of pituitary hormones. Corticotropin-releasing hormone (CRH) is thought to be the most important secretagogue for corticosteroids as the starter of the hypothalamus–pituitary–interrenal (HPI) axis in fish; hypothalamic CRH stimulates ACTH secretion, and cortisol secretion is controlled by the circulating ACTH level. Gonadotropin-releasing hormone (GnRH) is primarily known to be produced in hypothalamus and stimulates gonadotropin release from pituitary among vertebrates. In rainbow trout (*Oncorhynchus mykiss*), administration of GnRH increases proliferation, superoxide production during phagocytosis, and mRNA levels of TNFα in leukocytes (Yada 2012). Melanin-concentrating hormone (MCH) on the fish immune system has been investigated in relation to its stress and background adaptation (Harris and Bird 2000b). In vitro study using leukocytes isolated from HK of trout also revealed that MCH directly stimulated phagocytosis (Harris and Bird 1998). Prolactin-releasing peptide, a candidate stimulator of PRL secretion in fish, stimulates phagocytosis by HK leukocytes of Atlantic salmon (*Salmo salar*) (Romero et al. 2012).

Other possible endocrine factors affecting the fish immune function are gastrointestinal hormones, leptin, and thyroid hormone. In trout, somatostatin inhibited but substance P stimulated mitosis of peripheral blood leukocytes, and these effects were modulated by the administration of LPS or phytohemagglutinin (PHA) (Ndoye et al. 1991). Ghrelin increases but leptin decreases superoxide production in rainbow trout (Yada et al. 2006a, b; Mariano et al. 2012). Although interactions between thyroid function and immune system are established in mammalian species, only a few studies examined the effects of thyroid hormone on fish immune functions (Dorshkind and Horseman 2000; Verburg-van Kemenede et al. 2009; Yada and Tort 2016). Administration of thyroid hormone modulates leukocyte number in hypophysectomized sailfin molly *Poecilia latipinna* and thymus development in zebrafish *Danio rerio* (Ball and Hawkins 1976; Lam et al. 2005). Recently, Quesada-García

et al. (2016) revealed direct effects of thyroid hormone on the expression of several immune genes in trout using microarray and qPCR. Studies on extrapituitary hormones on fish immunity, such as thyroid hormone, in relation to nutrition metabolism and early development are awaited in future.

Regulation of Endocrine System by Immune Components

Cytokines are known to regulate the secretion of pituitary hormones through a modification of hypothalamic control in mammals (Wilder 1995; Johnson et al. 1997; Turnbell and Rivier 1999; Haedo et al. 2009). Because of a lack of knowledge of fish cytokines, there was little information on the role of cytokines in fish endocrine systems until the 1990s. Following discoveries of fish cytokines using molecular techniques, the regulation of the endocrine system by immune components especially in inflammatory cytokines has been examined in fish (Balm 1997; Weyts et al. 1999; Engelsma et al. 2002; Verburg-van Kemenede et al. 2009). The expression of cytokine genes in fish brain suggests the possibility that cytokines act as neuroendocrine factors to regulate hormone secretion from the pituitary gland (Metz et al. 2006; Iliev et al. 2007; Castellana et al. 2008; Wang and Secombes 2009; Wang et al. 2010; Øvergård et al. 2012).

Hormones are expressed in mammalian lymphoid organs and are thought to act as paracrine factors (Kooijman et al. 2000; Venters et al. 2001; Silva and Palmer 2011). Extrapituitary expression of GH, PRL, and POMC-derived peptides has been observed in various lymphoid tissues and leukocytes of fish (Yada and Nakanishi 2002; Engelsma et al. 2002; Mola et al. 2005; Verburg-van Kemenede et al. 2009). Ghrelin was originally discovered in rat stomach as an endogenous ligand for the GH secretagogue-receptor (GHS-R) and known to regulate GH secretion and appetite not only in mammals but also in lower vertebrates (Kojima et al. 1999; Unniappan and Peter 2005; Kaiya et al. 2012). Thus, ghrelin seems to be a likely candidate as modulator of GH gene expression in fish immune system. Administration of ghrelin increased superoxide production in rainbow trout phagocytic leukocytes, and immunoneutralization of GH by the addition of antisalmon GH serum into the medium blocked the stimulatory effect of ghrelin (Yada et al. 2006a, b). These results suggest that ghrelin stimulates fish leukocytes, at least in part, through GH secreted by leukocytes. Further studies on the peripheral expression of hormones are needed to evaluate their roles as paracrine and autocrine factors that might modulate fish endocrine and immune systems.

Immunosuppression

The anterior part of the kidney (HK or interrenal) of teleost fish is the major origin of corticosteroid hormones and catecholamines, rather than the adrenals in higher vertebrates. Interrenal cells, which produce corticosteroids and are comparable to the adrenal cortex, are located around the walls of the posterior cardinal veins in the HK. Aldosterone is the most effective mineral corticoid that exhibits actions on ion metabolism in mammals. However, the consensus is that most fish do not produce

aldosterone, and the main corticosteroid of the teleost, cortisol, seems to act not only as a glucocorticoid involving metabolism but also as a mineral corticoid (Bentley 1998; Mommsen et al. 1999; Bernier et al. 2009; Gorissen and Flik et al. 2016). The chromaffin cells scattered or in small clusters in the HK of fish are the homolog of the adrenal medulla in higher vertebrates and are the main source of circulating catecholamines. Cortisol and catecholamines are the most important transducers of the stress response and are regarded as stress hormones in fish. The regulatory system for the secretion of these stress hormones has been established as the HPI axis and the hypothalamus–sympathetic–chromaffin cell axis in fish (see aforementioned reviews).

Effects of Chemicals

Aquatic contamination by toxic substances affects fish health through such extensive and delicate surfaces as the gills and interferes with respiratory homeostasis (Heath 1987). Sublethal concentrations of pollutants suppress the fish immune system directly or indirectly by means of stress hormones (Heath 1987; Anderson 1996; Hoole 1997; Geeraerts and Belpaire 2010). Specific and nonspecific immune functions of fish are reported to be suppressed in the presence of metals, such as copper, aluminum, and cadmium. Copper seems to be one of those metals that cause serious immunosuppression in fish. Besides the lethal effect of low amounts of copper in environmental water, a sublethal concentration results in an increased susceptibility to disease in fish (Anderson 1996; Khangarot and Rathore 1999). In vitro suppression of several immune functions and expression of immune-related genes by copper indicated the direct effect of this metal on the fish immune system (Ellsaesser et al. 1986; Anderson et al. 1989; Morcillo et al. 2016). Aluminum also showed a direct inhibitory effect on phagocytosis (Elsasser et al. 1986). The effect of aluminum on the fish immune system is related to water acidification, since aluminum is generally present at higher concentrations in more acidic water (Brown and Sadler 1989). Depression of fish immune functions following administration of cadmium has been reported repeatedly (Varma and Jain 2016). Cadmium is known to interact with estrogen and inhibit the transcription of the estrogen receptor (Olsson et al. 1995; Le Guével et al. 2000; Mehinto et al. 2014). Cadmium seems to influence fish immune functions through the immunomodulatory action of estrogen at the receptor level.

Detailed descriptions of the endocrine disruption of the fish immune system with several estrogenic substances that mimic the physiological effects of estrogen or antagonize endogenous androgen have been presented in previously mentioned reviews (Anderson 1996; Sumpter et al. 1996; Sumpter 1998; von Ginneken et al. 2009; Casanova-Nakayama et al. 2011; Milla et al. 2011; Johnson et al. 2014). Estrogenic substances also showed direct actions on fish immune functions. Aromatic hydrocarbons, such as polychlorinated biphenyls (PCBs) and polynuclear aromatic hydrocarbons (PAHs), bisphenol, and pesticides, such as 1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and tributyltin (TBT), showed inhibitory effects on several immune functions in fish and resulted in increased susceptibility to disease (Rice et al. 1995; Anderson 1996; Quabius et al. 1997; Rice and Xiang

2000; Regala et al. 2001; Gushiken et al. 2002; Yin et al. 2007; Yang et al. 2015). In combination with cadmium, PCB126 reduces antibody production against a parasitic infection in the European eel *Anguilla anguilla* (Sures and Knopf 2004; Sures et al. 2006).

Pesticides are widely used for the improvement of agricultural production. However, there are growing concerns about water pollution by pesticides, although their persistence in the environment seems to be limited. Adverse effects of pesticides on fish immune responses have been reported in a number of fish species (Anderson et al. 1996). Organophosphorus pesticides (OPs) are a group of insecticides that include chlorpyrifos, malathion, diazinon, phosalone, and others. Immunotoxic effects of OPs on both humoral and cellular immune parameters of innate and adaptive immunity in fish have been reported (reviewed by Díaz-Resendiz et al. 2015). One of the most likely immunotoxicity mechanisms of OPs is the blocking of acetylcholinesterase (AChE) activity, resulting in the alteration of neuroimmune communication.

Suppression of both adaptive and innate immune responses by the excessive use of antibiotics, including oxytetracycline, florfenicol, and oxolinic acid, has been reported in carp, rainbow trout, turbot, and Atlantic cod (Barros-Becker et al. 2012). In contrast, Tafalla et al. (1999) reported that in vivo administration of oxytetracycline did not suppress innate immune responses in turbot, while macrophage function was inhibited by in vitro treatment. Furthermore, induction of inflammation in zebrafish larvae exposed to oxytetracycline has been reported (Barros-Becker et al. 2012). Thus, the results are contradictory depending on the type of assay and fish species used.

Effects of Environmental Factors

Temperature

It is well known that a change of temperature or difference of temperature affects immune function through other mechanisms, such as stress, in addition to the direct effect of lower temperature on immune functions (Fries 1986; Bly and Clem 1992; Manning and Nakanishi 1996; Schreck 1996; Le Morvan et al. 1998; Makrinos and Bowden 2016). Temperatures below the range at which optimal immune responses occur, but still within the physiological range, suppress both cellular and humoral specific immune functions (Manning and Nakanishi 1996). Earlier studies suggested that helper T cells, and not memory T cells or B cells, are sensitive to lower temperature (Bly and Clem 1992; Manning and Nakanishi 1996; Le Morvan et al. 1998). In contrast, nonspecific immunity, for example phagocytosis and nonspecific cytotoxicity, tend to be more resistant to low temperature than specific immunity (Ainsworth et al. 1991; Dexiang and Ainsworth 1991; Collazos et al. 1994; Kurata et al. 1995).

The heat shock proteins (HSPs) are key molecules for stress-related response to increased temperature in an immune system since they are known to modulate the binding of corticoid receptors to form homodimers with the promoter regions of the target genes (Pratt and Toft 1997; Richter and Buchner 2001). In fish, HSPs seem to

be under multiple regulations by the endocrine system. Heat-shock-induced expression of HSPs has been suppressed by the administration of cortisol (Deane et al. 1999; Ackerman et al. 2000; Basu et al. 2001; Sathiyaa et al. 2001). In contrast, adrenaline increased HSP70 level in hepatocytes of trout, and β-adrenoblocker eliminated the effect (Ackerman et al. 2000). In silver seabream *Sparus sarba*, GH and PRL reduced HSP70 expression in liver (Deane et al. 1999). Stolte et al. (2009) showed that HSP70 and corticoid receptor expressions were differentially regulated between immune tissues and cells of common carp *Cyprinus carpio* in response to different stimuli on immunity, such as LPS, zymosan, or infection with parasite. Although the involvement of HSPs in fish immunomodulation is complicated and still unclear, HSPs can mediate the stress response by temperature at the level of the corticoid receptor.

Salinity

The response following entry into different environmental salinities may be simply recognized as a disturbance in osmoregulation (McDonald and Milligan 1997; Wendelaar Bonga 1997). It is generally accepted that cortisol regulates fish ionic balance during adaptation from freshwater to seawater (McCormick 1995; Bentley 1998). In the rainbow trout, acute exposure to seawater results in a decrease in antibody production accompanying an increased plasma level of cortisol, while chronic or long-term exposure did not affect either immune function (Betoulle et al. 1995). In Mozambique tilapia *Oreochromis mossambicus*, increased salinity enhanced innate immune function, possibly through the modulatory actions of GH and PRL (Yada et al. 2002). Conversely, increases in the ratios of monocyte and neutrophil and in phagocytic activity were observed after acclimation to lowered salinity in Nile tilapia *O. niloticus* (Choi et al. 2013). The effects of salinity on immune functions have been reported in several fish species (Yada and Nakanishi 2002; Makrinos and Bowden 2016), although those effects vary with species as described earlier, suggesting that the difference is related to the adaptability of fishes to different environmental salinities.

Hypoxia and Acidification

Hypoxia or anoxia is usually accompanied by changes in water quality such as increased bicarbonate and ammonia. These changes provoke serious disturbance in ionic, osmotic, and acid–base regulation of fish blood as the environmental stressor (McDonald and Milligan 1997). In peripheral blood leukocytes, ratios of thrombocytes and lymphocytes decreased, but that of phagocytes was increased after acute hypoxic stress of flatfish *Limanda limanda* (Pulsford et al. 1994). Hypoxia was one of the first stressors examined using transcriptome analysis in fish and resulted in unexpected findings, such as myoglobin expression in nonmuscle tissues (Fraser et al. 2006; Prunet et al. 2008; de Souza et al. 2014). Air exposure of juvenile Japanese eel *Anguilla japonica* resulted in significant changes in the expression of numerous genes related to the immune system; however, genes related to endocrine regulation, including the HPI axis, have not been detected by gene ontology analysis following RNA-seq in stressed elvers (Yada et al. 2018).

Water acidification affects fish immune functions also through stress response in the endocrine system. Exposure of carp to acidic water resulted in an increased plasma level of cortisol and decreases in the respiratory burst and plasma Ig level (Nagae et al. 2001). Trout showed reduction in plasma lysozyme level following acid water exposure accompanied by significant elevation in the plasma level of cortisol (Yada et al. 2006a, b). Rising CO2 levels and global climate changes are predicted to result in ocean acidification in the near future; however, there is little information available on the possible influences of acidification on immune and endocrine systems in marine fishes (Pankhurst and Munday 2011; Makrinos and Bowden 2016).

Physical Stress

Physical disturbance in aquaculture, such as capture, handling, confinement, and transport, causes many physiological maladaptations, resulting in immunosuppression (Fries 1986; Barton and Iwama 1991; Wedemeyer 1997). Anesthetization suppresses mediation of the stress response and is used to mitigate physiological stress during handling and transportation. Indeed, appropriate concentration of anesthetics completely blocked a stress-induced elevation of the circulating cortisol (Barton et al. 1985; Gerwick et al. 1999; Sneddon et al. 2016).

Social Confliction

Social conflicts during the initial establishment of social rank in dominance hierarchies or territoriality also affect fish health, and aggressive behavior of the dominant fish causes stress and physical injury in defeated individuals (Schreck 1996). Impairments of both innate and adaptive immune functions in subordinate fish have been reported in a considerable number of species (reviewed by Yada and Nakanishi 2002). In most cases, increased levels of hormones such as cortisol, ACTH, MSH, and serotonin were observed in subordinate fish.

In fish culture conditions, crowding is one of the most common sources of stress. Crowding stress decreased numbers of immune cells in fish with an elevation of plasma cortisol level (Pickering and Pottinger 1987; Mazur and Iwama 1993). Both innate and adaptive immunity are suppressed in fish under crowded conditions (reviewed by Yada and Nakanishi 2002). These changes in immune responses by crowding seem to be mediated by the stress hormone cortisol.

Natural Changes Affecting the Immune Responses

Metamorphosis includes morphological and physiological changes that adapt juveniles to a new habitat. Endocrine control of fish metamorphosis has been reported in flatfishes with the importance of thyroid hormones and corticosteroids (Inui et al. 1995; Schreiber 2001). Thyroid hormone stimulated the shift of erythrocyte population from larval to adult types during the metamorphosis of the Japanese flounder *Paralichtys olivaceus* (Miwa and Inui 1991), while the effect on leukocyte population or lymphoid tissues has not been elucidated.

The parr-smolt transformation in anadromous salmonids involves morphological, behavioral and physiological changes (Hoar 1988; Barron 1986; Dickhoff et al.

1997). Transient reductions in immune responses including decreased numbers of lymphocytes, lowered plasma lysozyme activity, and lowered antibody production have been observed during the parr-smolt transformation of anadromous salmonids (McLeay 1975; Maule et al. 1987; Muona and Soivio 1992; Schreck 1996; Steine et al. 2001). Vaccination during the transformation resulted in lowered antibody titer compared with fish vaccinated earlier (Melingen et al. 1995). These changes in immunity coincided with an increased resting level of plasma cortisol and an enhanced response to stress (Barton et al. 1985; Schreck 1996). These facts imply the inhibitory regulation of the immune system by corticosteroid as the stress hormone, despite a progressive increase in plasma Ig level irrespective of the elevated cortisol level (Nagae et al. 1994).

Sexual Maturation

It is well known that fish show the suppression of immunity during sexual maturation. Immunosuppression during sexual maturation has been reported especially in salmonid species (Richards and Pickering 1978; Pickering and Christie 1980; Pickering and Pottinger 1987; Iida et al. 1989; Maule et al. 1996). A tendency toward a decreased plasma level of Ig was observed during the period of reproduction in trout, goldfish, and rock fish *Sebastiscus marmoratus* (Nakanishi 1986; Suzuki et al. 1996, 1997). Changes in the secretion of sex steroids are noteworthy endocrine events during sexual maturation, and the direct action of sex steroids on immune functions was also observed in fish (Bentley 1998; Blázquez et al. 1998; Yada and Tort 2016).

Other Natural Factors Affecting Immune Responses

In mammals, immune responsiveness against exogenous antigens, especially T-cell-mediated immunity, tends to decline with age, while immune reactivity against self-antigens increases, leading to an increase in autoimmune responses (Wick 1994). The most obvious aging effect is the involution of the thymus. In general, fish thymus shows involution with age, though in some long-lived species, the thymus does not appear to involute at all or continues to grow even after sexual maturity (see review by Tatner 1996). A marked involution of the thymus in relation to increasing age and sexual maturity has been described in an annual salmonid fish, the ayu *Plecoglossus altivelis* (Honma and Tamura 1984). Seasonal changes have been reported in the antibody response of the summer flounder (Burreson and Frizzell 1986) and in rock fish (Nakanishi 1986). A similar phenomenon has been noted in rainbow trout (Yamaguchi et al. 1980), although the effect of sexual maturation is not excluded since rainbow trout spawn in the autumn. A circadian rhythm has been found in immune activity against scale allograft of the gulf killifish *Fundulus grandis*, and allograft rejection is two to three times faster at night than in the daytime (Nevid and Meier 1993). It can be easily imagined that circadian variation in immune activity reflects rhythms of the neuroendocrine system in fish.

Immunostimulation

Immunostimulants such as β-glucans, prebiotics/probiotics, medicinal plants, and vitamins have been used in fish aquaculture to induce protection against a wide range of diseases, including bacterial, viral, and parasitic diseases. Among the many immunostimulants, β-glucans are most commonly used in aquaculture. β-glucans comprise a wide variety of structurally diverse molecules and are found in the cell walls of yeast, plants, seaweeds, mushrooms, and fungi. Administration of β-glucans has been shown to be effective not only in the increase of immune activities but also in the increase of protection against challenge by pathogens (reviewed by Dalmo and Bøgwald 2008; Petit and Wiegertjes 2016). The effects of β-glucans on innate immune parameters, including phagocytic capacity, oxidative burst, lysozyme, and complement activity, have been reported in many fish species. β-glucans have been mostly delivered orally as a practical application, although other routes of administration, such as injection and immersion, have been found to be effective. The efficacy of β-glucans varies with type, route of administration, and fish species. Although the exact mechanisms of glucan action on anti-infective immunity are not fully understood, oral administration of β-glucans can modulate microbial communities in the gut. β-glucans are thought to be internalized by phagocytosis, although the receptors involved have not yet been identified.

Both probiotics and prebiotics help keep gut bacteria healthy, but they serve different functions. Probiotics are defined by the World Health Organization as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host." Many species of bacteria, including *Lactobacillus*, *Lactococcus*, and *Enterococcus*, for example, are used as probiotics for aquaculture practices. Different probiotics can ultimately elevate phagocytic, lysozyme, complement, and respiratory burst activity as well as the expression of various cytokines in fish (Nayak 2010). Various factors like source, type, dose, and duration of supplementation of probiotics can significantly affect the immunomodulatory activity of probiotics. Prebiotics are indigestible carbohydrates that act as food for probiotics. They directly enhance innate immune responses including phagocytic activation, neutrophil activation, activation of the alternative complement system, and increased lysozyme activity (Seong et al. 2014).

A wide range of medicinal plants such as herbs, seeds, and spices in various forms have been used in aquaculture. Herbs contain many immunologically active components, such as polysaccharides, organic acids, alkaloids, glycosides, and volatile oils, that can enhance immune responses. They act as immunostimulants as well as growth promoters, and antimicrobial activities against bacteria, viruses, parasites and fungi have been reported (reviewed by Awad and Awaad 2017; Murthy and Kiran 2013). Medicinal plants can be used as alternatives to antibiotics since they contain phytochemicals, for example, tannins, alkaloids, and flavonoids having antimicrobial activity. Herbs can also act as immunostimulants, conferring the nonspecific defense mechanisms of fish and elevating the specific immune response. Medicinal plants have been widely used thanks to the ease of their application and traditional applications across human generations, although the exact mechanisms

of their functions remain unknown in most cases. In applying herbs to aquaculture, the side effects of overdose should be considered.

Research on the nutrition and immune function of fish is expanding to define the role of specific nutrients in disease resistance in fish. The potential impact of certain vitamins, essential fatty acids (FAs), and minerals on the immune response has been examined (Oliva-Teles 2012). Dietary supplements are also being evaluated for their antioxidant potential, as fish are potentially at risk of peroxidative attack because of large quantities of highly unsaturated FAs in both fish tissues and diets.

Immunomodulatory effects of ascorbic acid (vitamin C) have been reported in terms of growth, serum concentration, enhancement of nonspecific immune responses including oxidative respiratory burst, alternative complement activity, myeloperoxidase (MPO) content, and natural hemagglutination titer. The efficacy of high doses of ascorbic acid in the treatment of several diseases has also been reported, although the efficacy varies with the viral strains and fish size (Ishikawa et al. 2013).

Future Directions

Since 1990 genomic research has greatly advanced owing to the development of polymerase chain reaction technique and progress in the genome analysis of model fish resulting in the identification of considerable numbers of immune genes. This has led to great advances in understanding fish immunology in the early twenty-first century, as demonstrated by increased numbers of reports on identification and characterization of immune genes along with expression analysis in tissues. In particular, draft genome sequences of fugu and zebrafish greatly contributed to the discovery of new immune genes because the homology of fish genes is low (e.g., approx. 10% for *ifng*) and synteny analysis is essential to confirm the certainty of identifications. Techniques to produce recombinant protein also greatly help to analyze gene function. Recent new technologies for next-generation sequencing (NGS) and global expression analyses, including microarray, proteomics, and metabolomics, are accelerating the advancement of research and are helping to understand more in-depth mechanisms underlying fish immune responses. Furthermore, aquaculture is one of the fastest growing sectors, and the number of researchers involved in fish immunology is increasing internationally. Thus, research on the immune system seems promising. However, experimental tools for analyzing fish immune systems are limited, and this may hamper further research developments. Here, we will propose subjects to be developed *in terms of* immunological tools and adjuvanted vaccine.

Development of Immunological Tools

Polyclonal and monoclonal antibodies (mAbs) against the cell surface markers of T cells are now available in several fish species, as mentioned in the previous section.

However, mAbs are only available for a few species, and fish immunologists are working on many different fish species. Fish are so diverse, and a cross reaction of antibodies between species cannot be expected. To assist further studies mAbs for immune cells should be produced in fish species important for aquaculture as well as model fish such as zebrafish and medaka. The development of new techniques to produce antibodies would also be helpful. The production of antibodies against specific motifs or epitopes conserved across species should be explored.

Zebrafish have been used as a model for embryogenesis for more than 100 years. Recently these fish have emerged to the forefront of biomedical research to analyze human development, neurobiology, genetics, and toxicology. Furthermore, zebrafish are attracting attention as a model for human infectious disease, immunology, and cancer (Trede et al. 2004) as well as for fish diseases (van der Sar et al. 2004; Sullivan and Kim 2008). Zebrafish emerge as ideal models thanks to the ease of mutagenesis and production of transgenic individuals. Recent breakthroughs in gene editing with CRISPR/Cas9 have enabled rapid and easy production of gene knockouts. Moreover, live imaging techniques using transgenic embryos and even transparent adult zebrafish would aid considerably. These could then be employed as fluorescently tagged cells or proteins. The result would allow unprecedented access to cells and tissues and would lead to numerous important discoveries on in vivo functions of immune genes. These techniques after further development in zebrafish can be expanded to other fish species.

Development of Adjuvant for Fish Vaccines

The use and misuse of antibiotics have resulted in the development and spread of antibiotic resistance. Antibiotic resistance is also a food safety problem in terms of the spread of resistant bacteria and resistance genes from fish to humans. Furthermore, antibiotics are not effective against viruses. Thus, the development of vaccines is important in fish aquaculture from the point of view of food safety and effective control of fish diseases.

A considerable number of fish vaccines have been commercialized worldwide. However, most vaccines are inactivated bacteria for extracellular bacteria, and only a few are available for viruses and intracellular bacteria. Vaccines based on inactivated pathogens or recombinant antigens alone are insufficient to confer a high level of protection against diseases caused by viruses and intracellular bacteria, against which defense is largely dependent on cell-mediated immunity. Live vaccines are effective at inducing CMI and are widely used against diseases caused by viruses and intracellular bacteria in humans and animals. In fish, however, live attenuated vaccines are not recommended, for several reasons, including the rapid propagation of microorganisms in water, lack of information on the distribution and infection mechanisms of pathogens in natural water, difficulty of isolation in open water, and the potential for reversion to virulence. Thus, the development of adjuvants and immune stimulants added to inactivated or subunit vaccines is required.

Oil adjuvants with fewer side effects are currently used in fish vaccines. However, new adjuvants or delivery methods of antigens should be explored. Recently, next-generation adjuvants targeting TLRs involved in the recognition of viral DNA or RNA have been reported. A combination of synthetic CpG oligonucleotides (ODNs) (TLR9 ligand) and polyI:C (TLR3/22 ligand) has been effective in producing pro-inflammatory cytokines/chemokines and type-I IFNs in protecting against SAV in Atlantic salmon (Strandskog et al. 2011). Improvements are needed in antigen delivery methods that will allow antigens to be effectively recognized by APCs or would attract effector cells. Interestingly, Somamoto et al. (2015) demonstrated that a "per-gill infection method" was effective at inducing both local and systemic adaptive immunity against viral infection.

As mentioned earlier, adjuvants that induce CMI or Th1-directing adjuvants that combat viruses and intracellular bacteria should be explored. Recently, Matsuura et al. (2017) reported on the enhanced expression of Th1 cytokine genes including IFNγs, IFNγrels, and IL-12 both in vitro and in vivo following stimulation by heat-killed *Enterococcus faecalis* in ginbuna crucian carp. IL-12 is a heterodimeric cytokine composed of p35 and p40 and known to play a crucial role in promoting CMI through Th1 differentiation and IFN-γ production. Matsumoto et al. (2016) have shown the upregulation of IFN-γ and downregulation of IL-10 in kidney leukocytes of amberjack stimulated by recombinant IL-12. DNA vaccine against infectious hematopoietic necrosis (IHN) virus has been commercialized in Atlantic salmon. This is the first DNA vaccine in farmed animals. Cytokine adjuvants, including IFN-γ and IL-12, are in progress in many laboratories.

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Reptilia: Humoral Immunity in Reptiles

Laura M. Zimmerman

Introduction

Reptiles are a diverse taxon that includes over 9500 species (Pincheira-Donoso et al. 2013). They diverged from amphibians over 300 million years ago and were the first to be able to live a fully terrestrial life (Raven et al. 2008) (Fig. 1). Early on, reptiles branched into two distinct lineages: one that would give rise to the Squamata and Tuatara, and the other to the Archosaurs (which includes the reptilian order Crocodilia along with birds) and their sister group Testudines (Fig. 2; Rest et al. 2003; Le et al. 2017).

The four orders of reptiles vary greatly in a number of characteristics including size, shape, habitats, and life history (Pincheira-Donoso et al. 2013). Crocodilians are the closest living relatives of birds and are typically large, aggressive, and found in pathogen-rich environments. Despite the potential for injury and infection, they tend to live relatively healthy lives (Merchant et al. 2006) and can have life spans ranging from 50 to 75 years (Fig. 3; Densmore 2001). The order Testudines includes turtles and tortoises and of course is most recognizable by the shell that encloses the pelvic and shoulder girdles (Fig. 4). The Testudines have an impressive life span, with some living up to 150 years (Castanet 1994). Others such as the red-eared slider (*Trachemys scripta*) and the Pacific leatherback turtle (*Dermochelys coriacea*) are estimated to have maximum life spans of around 30 years (Spotila et al. 2000; Ernst and Lovich 2009). The majority inhabit freshwater environments, but some live in marine and terrestrial habitats as well. The order Tuatara only includes one species of Sphenodon that is restricted to New Zealand (Fig. 5). The nocturnal

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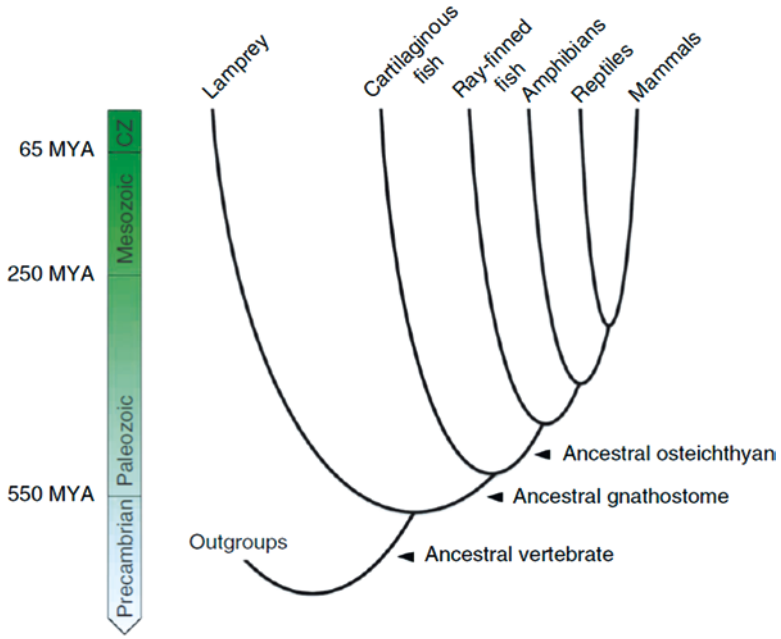

Fig. 1 Phylogenetic relationship of the vertebrates. Reptiles include reptiles and birds. Some lineages were removed to simplify the tree. (From Smith et al. 2013)

Tuatara are small, ranging in size from 0.45 to 0.65 m, and are known for a low fecundity. They also have impressive life spans of around 100 years (Mitchell et al. 2010). The order Squamata includes lizards (Fig. 6) and snakes (Fig. 7). This is a highly diverse group that can live in a variety of habitats, including aquatic, terrestrial, subterranean, and arboreal habitats. Both have relatively short life spans when compared with other reptiles: lizards typically have life spans of about 1–2 years, though some can have life spans of around 10–20 years (Castanet 1994). Snakes typically have life spans of 7–15 years, but some can live as many as 50 years (Castanet 1994). Interestingly, there are both oviparous and viviparous squamates.

All of these wide-ranging characteristics provide unique challenges for that taxa's immune system. Understanding how reptiles use their immune responses to face these challenges can provide insight into a number of fields and further elucidate the connections between immunology, evolution, ecology, and physiology while contributing to conservation of species. Reptiles are important from an evolutionary standpoint, given that they are the only ectothermic amniote. The diversity of life-history characteristics also allows for contributions to the field of ecological immunology, a field that is concerned with understanding the causes and consequences of immune variation in natural populations (Downs et al. 2014).

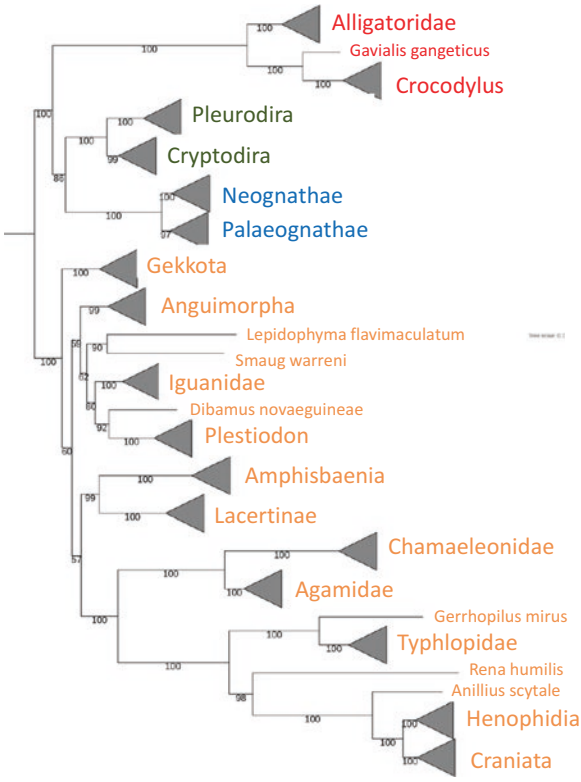

Fig. 2 Phylogenetic relationships of reptiles. Taxa are color-coded by order: green for Testudines, red for Crocodilia, orange for Squamata, and blue for Aves. Tuatara are not included in the phylogenetic tree [due to the endangered status of the Sphenedon, tissue is not available for most phylogenetic analyses; it is believed, however, that the Tuatara form a sister group with Squamata (Rest et al. 2003)]. (Modified from Le et al. 2017)

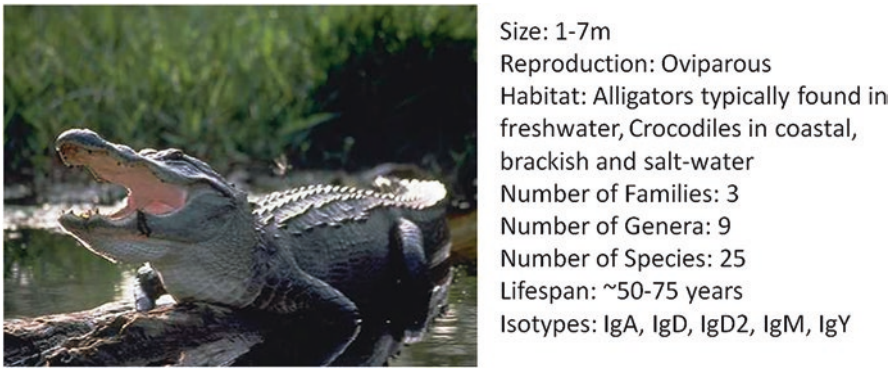

Fig. 3 Crocodilia. The order first appeared 83.5 million years ago and includes the crocodiles, alligators, caimans, and gharial. Their characteristically aggressive behavior may have led to the evolution of an immune system that can respond to a wide range of pathogens (Merchant et al. 2016). (Photo: *Alligator mississippiensis*, US Fish and Wildlife Service)

Size: 0.06-2m
Reproduction: Oviparous
Habitat: Majority freshwater, also found in marine and terrestrial
Number of Families: 14
Number of Genera: 93
Number of Species: 327
Lifespan: 30-60 yrs, Some live ~150 yrs
Isotypes: IgD, IgD2, IgM, IgY, ΔY

Fig. 4 Testudines. The order includes turtles and tortoises. Marine turtles, such as the green sea turtle pictured here, face an uncertain future due to a number of factors including climate change, ocean pollution, poaching, and disease. Knowing how these factors impact the immune system of turtles is necessary for conservation management. (Photo: *Chelonia mydas*, US Fish and Wildlife Service)

Size: 0.45-0.65m
Reproduction: Oviparous, low fecundity
Habitat: Restricted to New Zealand
Number of Families: 1
Number of Genera: 1
Number of Species: 1
Lifespan: ~100 years
Isotypes: Unknown

Fig. 5 Tuatara. Though tuatara flourished 200 million years ago, only one species remains today. Because of their small population size, immunological studies in the tuatara often include a population genetics component and are used to inform conservation management. (Photo: Bernard Spragg, www.commons.wikimedia.org)

Size: 2cm – 3m
Reproduction: Most oviparous
Habitat: Terrestrial, subterranean, and arboreal habitats
Number of Families: 35
Number of Genera: 498
Number of Species: 5,634
Lifespan: 1-2 yrs typically, as high as 20 yrs
Isotypes: IgD, IgD2, IgM, IgY, ΔY

Fig. 6 Squamata—lizards. The suborder Lacertilia, more commonly known as lizards, includes a number of groups including the geckos and iguanas. An interesting aspect of some lizards is the existence of different color polymorphisms that correlate to variations in life history and immunity. In the wall lizard (top), orange morphs had higher parasite prevalence and infection intensities and tended to have lower humoral immune function than white morphs. (Calsbeek et al. 2010) (Photo: *Podarcis muralis*. www.commons.wikimedia.org)

Fig. 7 Squamata—snakes. Many snakes have skulls with highly mobile jaws that enable them to swallow prey much larger than their heads. The live prey of snakes can inflict damage on the snake or be covered in potentially pathogenic microbes. Thus, snakes may have to upregulate their immune response after eating (Madsen et al. 2007; Luoma et al. 2016). (Photo: Western terrestrial garter snake [*Thamnophis elegans*], James Bettaso, US Fish and Wildlife Service)

Despite the many potential contributions to be made by examining the immune responses of reptiles, relatively little is known about their immune system. The humoral immune system is an excellent place to begin investigations because it includes both innate and adaptive components. Traditionally, the dominant research focus in humoral immunity is the production of specific antibodies by B cells. However, studies in reptiles have indicated that this focus on specific antibodies may not accurately explain how they use the humoral system in immune defense. It has been demonstrated that reptiles have a slow humoral response to novel antigens, if they make any response at all. In addition, they tend to have antibodies capable of binding the novel antigen even before exposure. This has led to the hypothesis that reptiles utilize a broader, non-specific antibody response, known as natural antibodies (NAbs), as part of their immune defense. Further, phagocytic B cells have been identified in reptiles, and this may also contribute to their broad humoral defenses. In this chapter, after a brief description of reptilian lymphoid tissue and immunoglobulins (Igs), I review these three aspects of humoral immunity in reptiles: specific antibody production, non-specific antibody production in the form of NAbs, and phagocytic B-cell function (Fig. 8). Further, because reptiles are ectothermic, the effects of temperature and season on these functions are also discussed along with the potential impact of climate change. Finally, because reptiles tend to be long-lived, the effect of age on reptilian humoral immunity is discussed.

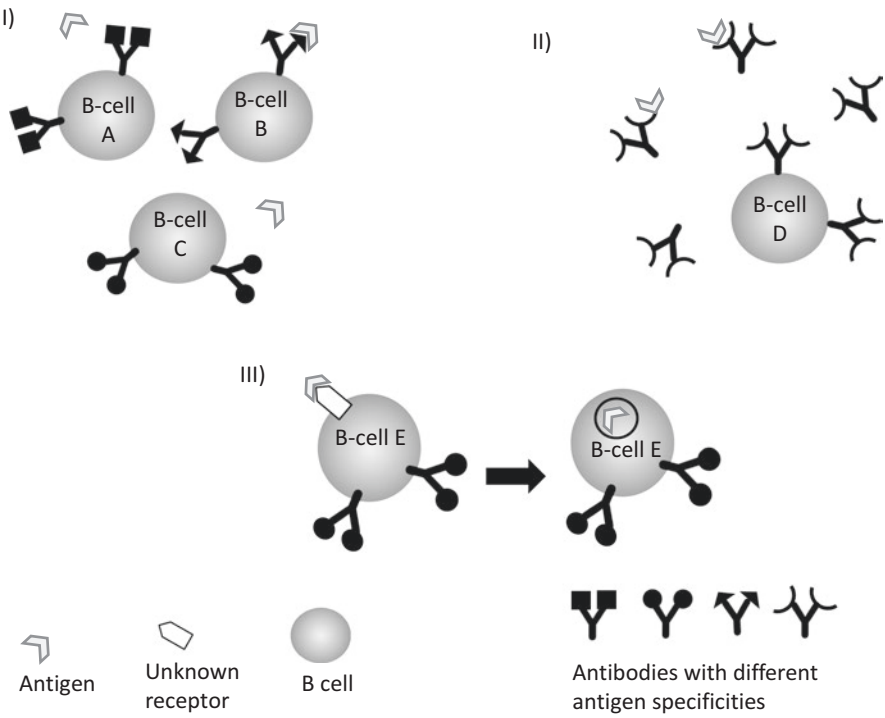

Fig. 8 Functions of B cells in reptiles. (**I**) Antigen can activate B cells by binding directly to antigen-specific surface immunoglobulins (Igs). In this case, only antigen-specific B cells (B cell B) are activated to produce Ig. (**II**) Natural antibodies can be secreted in the absence of antigen stimulation. In this situation, B cell D would spontaneously secrete low affinity antibodies with the ability to bind to the antigen. (**III**) B cells of some reptiles have demonstrated phagocytic activity. In this case, B cell E has phagocytosed the antigen and the phagosome can then fuse with the lysosome to destroy the antigen. The receptor that mediates phagocytosis in B cells is currently unknown, but in mice it has been demonstrated to be independent of the B cell receptor

Lymphoid Tissues

Primary lymphoid tissues in reptiles include the thymus and bone marrow. The thymus is the site of T cell maturation in reptiles. Similar to mammals, it typically contains two pairs of lobes (though in some reptiles it is only one), surrounded by a connective tissue capsule (Saad and Zapata 1992). The thymus is separated into a cortex and medulla, the boundaries of which change seasonally and with ability to mount an immune response (Rooney et al. 2003). The structure of the thymus varies with the breeding season, with the thymus being well-developed in the non-breeding season and involuted during the breeding phase (Hareramadas and Rai 2006; Lal et al. 2009). This is most likely mediated by sex steroids (Hareramadas and Rai 2001, 2006; Lal et al. 2009).

Bone marrow is the major site of hematopoiesis in reptiles, and consists of a stroma formed by venous sinuses and reticular cells (Zapata et al. 1981; Sano-Martins et al. 2002; Dbrowski et al. 2007). In some species of reptiles, erythropoiesis has been described in both spleen and bone marrow. This is believed to reflect the reptiles' position in evolution between amphibians, which use the spleen as the main site of hematopoiesis, and the birds and mammals, which exclusively use the bone marrow (Dbrowski et al. 2007).

Secondary lymphoid tissues include the spleen and gut-associated lymphoid tissue (GALT). Reptiles do not form germinal centers in their secondary lymphoid tissue (Zimmerman et al. 2010b). The reptile spleen contains both a white pulp and red pulp, which is similar to the mammalian spleen and it has been suggested that this type of organization first appeared in reptiles (Neely and Flajnik 2016). The red pulp is more abundant than the white pulp and is formed of vascular sinuses filled with red blood cells and lymphocytes (Pitchappan and Muthukkaruppan 1977; Kassab et al. 2009). No follicles are observed in the white pulp (Hussein et al. 1978). The spleen is divided into two discrete areas: the periarteriolar lymphoid sheaths (PALS) and the peri-ellipsoid lymphoid sheath (PELS) at the periphery of the PALS. The structure of the PALS and PELS can vary in structure between species (Neely and Flajnik 2016). In turtles, no marginal zones are found and, instead, ellipsoids are embedded in the PELS, which are capillaries encircled by a cuff of reticular tissue. Dendritic cells trap antigens in these ellipsoids and present antigen to lymphocytes that migrate into the ellipsoid from the bloodstream (Kroese and van Rooijen 1983; Bao et al. 2009).

GALT has been found in some reptiles at locations ranging from the esophagus all the way to the cloacal opening (Borysenko and Cooper 1972; Hussein et al. 1978). Reptiles do not form Peyer's patches and do not have M cells. In the diadem snake, *Spalorosphis diadema*, GALT aggregates that are similar to Peyer's patches were found; however, they were smaller than Peyer's patches (Hussein et al. 1979a). In the Chinese soft-shelled turtle, *Pelodiscus sinensis*, intragastric challenge with lipopolysaccharide (LPS) resulted in an increase in intraepithelial lymphocytes and goblet cells in the small intestine 48 h later (Xu et al. 2016).

Reptiles do not have lymph nodes, although lymph node-like aggregates were found in the snapping turtle (*Chelydra sepentina*) at anatomical locations similar to where lymph nodes are found (Borysenko and Cooper 1972). Interestingly, reptiles appear to lack the cytokine lymphotoxin A (LT-α), which is part of the tumor necrosis factor (TNF) family of molecules. They do, however, have other members of the TNF family, including TNF-α (Zimmerman et al. 2014). In mammals, LT-α is involved in the formation of secondary lymphoid tissues. In mice that lack LT-α, lymph nodes and Peyer's patches fail to develop. While the spleen is found in these mice, its organization differs from mice that produce LT-α (Wu et al. 1999; Schneider et al. 2004). Further, high-affinity antibody responses are not produced in these mice, and this response is comparable to that in ectothermic vertebrates (Lane et al. 2009). This suggests that the appearance of members of the TNF family such as LT-α was pivotal in the evolution of high-affinity antibody responses that are typical in mammals (Lane et al. 2009).

Immunoglobulins of Reptiles

All jawed vertebrates produce IgM. It typically has a low affinity, but can still be effective due to its multivalency (Zimmerman et al. 2010b). The J chain links monomeric IgM and has been sequenced in reptiles (Iwata et al. 2002). Several classes of IgM have been found in both squamates and crocodilians. It is currently unclear how these classes of IgM are expressed and the role they may play in B cell differentiation and antibody responses.

Except for birds, all jawed vertebrates also have IgD; however, its function in reptiles remains unknown. Two subclasses of IgD—IgD1 and IgD2—have been found in reptiles. In the leopard gecko (*Eublepharis maculaius*), IgD1 was found in levels similar to that of IgM in a variety of tissues, while IgD2 was found in the same tissues but at lower levels (Gambon-Deza and Sánchez-Espinel 2008). This provides excellent opportunities for further studies on the function and evolutionary history of IgD.

The isotype IgY can be found in amphibians, birds, reptiles, and an ancestral mammal, the monotremes. It is believed that IgY is the evolutionary precursor to the mammalian IgG and IgE isotypes (Brown 2002). IgY lacks the flexible hinge region that IgG contains (Zhang et al. 2017). Like IgG, IgY can be transferred from the mother to the developing embryo, but, because most reptiles are oviparous, this occurs via the yolk (Warr et al. 1995; Hassl 2005a, b). Both a full-length form and a truncated form of IgY have been identified in turtles, tortoises, and lizards. The function of the truncated form is unknown, but it is suggested that it allows for pathogen neutralization without inducting inflammation (Zhang et al. 2017). In the red-eared slider turtle, *T. scripta*, a distinct sequence for the truncated IgY was found, with expression being primarily found in the large intestine (Li et al. 2012). Following the divergence of the squamates from the archosaurs, but before the divergence of the crocodilian, turtle, and bird lineages, three subtypes of IgY emerged in the archosaurs. Two of these were then lost in the birds and turtles (Magadán-Mompó et al. 2013). In the squamate lineage, two subtypes emerged (Olivieri et al. 2016). Further work is needed to determine if there are functional differences in these subtypes of IgY, perhaps like that of IgG and IgE.

The isotype IgA has been identified in crocodilians. Similar to birds, it is found in an inverted transcriptional orientation, suggesting that this inversion took place before the divergence of birds and the crocodilians (Magadán-Mompó et al. 2013). However, it has not been found in turtles, lizards, and snakes, suggesting it was likely lost in these groups (Magadán-Mompó et al. 2013). Two isotypes of an IgA-like gene that evolved separately from IgA proper were identified in the leopard gecko. One isotype was found in lung tissue while the other was found in the intestine. Neither was found in other tissues (Gambon-Deza and Sánchez-Espinel 2008). In lizards, which lack the *IgA* gene, a second class of IgM was identified in mucosal-associated lymphoid tissue, indicating that this may serve the same function as IgM (Olivieri et al. 2016).

Chelonians and crocodilians have two light chain isotypes: λ and κ. Squamates produce only the λ isotype, except for members of the family Iguanidae which also

have λ and κ (Olivieri et al. 2016). The σ light chain isotype is found in boney fishes, cartilaginous fishes, and amphibians, and was most likely lost in all other vertebrates after the divergence from amphibians. Interestingly, more *VL* genes were found in *Alligator sinensis* than in *Anolis carolinensis*, along with higher rates of V–J combinatorial diversity (Olivieri et al. 2016). While functional studies are still needed, this extra diversity could help crocodilians recognize a wider range of pathogens. Recognizing a broad range of pathogens could be a common theme of the crocodilian immune response, as two distinct isoforms of the complement component C3 are found in crocodilians as well (Merchant et al. 2016).

Kinetics of Specific Antibody Response

Reptiles differ greatly from mammals in the kinetics of the antibody response, potentially due to their lack of germinal centers (Hsu 1998). Like mammals, reptiles have a latent period of about 1 week before antibodies can be detected. However, mammalian antibody production peaks around 2 weeks post-exposure, while reptilian antibody production typically does not peak until 6 or 8 weeks post-immunization (Fig. 9; Zimmerman et al. 2010a). It has even been demonstrated that the peak can occur up to 27 weeks later (Sandmeier et al. 2012). Reptiles also tend to have a prolonged IgM response before isotype switching to IgY (Zimmerman et al. 2010b).

The primary response in reptiles can be detected as many as 34 weeks after immunization. After a second exposure, the latent period is shortened but affinity of the antibodies and the titer are not increased (Zimmerman et al. 2010b). The isotype of the secondary response in reptiles is unknown. The lack of a memory response could be a result of a lack of germinal centers in reptiles (Hsu 1998). However, evidence for somatic hypermutation has been found (Turchin and Hsu 1996).

In the Atlantic cod, the lack of a vigorous antibody response is in part explained by the lack of the CD4 molecule on T cells (Star et al. 2011). However, this does not appear to be the case in reptiles. The CD4 molecule has been identified in the genome of a number of reptiles including three species each of alligators and turtles, as well as in two squamates (https://www.ncbi.nlm.nih.gov/gene). However, the

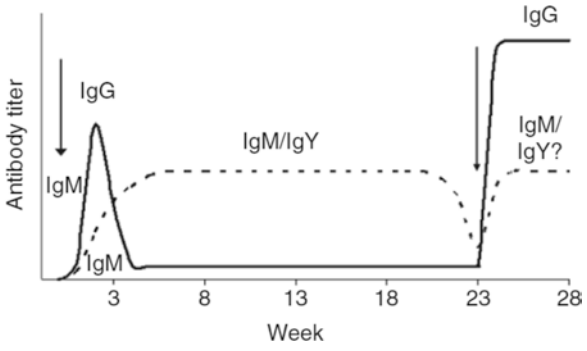

Fig. 9 Kinetics of the antibody response in reptiles and mammals. The arrows represent antigen exposure, the solid line represents the mammalian response, while the dotted line represents reptiles. (From Zimmerman et al. 2010b)

role of CD4+ T cells and other T cell subsets in the specific antibody response of reptiles is currently unexplored, mostly due to the lack of T cell reagents for use in reptiles. Hopefully, the increase in sequenced genomes of reptiles will lead to the development of T cell reagents to further explore the role of T cells.

Natural Antibodies

In some reptiles, no detectable increase in antigen-specific antibody titers has been detected, even after repeated exposure to a novel antigen. In these cases, pre-existing antigen-specific antibodies were also detected that may have masked epitopes on the antigen (Madsen et al. 2007; Zimmerman et al. 2013a). These antibodies are a type of non-specific antibody termed NAbs that function at the intersection of innate and adaptive immunity. In mammals, NAbs act as a first line of defense and also carry out housekeeping functions (Ochsenbein and Zinkernagel 2000). NAbs are defined by a series of characteristics including polyreactivity, low affinity, and germ-line encoding, and are both spontaneously produced prior to exposure and in response to antigen exposure (Baumgarth et al. 2005). They also tend to increase with age (Frasca et al. 2008). NAbs have been identified in plasma samples from a variety of reptiles, including alligators, water pythons, garter snakes, red-eared slider turtles, and desert tortoises (Zimmerman et al. 2013a). NAbs can also be detected in cloacal swabs from the slider, though the isotype of these NAbs are unknown (Stromsland and Zimmerman 2017).

The next step in NAb research in reptiles is to determine if NAb levels correlate with parasite infection, health of the reptile, survival, and fitness. Evidence in birds suggests that antibody levels against novel antigens predict parasite fitness, even if the antigen was not taken from parasites (Owen et al. 2014). In red-eared slider turtles, higher amounts of mucosal antibodies correlated with lower amounts of intestinal parasites. Interestingly, antibody levels in the plasma samples demonstrated no relationship to the amount of intestinal parasites (Stromsland and Zimmerman 2017)

Phagocytic B Cells

NAbs border the intersection of innate and adaptive immunity. Another component of the reptiles' more innate-like humoral immune response is phagocytic B cells, which were first identified in fish and amphibians before being identified in a reptile, and then more recently in mice and humans (Li et al. 2006; Zimmerman et al. 2010c; Parra et al. 2012; Zhu et al. 2016). Studies in fish and mice have demonstrated that the B cell internalizes the particle, then the phagosome fuses with lysosomes in order to degrade the particle and kill the invading microbe (Li et al. 2006; Parra et al. 2012). After degradation, the B cell can process the antigen and present it to $CD4^+$ T cells and thus activate the adaptive immune system. In addition, phagocytic B cells in mice have also been demonstrated to be able to produce reactive

oxygen species (Kovacs et al. 2015). In mice, B-2 cells had very minimal phagocytic ability while B-1 cells had the highest phagocytic function (Parra et al. 2012). Currently, the receptor that mediates phagocytic activity is unknown, but it was demonstrated in mice that this process occurs independently of the specific B cell receptor. This raises the possibility that after phagocytosis the B cell could release polyreactive NAbs (Parra et al. 2012).

The first reptile in which phagocytic B cells were identified was the red-eared slider turtle (Zimmerman et al. 2010c) (Fig. 10). Lymphocytes are the most common leukocyte in the slider, composing 38–45% of all leukocytes, though this number does include both T cells and B cells (Zimmerman et al. 2013b). The percentage of B cells in peripheral blood leukocytes that were phagocytic in the slider was reported as ranging from 14% to 25%. Previous studies in fish have reported percentages as high as 55% in the rainbow trout (*Oncorhynchus mykiss*) and 36% in Atlantic salmon (*Salmo salar*) (Li et al. 2006; Øverland et al. 2010). The percentage reported in the red-eared slider is closer to that reported for Atlantic cod, *Gadus morhua* (19%) and *Xenopus laevis* (14%) (Li et al. 2006; Øverland et al. 2010). In mice, only 1.6% of B cells in peripheral blood leukocytes were phagocytic. However, 11–14% of B-1 cells from the peritoneal cavity were phagocytic (Parra et al. 2012), supporting the idea that the B cells from peripheral blood leukocytes in red-eared sliders are B-1-like cells. Similarly, in fish, the percentage of B cells that are phagocytic can differ based on the anatomical location of the B cells. For example, in

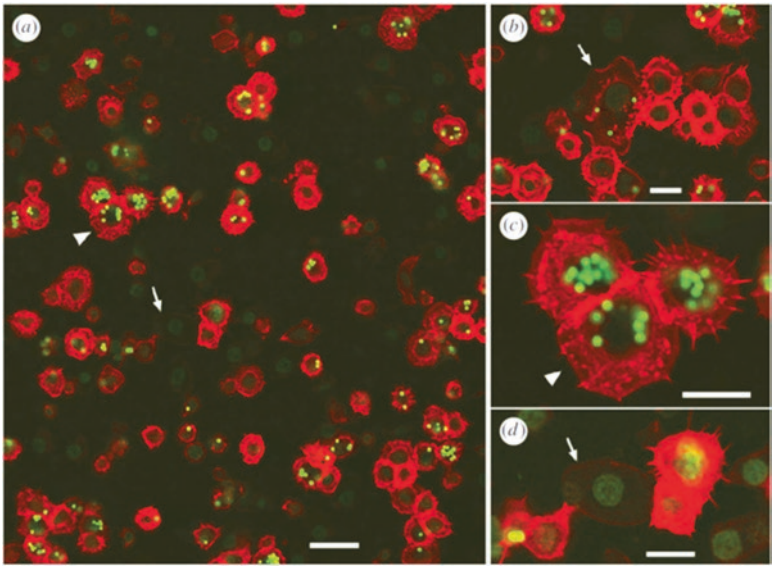

Fig. 10 Phagocytic B cells from the red-eared slider turtle, *Trachemys scripta.* Anti-light chain is red, while both beads and SYBER™ green DNA stain are green. Leukocytes were incubated with 1 μm fluorescent beads for 3 h before being analyzed using confocal microscopy. (From Zimmerman et al. 2010c)

Atlantic cod, 19% of B cells from peripheral blood leukocytes were phagocytic while 31% from the head kidney (a lymphatic organ in fish) were phagocytic (Øverland et al. 2010). Further work is needed in red-eared sliders to determine if there are also differences in phagocytic function in B cells from different anatomical locations.

The identification of the B cells that are phagocytic opens new avenues into understanding how disease may impact reptiles. Fibropapillomatosis (FP) is a disease found in sea turtles that is likely caused by the Chelonid herpesvirus 5 and results in the development of debilitating skin tumors. The progression of FP is believed to be influenced by a number of factors including pollution, genetics, and the immune response (Jones et al. 2016). The phagocytic capacity of lymphocytes from green turtles with and without FP were recently compared. No differences were found in phagocytic ability between infected and non-infected turtles; however, phagocytosis was increased by addition of Zymosan A (Rossi et al. 2016). FP is a skin disease, and it will be interesting to see if the phagocytosis of B cells plays a stronger role in diseases that affect other areas of the body.

Recently, phagocytic B cells have also been identified in three species of Brazilian snakes. Lymphocytes were able to phagocytose particles up to 3 μm but engulfed fewer particles than heterophils and azurophils (de Carvalho et al. 2017). Further work is needed to learn more about the phagocytic capacity of B cells in other reptiles.

Temperature and Season

Because they are ectotherms, temperature influences all of the biological processes of reptiles, including the immune system. Typically, reptiles are active over a wide range of temperatures, with a preferred temperature at which activity is greatest. The immune system mirrors this, with immune responses occurring over this range of temperature but peak responses at the preferred temperature (Zimmerman et al. 2010b; Butler et al. 2013). This inverted U-shaped response curve has been reported for a number of immune responses in reptiles, including complement activity, phagocytosis by macrophages, and acid phosphatase activity (Mondal et al. 2001; Merchant et al. 2005; Dang et al. 2015).

Temperature may influence the kinetics of the specific antibody response. In the lizard *Tiliqua rugosa*, maximum titers were identical at 30 °C and 25 °C (Wetherall and Turner 1972) but the peak titer was reached 22 days later for the lizards held at 25 °C. This delay in peak titers when held at lower temperatures has also been identified in fish (Hrubec et al. 1996; Mikkelsen et al. 2006). However, there may be a minimal temperature that must be reached. In the study cited by Wetherall and Turner, maximum titers reached by lizards at 20 °C were both lower and reached later than lizards at the two higher temperatures (Wetherall and Turner 1972).

Temperature could affect a variety of immune components involved in specific antibody responses, such as T cell function and cytokine production (Mondal et al. 2001). The effect of temperature on B cells themselves from the red-eared slider

found both antibody production and phagocytic capacity of B cells to be influenced by temperature. The preferred temperature of sliders is 28–29 °C, and impaired antibody production with or without antigen stimulation as well as the percentage of B cells that were phagocytic was impaired at temperatures below 29 °C (Zimmerman et al. 2017). However, this study did not control for the body temperature of the turtle before the blood sample was taken. A different result could be possible if the turtles were held at a constant temperature for an extended period before the assay was conducted. The immune responses of ectotherms are often depressed after prolonged exposure to cold, but can ramp up in response to warming temperatures before dropping to a lower level even if temperatures remain high. This peak in immune responses that occurs after prolonged exposure to cold temperatures can even be higher than when the animals are held at a consistently warm temperature (Fig. 11; Maniero and Carey 1997; Zimmerman et al. 2010a).

The temperature at which eggs are incubated can also influence the immune function of reptiles. However, understanding the effect of temperature on immune function in oviparous reptiles is complicated by the fact that many have temperature sex determination and so temperature is confounded with sex. In many turtles, including the red-eared slider turtle, low incubation temperatures tend to produce males while higher temperatures produce females. Red-eared sliders incubated at lower temperatures had an increased number of B cell clusters in their GALT. However, it is unclear if this represented differences in proliferation or just distribution because the number of B cells at other anatomical locations was not determined (Marrochello 2016). In the soft-shelled turtle, which has genetic sex determination, lower incubation temperatures also led to higher IgM, IgD, and CD3γ expression. This increased expression may help explain why the hatchlings from lower incubation temperatures also had significantly lower mortality when faced with bacterial challenge (Dang et al. 2015). Further studies are needed to see if this trend of higher humoral immunocompetence at lower incubations holds for other reptiles and to describe the mechanisms that cause it.

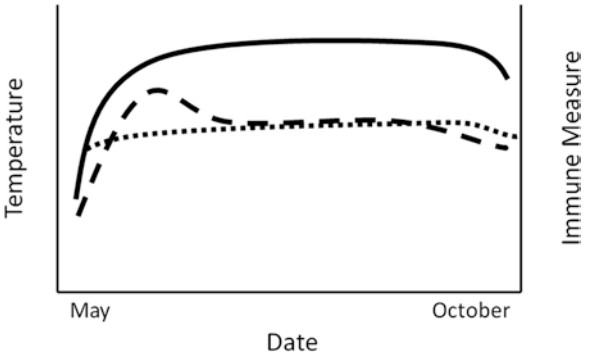

Fig. 11 Ectothermic immune responses to temperature. The solid line represents the temperature across the active season. The dashed line represents an immune response with rising temperatures after a prolonged exposure to colder temperatures. The dotted line is the same response if the animal was not first exposed to the cold

Immune responses of reptiles often change seasonally as the temperatures change; however, studies suggest that seasonal variation in immune responses of reptiles may be at least partly independent of temperature changes. For example, an early study in lizards (*Scincus scincus*) found that antibody responses were more vigorous in the fall (autumn) when compared with the spring even though the temperature was the same (Hussein et al. 1979b). Seasonal variation in lymphoid tissue may explain this finding. Typically, the thymus and white pulp of the spleen is involuted during the winter and well-developed in the spring and fall. The state of these tissues in the summer varies by species (reviewed in Zimmerman et al. 2010b). The effect of season on GALT is species specific. In some species, it is unaffected by seasonal variation, while in others it follows a similar pattern of involution and development as the thymus and spleen (Hussein et al. 1978, 1979a, b; Zimmerman et al. 2010b).

NAbs can also vary seasonally, potentially as a result of varying exposure to pathogens, though this hypothesis has not been explicitly tested in reptiles. Antibody levels increased over the active season in the red-eared slider turtle. In a different study conducted at the same time, *Salmonella* prevalence also increased in the same population (Zimmerman et al. 2010a). Studying populations of the same species at different latitudes may help determine if seasonal variation in pathogen prevalence is driving seasonal patterns. Lower latitudes are believed to have greater pathogen virulence, diversity, and abundance (Adelman et al. 2010). Seasonality of phagocytic capacity of B cells has yet to be determined, but variation in this function could also lead to variation in NAbs if polyreactive antibodies are released after phagocytosis.

Besides temperature, other factors have been put forth that can help explain seasonal variation in immune responses in vertebrates, including photoperiod, hormones, and nutrient and water availability (Sandmeier et al. 2016). It is likely that for each reptile, depending on its life history, a different combination of these is responsible for seasonal variation in humoral immune responses. Determining the influence of each factor will contribute to understanding host–parasite dynamics, the potential impacts of climate change, and conservation management.

Climate Change

Given the impact of temperature and season on the physiology of reptiles, it is reasonable to expect climate change to impact reptilian populations. Climate change will compound a situation where one-fifth of reptiles are already estimated to be threatened with extinction due to factors such as habitat loss, invasive species, and harvesting (Bohm et al. 2016; Winter et al. 2016). Further, as climate change progresses, species with temperature sex determination may experience changes in sex ratios that could limit reproductive output (Mitchell and Janzen 2010). However, a minimal number of studies have examined the effects of climate change on reptiles in general, and only a handful have examined immune responses. It is of upmost importance to expand not only the number of studies, but also to include a wide range of species that fully covers the diversity of life-history traits and evolutionary history of reptiles (Table 1).

Table 1 Number of families per order in which climate change has been investigated

Order	Families per order (*n*)	Families studied (*n*)	Families studied per order (%)
Crocodilia	3	0	0
Tuatara	1	1	100
Squamata	66	16	24
Testudinata	18	5	28

From Winter et al. (2016)
Data from 104 studies on amphibians and reptiles conducted in the previous decade were used and biases were found with respect to geography and taxonomy that makes global conclusions on the potential effects of climate change impossible. Further studies are needed to expand the research into more regions and taxa

The effects of climate change on the immune system may differ between reptiles found at higher and lower latitudes (Deutsch et al. 2008). At lower latitudes, reptiles are closer to their critical thermal maximum, and, further, ectothermic organisms in tropical environments may also be adapted to a more narrow range of temperatures. This may make them more sensitive to small changes in temperatures (Rohr et al. 2011). At higher latitudes, temperatures may not reach the critical thermal maximum, especially since climate change is expected to increase the minimal daily temperatures more than the maximum (Clarke and Zani 2012).

There is also the possibility that a novel climate could cause a stress response and thus lower the immune response. In order to investigate this possibility, a common garden study was carried out using painted turtles. Individuals from four different populations from across the geographic range of the painted turtle (sites were Washington, Illinois, New Mexico, and Iowa in the USA) were housed in a common garden in the location of one of the populations (Iowa). Turtles were sampled four times over the course of the year. Corticosterone levels did not vary between the four populations, suggesting that the turtles did not produce a stress response due to the novel climate. Further, the novel climate did not appear to depress immune function (Refsnider et al. 2015). Though the adults did not appear to suffer negatively from the novel climate, further study is needed to determine if hatchlings or juveniles would suffer negative consequences.

Even if reptiles can maintain their body temperature within their thermal preference and thus limit the negative effects on the immune system, climate change could still influence the health of reptiles by impacting the growth of the pathogen (Jackson and Tinsley 2002). In addition, higher temperatures could increase metabolism and thus lower body mass. This could reduce the amount of resources available to the immune system (Downs et al. 2014). Even though direct links to climate change have not been established, disease can be especially dangerous for populations already impacted by other anthropogenic changes such as habitat fragmentation and pollution (Jones et al. 2016). A recent case study of a decline in an isolated population of timber rattlesnakes in New Hampshire, USA, illustrates how disease and anthropogenic changes can interact (Clark et al. 2011). The population represents the last remaining population of timber rattlesnakes in the state, and genetic analysis

indicates that the population is suffering from inbreeding depression. Following abnormally high summer rainfall, an unknown skin infection caused significant mortality in the population. The warm, wet conditions may have increased the growth of the pathogen, which is believed to have been a fungus. In addition, the population may not have been able to behaviorally regulate its body temperature to produce a sufficient immune response due to high amounts of cloud cover. Inbreeding depression may have also negatively impacted the immune response. A study in Galapagos hawks found that more inbred populations had lower NAb levels (Whiteman et al. 2006). Potentially, if the same relationship between NAbs and inbreeding depression holds in snakes, the timber rattlesnakes in the isolated population may not have had high enough NAb levels to control the fungus compared with other less inbred populations of timber rattlesnakes that did not suffer the same levels of mortality (Clark et al. 2011).

Aging in Reptiles

Reptiles are an intriguing taxon in which to study aging. They typically have long life spans and, in addition, often demonstrate indeterminate growth. This means they grow throughout their lifetime, and, therefore, the larger they are, the older they likely are. This facilitates examining the impact of age on natural populations of reptiles. Further, studies in a variety of taxa, including reptiles, vascular plants, and corals, demonstrate that indeterminate growers may exhibit fundamentally different patterns of senescence than do determinate growers (Jones et al. 2014). The different selection pressures faced by indeterminate growers is illustrated in turtles. Older, and therefore larger, turtles produce larger clutches of larger eggs than younger, smaller individuals (Congdon and Gibbons 1985). Therefore, old females continue to gain reproductive fitness as they age (Paitz et al. 2007). Thus, turtles and other reptiles with indeterminate growth may benefit from an immune strategy that can maintain its function with increasing age.

This strategy is possible because not all components of the immune system, especially in the humoral immune system, respond to aging the same way. In mammals, specific antibody responses tend to decrease with age (Frasca and Blomberg 2011) while NAbs tend to increase with age (Frasca et al. 2008). In humans, the increase in NAbs is not considered sufficient to compensate for the decrease in specific antibodies, and there is an increase in morbidity and mortality with age (McGlauchlen and Vogel 2003).

Reptiles also follow a similar pattern of senescence in the humoral immune response. A number of studies have demonstrated decreased specific immune responses with increasing age in reptiles (Ujvari and Madsen 2005) and an increase in NAbs with age (e.g., Zimmerman et al. 2010a; Groffen et al. 2013; Mestre et al. 2017). However, because reptiles seem to rely less on the specific antibody response, an increase in NAbs may be able to compensate for the decrease in the specific antibody response (Ujvari and Madsen 2011). Potentially, the increasing amount of NAbs may even be considered a positive consequence of aging. This idea was

recently supported by a study in the red-eared slider turtle, where a higher total mucosal Ig level and increasing age was associated with fewer intestinal parasites (Stromsland and Zimmerman 2017). Similar studies on a diversity of reptiles and types of parasites is needed to understand the role NAbs play in protecting host health across different ages.

In mammals, specific antibody responses decrease with age for a number of reasons, including impaired T cell function, reduced or dysregulated cytokine production, functional deficits in B-2 cells (the B cell subset that produces the antibodies), and involution of the thymus (McGlauchlen and Vogel 2003; Taub and Longo 2005; Frasca et al. 2008). NAbs increase with age in part because they are produced by a subset of B cells termed B-1 cells that tend to maintain their function with increasing age. These are typically found in the peritoneal cavity, though this is not the case in all species. In reptiles, little is known about T cell functioning or cytokine production, but impairment of these functions could also explain the decrease in specific antibody responses in reptiles. While most focus on lymphoid tissue is on seasonal variation, an early study on *Calotes versicolor* suggested the thymus may also involute with age (Rao 1955). However, evidence from the red-eared slider turtle suggests that all of their B cells may be B-1-like and maintain their function with age. Neither the number of B cells that were phagocytic, the amount of antibody produced, nor the number of B cells that produced antibody decreased with age (Zimmerman et al. 2013b, 2017). Expanding this research into other reptiles will allow us to determine if this maintenance of B cell function is a feature of the red-eared slider, chelonians, or reptiles in general.

Conclusion

Reptiles are underrepresented in the humoral immunity literature, but a number of intriguing paths have been opened. It has been established that reptiles tend to have a slower, less robust humoral immune response to novel antigens than mammals. Thus, they may rely more heavily on a NAb response. While the presence of NAbs has been detected in a wide variety of reptiles, further study is needed to understand how these NAbs are used in immune defense against potential pathogens. The B cells of reptiles have been found to have the capacity to phagocytose antigens. This opens many interesting avenues of research, and examining this function in each of the four orders of reptiles should be a priority. However, because reptiles are ectothermic, temperature must be considered when designing studies into their humoral responses. Understanding the humoral immune responses of reptiles could be vital to protect species from the negative impacts of climate change and other anthropogenic effects and changes in pathogen pressures. Further, because of their long life spans and indeterminate growth, reptiles are an interesting taxon in which to study the aging of the immune system. Recent studies suggest that due to their reliance on NAbs instead of specific antibodies they may suffer fewer negative consequences of aging. In order to push forward with these questions and lines of research, more specific reagents for cellular studies will need to be made available

for a wide range of reptiles that includes all four orders. In addition, analyzing gene expression will be necessary as well. Hopefully, the increasing number of genomic sequences will facilitate this process. Overall, reptiles are an intriguing group in which to study the immune system and many avenues are available to make contributions to a number of fields such as evolutionary biology, eco-immunology, conservation, and climate change biology.

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Reptilia: Cellular Immunity in Reptiles: Perspective on Elements of Evolution

Soma Mondal Ghorai and Manisha Priyam

Introduction

In the course of evolution, squamates and rhyncocephalids became the first class of vertebrates to have faced the challenges of a full-blown terrestrial mode of life. These conditions must have had a significant impact on how reptiles divided their resources for various self-maintenance activities, including immune function (Zimmerman et al. 2010). Thus, it is intriguing to see how they adapted to the contrasting and diverse changes and managed to survive on land. Reptiles possess an extensive innate immune system to combat a variety of pathogens with a diverse group of molecules and cells like leukocytes, lysozymes, antimicrobial peptides, and the complement pathway. Being ectotherms, their immunity heavily relies on ambient temperature. They are known to exhibit both sex- and temperature-dependent variations in immune reactivity. While they share similar traits with mammalian immunity concerning functions of lymphoid organs (spleen, bone marrow, thymus, and gut-associated lymphoid tissue), their distinguishing features are lack of lymph nodes and germinal centers (Saad and Zapata 1992; Kvell et al. 2007). The reptilian defense system is composed of both innate and adaptive immune components. Despite the similarities in mammalian and reptilian adaptive immunity components, numerous reports suggest the robustness of innate immune response over adaptive response in reptiles (Zimmerman et al. 2010). The latency period for antibody levels to reach the maximal limit in reptiles is around 6–8 weeks postimmunization, unlike in mammals, where it is 1 week (Marchalonis et al. 1969;

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Work et al. 2000; Origgi et al. 2001). Moreover there is no increase in binding affinity and antibody titer during a secondary response in reptiles, underscoring once again that, though adaptive immunity is more complex in higher vertebrates, innate immunity is more hardwired in the lower cadres of phylogeny. Cellular immunity does not involve antibodies or complement but is imparted with the help of cells like macrophages, antigen-presenting cells, natural killer cells, and cytotoxic T cells. Cellular immune responses depend on direct interactions between cytotoxic T cells and cells bearing the antigen that these cells recognize. Hence, cellular immunity constitutes an interplay between the immune cells from both innate and adaptive immune systems. This chapter aims to highlight the various aspects of cellular immunity in reptiles, its evolution, and the various components involved.

Cellular Immunity in Reptiles

Most of the cellular components of innate immunity, like macrophages, heterophils/neutrophils, basophils, and eosinophils, are present uniformly across amphibians to mammals (Zimmerman et al. 2010). Cellular components of innate immunity in reptiles include basophils, eosinophils, heterophils, monocytes, and macrophages.

Heterophils are the functional equivalent of neutrophils in sauropsids, and they are reported to mediate inflammatory responses (Montali 1988). They lead to the formation of heterophilic granulomas in response to extracellular pathogens, which ultimately undergo degranulation followed by necrosis to stimulate macrophages. On the other hand, intracellular pathogens induce the formation of histiolytic granuloma wherein macrophages accumulate and become necrotic. Hence, unlike mammals, there is no formation of pus, just a caseous mass. Basophils, on the other hand, function similarly to mammals by releasing histamine on stimulation by an antigen (Montali 1988).The defensive role of eosinophils has not yet been identified in reptiles. IL-8 is a chemokine produced in response to endogenous proinflammatory cytokines and exogenous stimuli, such as lipopolysaccharides, and triggers the release of lysozomal enzymes and respiratory burst from macrophages, T lymphocytes, epithelial cells, and vascular endothelial cells (Loetscher et al. 1994). IL-8 is identified in birds (Wu et al. 2008), and recently an IL-8 homolog was identified and sequenced in the Chinese soft-shelled turtle (*T. sinensis*), indicating the active cellular role of heterophils/neutrophils in reptiles.

Though innate immunity forms the first line of defense and generates the most immediate response, it further activates the adaptive immune components to combat pathogenic infection. The mammalian hemopoietic cellular immune response consists of the lymphoid and myeloid lineages, mainly including the T-cell system, the mononuclear-phagocytic system, and the natural killer (NK) system. A unique ability for somatic hypermutation and gene arrangements helps achieve a myriad of antibodies (Abs) and their receptors in the lymphoid lineage, which forms the main component of the adaptive immune response. Reptilian cellular innate immunity is well established and forms the primary defense system in reptiles, but the mechanisms mediating the cellular acquired immune responses are quite different than in mammals. As in mammals, the adaptive response in reptiles is composed of both

cell-mediated and humoral responses, but there is far less available literature on cell-mediated immunity, especially the B- and T-cell systems and NK cells.

Ontogeny of Various Cells of Cell-Mediated Immunity

Hematopoietic cells in all vertebrates can have either extraembryonic (yolk sac) or intraembryonic (within the embryo itself) origins. In reptiles, the yolk sac is known as the earliest hematopoietic organ for the production of all types of immune cells. Though studies on the ontogeny of immune cells in reptiles are still at a primitive stage, the monocyte–macrophage system of the innate immune system is highly conserved evolutionarily and is derived from the mesoderm (Fig. 1). Studies have shown that in the lizard *Chalcides ocellatus*, B cells are derived from extraembryonic liver and differentiate into spleen cells at day 40–41 of the embryo (El Deeb et al. 1985)

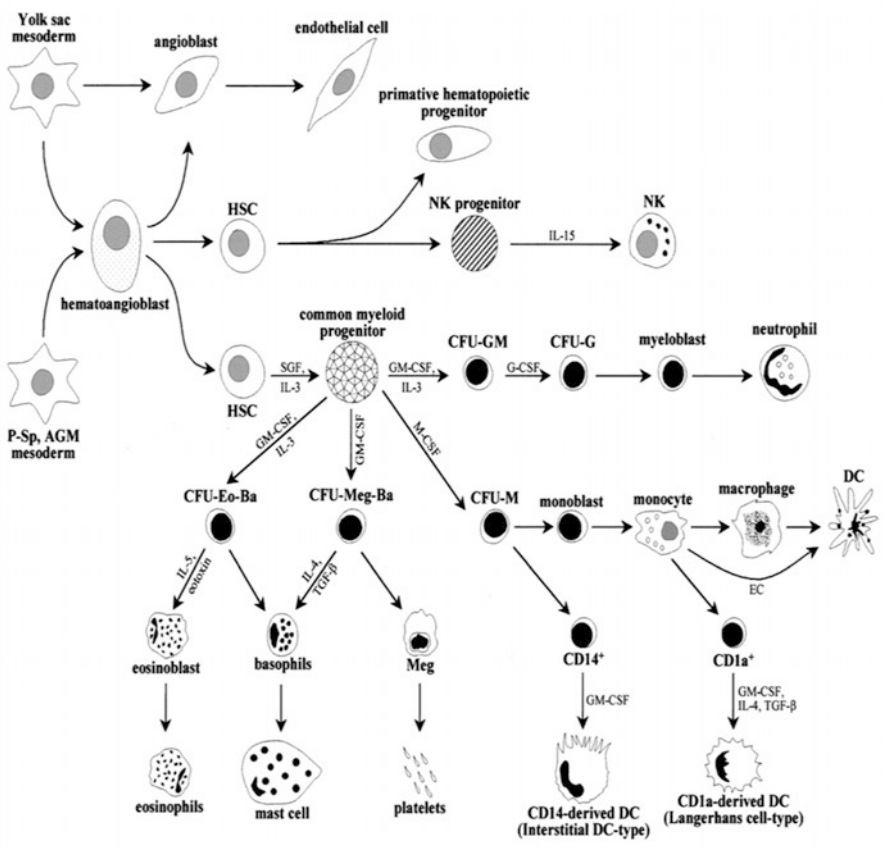

Fig. 1 Proposed pathway for ontogeny of various immune cells. Depicted is the mesodermal origin of the highly conserved monocyte–macrophage system of the innate immune system. (Source: Ghorai and Priyam)

B Cells and Ig Heavy-Chain Isotypes

B cells identified in *Trachemys scripta* have been reported to show phagocytic activity, a trait seen in lower vertebrates (fish and amphibians). This also underscores once again the hypothesis that B cells originate from a phagocytic ancestor (Zimmerman et al. 2010). Immunoglobulins (Ig), among nonmammalian vertebrates, underwent multiple evolutionary changes owing to repeated insertions and deletions in the sequences of Ig heavy-chain C-region genes, resulting in isotypes unique to reptiles (Fig. 2).

Diversity in the heavy chain of Igs is created by a series of somatic gene rearrangements of variable (V), diversity (D), and joining (J) genes, while the light chain is formed from a rearrangement of V and D gene segments. Reptiles show a high level of genetic diversity and are arranged as a single locus with multiple heavy-chain variable region genes (Turchin and Hsu 1996). Thus, reptiles differ from birds, cattle, and rabbits, which show limited combinatorial diversity and use gene conversion with a series of upstream pseudogenes to generate higher amounts of antigen-binding diversity (Litman et al. 1999; Arakawa et al. 2004). Out of five mammalian Ig classes, only IgM has been reported in reptiles (Natarajan and Muthukkaruppan 1985). Instead, IgY, the ancestor to mammalian IgG and IgE

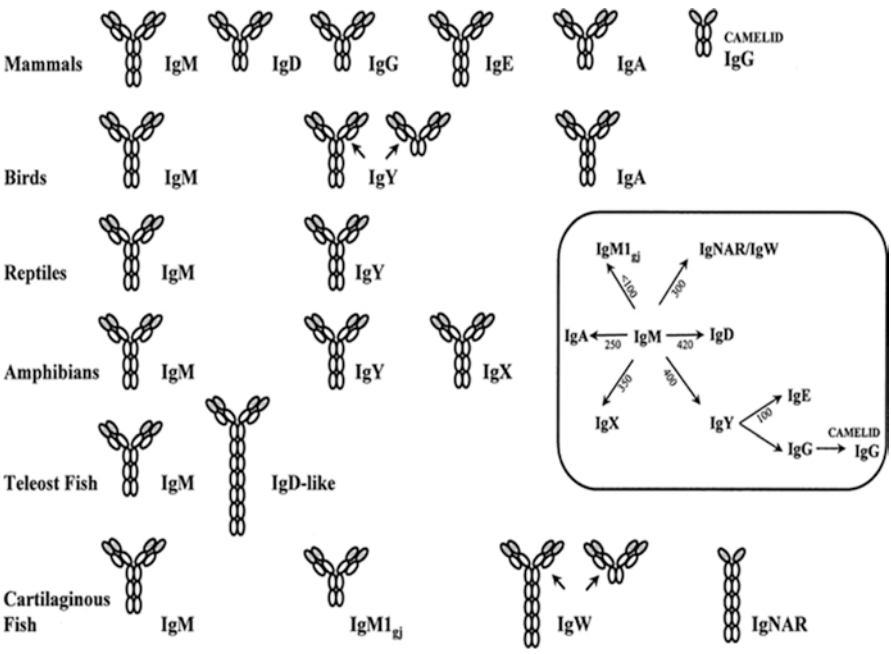

Fig. 2 Ig heavy-chain isotypes of jawed vertebrates. Among nonmammalian vertebrates Igs underwent many evolutionary changes owing to repeated insertions and deletions in the sequences of Ig heavy-chain C-region genes rather than in V domains, resulting in isotypes unique to reptiles. (Source: author)

(Brown 2002), has been detected as the major defense player in reptiles courtesy of its longer half-life and greater secretion in comparison to IgM (Warr et al. 1995). Two forms of IgY are secreted in testudines and squamates—7.5S form and a truncated 5.7S form—of which the function of the latter remains to be elucidated (Wei et al. 2009). Despite identification, the functional status of IgA and IgD remains unclear in the case of reptiles. While IgD has been identified in gekkonids, *Eublepharis macularius* and *A. carolinensis* (Deza and Espinel 2008), IgA seems to be present only in *E. macularius* (Wei et al. 2009). The demonstration of IgD in lizards makes clear that IgD, an Ab once thought to be present only in primates, shows an evolutionary continuity from fish to mammals. The absence of IgD in certain taxa (e.g., chickens, ducks, and rabbits) is attributed to the loss of the δ gene from the Ig heavy (IGH) locus. IgA appears to be present in some reptiles, such as the gecko (Deza et al. 2007), but not in the green anole. The absence of IgA in the anole most likely represents the loss of the α gene from the IGH locus of this species. The IgY(ΔFc) of the lizard appears to be a structural equivalent of a F(ab′)2 fragment, with a VH-CH1-CH2 domain structure of the H chain. The IgY(ΔFc) of the lizard and duck arises from different genetic events and is an example of convergent evolution. This would argue for a selective advantage of Ab responses of lizards, sea turtles, and ducks due to the presence of IgY(ΔFc), but the biological significance of IgY(ΔFc) expression in any of these species is yet to be established (Fig. 3) (Wei et al. 2009).

This pattern may be related to the branching in the phylogeny of North American and Eurasian lizards. Reptiles, like mammals, also exhibit isotype switching and somatic hypermutation within variable regions of Igs (Turchin and Hsu 1996). The humoral response in reptiles is also augmented by natural antibodies (NAbs). These are germline-encoded Abs belonging to the IgM isotype repertoire and are produced by B cells in the absence of pathogens (Ochesenbein and Zinkernagel 2000). They have been identified in alligator (Longenecker and Mosmann 1981), snakes (Madsen et al. 2007; Madsen and Ujvari 2011), and turtles (Zimmerman et al. 2010). Though they have a low binding affinity, they have been found to be effective against bacterial and viral infections by stimulating both innate (complement activation) and adaptive immune networks (B-cell activation) (Ochesenbein and Zinkernagel 2000). Hence, they compensate for the latency of adaptive immune response in the reptilian system.

Natural Killer Cells or Natural Cytotoxic Cells

NK cells or natural cytotoxic (NC) cells are involved in the execution of antiviral innate responses (Sun and Lanier 2009; Sun et al. 2009). In higher vertebrates, recognition of virus-infected cells by NK cells is mediated by Ig- or lectin-type NK receptors (NKRs). NKR genes range from single copy to multigene families and show considerable variation among species, highlighting the fact that they underwent coevolution along with pathogens, mainly viruses. It signifies that despite being present in a higher vertebrate (mammals), the organisational and informational complexity of NK cells in lower vertebrates is confounding. Reptiles are

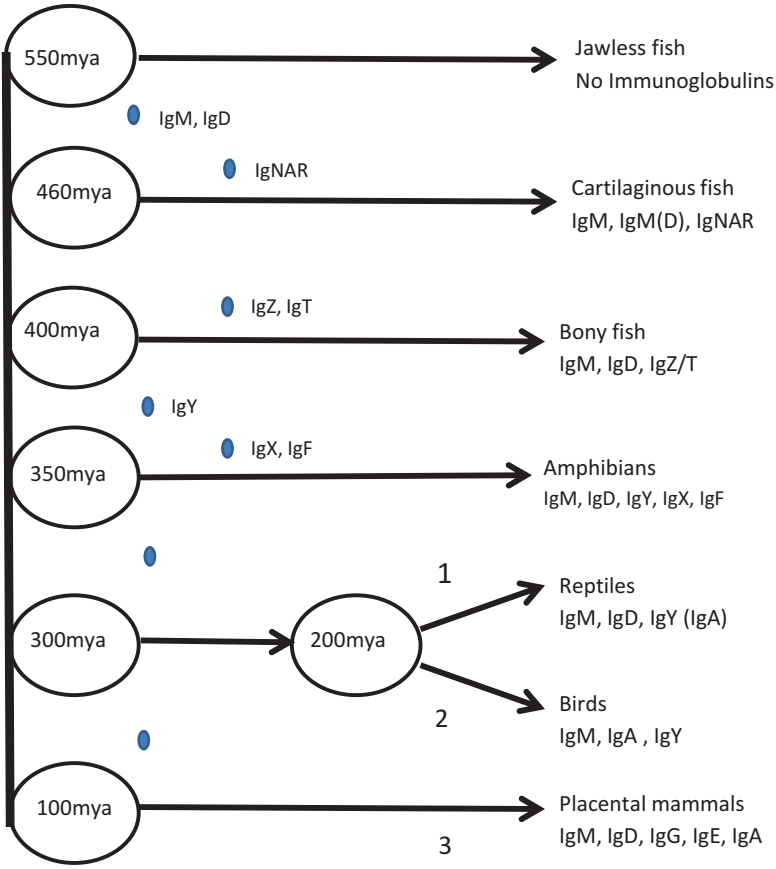

Fig. 3 Phylogeny of IgH isotypes in jawed vertebrates. Approximate times (unit, million years) of vertebrate divergence are shown in ovals. The filled circles indicate the first appearance of an Ig isotype. Genetic events: 1, IgA was lost during speciation of reptiles, being retained in some species such as the gecko; 2, IgD was lost during the emergence of birds; 3, IgG and IgE evolved from IgY, and hinge regions for IgD, IgG, and IgA were developed. (Source: author)

known to be silent carriers of pathogens, courtesy of their slow pace of metabolism. Viral infections are commonly noted in reptiles, for example, chelonians are commonly detected with herpesviruses, and lizards and snakes are affected by adenoviruses, reoviruses, and paramyxoviruses. Picornaviruses and iridoviruses are detected in the sera of snakes and tortoises after they get infected by them (Marschang 2016), but conversely immune tolerance is lacking in turtles and tortoises (McArthur et al. 2004). Transmission of viral pathogens from reptiles to humans has been rare due to thermoregulatory differences between reptilian and human hosts. But with certain zoonotic pathogens like arboviruses that are pathogenic to humans, such as West Nile fever virus, and direct loss to threatened reptilian species, it has become imperative to characterize and learn about their pathogenicity, diagnosis, and mechanism of action (Ariel 2011). NKRs are

germline-encoded, and selection pressure on receptor specificities to transduce signals may occur at the population level rather than individual species. NK-like cell like activity such as spontaneous killing of allogeneic cells by non-T-cell-receptor (TCR)-expressing cytotoxic cells have been demonstrated in nonmammalian species such as chicken (Straub et al. 2013), *Xenopus* (Horton et al. 1996), and catfish (Shen et al. 2004; Yoder 2004), but no such reports are found in reptiles. Though NK-like cells are known in reptiles, how they are involved in immune surveillance in the aforementioned viruses remains unknown. Therefore, reptiles present a very ambiguous picture of host-defense strategies (Sandmeier and Tracy 2014).

Apart from natural cytotoxic cells, other cellular activities involved can be attributed to cytokines like interferons (IFNs) released from activated NK cells, macrophages are also known to play an important role in host resistance against viral infection either directly or indirectly through binding heterodimeric receptors consisting of two chains, IFNAR1 and IFNAR2 (Pestka et al. 2004; Decker et al. 2005; Trinchieri 2010). Work on mammalian NK cells revealed that these cells might be representatives of protoadaptive immunocytes and equivalent to primitive B or T lymphocytes because they can alter their behavior based on major histocompatibility complex (MHC)-1 inhibition or stimulation (Cooper and Yokoyama 2010). Therefore, NK cells influence adaptive immune responses by promoting the priming of $CD4^+$ T helper type 1 cells by secreting interferon gamma (IFN-γ) (Krebs et al. 2009). IFN-γ, the only type-II IFN, consists of two receptor complexes, IFN-γ R1 and IFN-γ R2 (Bach et al. 2007), and is a key cytokine in the regulation of immune responses (Savan et al. 2009). IFN-γ has been widely identified in different classes of vertebrates, including teleost fish, amphibians, avians, and mammals (Zucker et al. 1992; Digby and Lowenthal 1995; Zou and Secombes 2011; Qi et al. 2010). A recent report showed IFN-γ and IFN-γ receptor (IFN- γR) genes in a nonavian reptile, the North American green anole lizard (*Anolis carolinensis*) (Chen et al. 2013). Like their counterparts in other jawed vertebrates, lizard IFN-γ, IFN-γR1, and IFN-γR2 show conserved features in genomic organizations, gene loci, and protein sequences, which thus suggests their function in the cellular immunity of reptiles. NK cells are known to eliminate activated T cells if these do not express sufficient amounts of classical or nonclassical MHC class I molecules. Thus, NK cells do not lyse self-cells exhibiting inhibitory MHC-1 molecules but lyse autologous target cells like viral-infected or tumor cells with MHC-1 surface receptors and self-cells exhibiting mismatch MHC-1 (Kumar and McNerney 2005; Höglund and Brodin 2010). In vertebrates the mechanism of action of NK cells is now known to be governed by MHC-I cell surface receptors, but NK cell–MHC-I involvement in T-cell function remains abstruse in reptiles.

Reptilian MHC Genes: A Paradigm Shift from Eutherian's MHC

The classical class I and II MHCs are a highly variable multigene family of genes that play a pivotal role in host–pathogen interaction and are ubiquitous in all jawed vertebrates (Hedrick and Miller 1994). They encode cell-surface glycoproteins of

either bacterial or viral origin and present them to T cells (Klein 1987). In eutherian vertebrates, the MHC region is large and dense and is located within a closely linked region of the genome (Kelley et al. 2005). The gene order comprises a class III MHC region containing cytokine and complement factor genes juxtaposed between class I and II MHC regions. Peptides for antigen processing like TAP1, TAP2, PSMB8, and PSMB9, which are loaded onto class I molecules, are found in the MHC class II region. Conversely, there are major differences in MHC structures and complexity between nonmammalian and mammalian vertebrates (Kelley et al. 2005). In most nonmammalian vertebrates, class I (containing antigen processing genes) and class II genes are located adjacent to one another with no intervening class III region (Kaufman et al. 1999; Ohta et al. 2006).

To understand the evolution of MHC and their related genes, studies on reptiles are essential as they occupy a key phylogenetic position as a sister group to both birds and mammals. Reptiles represent very diverse groups that show vast differences in morphological, reproductive, and developmental characteristics among their clades. Four reptilian clades, Squamata (lizards and snakes), Rhynchocephalia (tuatara), Crocodylia (crocodilians; birds form a monophyletic group with this clade, Archosauria), and Chelonia (turtles), diverged early in amniote evolution, around 2,502,280 million years ago (Hugall et al. 2007), and thus analysis of MHC structure in nonavian reptiles will contribute to filling an important gap in reconstructing the evolutionary history of the amniote MHC, though it has been poorly represented to date. A recent study (Green et al. 2014) of MHC organization in the saltwater crocodile (*Crocodylus porosus*) revealed a structure that is intermediate between eutherian mammals and birds, with larger genes and linkage between class I genes and the framework gene TRIM39 as in mammals but also a linkage between class I and TAP genes as in birds (Jaratlerdsiri et al. 2014a). Conversely, cDNA sequences and southern blot-restriction fragment length polymorphism studies on squamate MHC genes indicated a higher number of hybridizing bands, for example, 4 expressed loci in the Ameiva lizard (Grossberger and Parham 1992), 4–11 hybridizing bands for geckos (Radtkey et al. 1996), and an average of 11 bands for sand lizards (Olsson et al. 2003). In contrast, in Rhynchocephalia (tuatara), an evolutionarily divergent reptile with a genome-level characterization of MHC organization, a large MHC region with a high repeat content was revealed. A total of 7 class I sequences and 11 class II b sequences were observed, but some appeared to represent pseudogenes. Chromosome 13q appears to contain the core MHC, as clones containing classical class I, class II beta, and class II alpha chain genes map to here, but additional class I genes were located on chromosome 4p. MHC loci are highly polymorphic and contain fewer genes than lizards, indicating ancient radiation among reptiles (Miller et al. 2005). However, the evolutionary relationships among sequences from different reptilian orders cannot be resolved, reflecting the antiquity of the major reptile lineages.

T Lymphocytes in Reptiles

Remnants of an adaptive immune system can be seen even in jawless vertebrates (lampreys and hagfish) because they possess VLRs (a primitive form of IGH chain in the case of B cells and the TCR β-chain in the case of T cells (Boehm 2011). Therefore, it is evident that reptiles should possess both humoral and cell-mediated immunity. In jawed vertebrates, there are distinct anatomical sites for B- and T-cell development, the former in the bone marrow or fetal liver and the latter in the thymus. The somatic hypermutations and negative selection of TCRs occurs in the cortex and medulla of the thymus, respectively. In reptiles, the thymus is usually lobulated and is located in the cervical region near the carotid arteries and may have parathyroid glands adjacent to the ultimobranchial bodies, though there is little evidence of thymus regeneration. Evidence exists for functional T cells in all groups of reptiles, namely, the squamates, sphenodons, crocodilians, and testudines (Burnham et al. 2005). Categories of T cells (cytotoxic T cells and regulatory T helper cells) have also been reported in the thymus of the lizard *Calotes versicolor* (Manickasundari et al. 1984). El Deeb et al. (1985) indicated the presence of T cells in 40- to 41-day-old embryos using T-cell Ab. El Masri et al. (1995) attempted to identify T lymphocytes in reptiles using peanut agglutinin, though this protein shows nonspecific cross reactivity to various other cells including NK cells and nonlymphoid cells such as myoblasts, epidermal cells, and keratinocytes. Later, several in vitro studies on mitogens showed immune responses of reptilian T lymphocytes similar to that in mammals (Work et al. 2000; Ulsh et al. 2000; Keller et al. 2005a, b). Reptile T cells have been demonstrated to show a MHC-dependent response similar to that of mammals (Farag and El Ridi 1990). T-lymphocyte function is usually measured in terms of delayed-type hypersensitivity (DTH) response, and therefore, inoculation by phytohemaglutinin (PHA)/ConA and others can help realize T-cell activity. A seasonal pattern in T-cell function was observed, and lymphocyte proliferation is also affected by environmental pollutants (Keller et al. 2005a, b, 2006). T-cell response in reptiles to mixed lymphocyte reaction or T-cell mitogens like ConA or PHA was seen to be heightened in spring and autumn and diminished during summer and winter (El Ridi et al. 1987; Muñoz and De la Fuente 2004). Thymic involution was reported in the presence of steroidal hormones, implying that T-cell suppression occurs during stress and indicating a tradeoff between immunity and reproductive vigor (Hareramadas and Rai 2005, 2006; Sacchi et al. 2014). Despite their key phylogenetic position, reptiles are the only class of vertebrates in which T lymphocytes have not been characterized at the molecular level. Reptiles that are listed as endangered often suffer from debilitating diseases like neoplastic disease (fibropapillomatosis), the main cause being cellular immunosuppression (Work et al. 2000; Jones et al. 2016). T lymphocytes interact with antigen receptors through the TCR/CD3 complex, which is found in all modern jawed vertebrates (Gouaillard et al. 2001). CD3+ T lymphocytes are ubiquitous throughout the vertebrate lineage. CD3+ T lymphocytes are detected in numerous nonmammalian species like *Anas platyrhynchos* (Bertram et al. 1996), the porgie *Pagrus auratus* (Cook et al. 2001), and the amphibian *Xenopus laevis* (Göbel et al. 2000) using Abs against CD3+

receptors. A single report on a lizard (*C. ocellatus*) revealed that surface antigen expression changes during the emigration of T lymphocytes from the thymus to the spleen when treated with antisera (Jaffredo et al. 2005). Surprisingly, CD3+ T lymphocytes were exhibited in marine turtle thymus (Muñoz et al. 2009), and their distribution pattern was similar to those in mammals and birds (Bertram et al. 1996), although no data are available concerning the TCR structure in reptiles.

Understanding Reptilian Immunity by Genomics and Transcriptomics

Nonavian reptiles are divided into four extant orders: Crocodylia (crocodiles and alligators, approximately 25 species), Sphenodontia (tuatara, two species), Squamata (lizards and snakes, approximately 7900 species), and Testudines (turtles, approximately 300 species). The clade's most recent common ancestor is thought to have lived around 275 million years ago, and birds (class Aves) are nested within reptiles (class Reptilia). Characteristic features, like diverse sex determination, exothermicity, and extreme physiology, place the nonavian reptiles at the primeval origins of amniote (mammals, birds, and nonavian reptiles) evolution and development (St. John et al. 2012; Green et al. 2014). The study of reptilian genomes is thereby essential to understanding the patterns of genomic evolution across amniotes. Genomic resources have been gathered for the past few years to understand reptile immune components and pathways. Reptiles are widely used as pets and food in various parts of the world, which has allowed them to pass on epizootic diseases, food-producing animal complexes, or zoonotic diseases to humans. Despite increasing interest in the reptilian genome, genomic resources remain very limited, and research should now focus on identifying the critical gaps to generate improved diagnostics, vaccines, and pharmaceuticals to reduce reptile-related diseases and death. In terms of exploration of genomic and molecular resources, this has been the least privileged class. However, the *Anolis carolinensis* (green anole) genome sequencing project has been a major landmark in the arena of reptile genomics. Its genome assembly has yielded 17,472 protein coding genes and 2924 RNA genes (Alföldi et al. 2011).

Though genomic and transcriptomic data are being added within the reptilian clade, analysis of comparative aspects of development and physiology demands greater resources and insight. Genomic studies of the Burmese python (*Python molurus bivittatus*) showed positive selection for metabolic, developmental, and mammalian disease genes (Castoe 2012; Shaffer et al. 2013), whereas the genomics of softshell turtle (*Pelodiscus sinensis*) and the green sea turtle (*Chelonia mydas*) suggested a common body plan (Wang et al. 2012). Studies on molecular markers like endogenous retroviruses, which are the most ancient infections, are limited to a host range of crocodiles and turtles, suggesting a restricted evolution of retroviruses in these orders (Chong et al. 2014). Conversely, endogenous viral elements (EVEs) were annotated from Hepadnaviridae, Bornaviridae, and Circoviridae in the speckled rattlesnake, *Crotalus mitchellii*, which established multiple host switches of

viruses between mammals and reptiles (Gilbert et al. 2014). Knowledge of reptilian immunity has been largely disseminated through such a handful of reports available on crocodilians, testudines, and squamates and one for the only living species of tuatara (Sphenodon punctatus) of the order Sphenodontia (Miller et al. 2015). Thus, the recent focus is aimed at studying the transcriptome of various reptilian orders so that a decent amount of data is generated for genome annotation, molecular marker development, and studies of development, adaptation, and evolution among amniotes. For several decades, the vast majority of what was known about squamate genes and proteins was focused on venom proteins and the transcripts that encode them. The majority of snake gene sequences in Genbank, for example, are from venom gland cDNA sequencing. Studies of venom gland transcriptomes have, however, lacked context due to the lack of transcriptomes from other tissues and from other squamate reptiles (Shaney et al. 2014). Another genomic study on king cobra venoms revealed multiple genome-level adaptive responses to natural selection processes (Vonk et al. 2013).

However, "omics" approaches have not yet been used extensively to delineate the intricacies of the reptilian immune system. Miller et al. (2015) provided the partial transcriptome data of tuatara and revealed immune-related genes for MHC and Toll-like receptors (TLRs). Transcriptome analysis in saltwater crocodile, alligator, and gharial showed an additional number of MHC genes compared to aves but similar to mammals. This sort of gene duplication may be due to clustering of MHC genes into various clades and subclades rather than in species, thereby imparting complexity to orthologous MHC genes (Jaratlerdsiri 2014). Green anole TLR5 (Voogdt et al. 2016) and IFN-γ and IFN-γR (Chen et al. 2013) have been reported to exhibit conserved patterns across jawed vertebrates with respect to their gene loci, genomic organization, and protein sequence. Priyam et al. (2016) analyzed and predicted, on the basis of transcriptome data of *Helmidactylus flavividis*, nine PRRs consisting of (1) TLRs, (2) C-type lectin receptors (CLRs), (3) retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and (4) nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Fig. 4). Determining how these variations of the reptilian genome have led to the structuring of innate immunity in this class has created an arena of interest. The annotation of reptilian orthologs of immunity genes would allow the synthesis of reptile-specific biological reagents and help to detect the uniqueness of its immune network. This would also widen the scope for finding novel genes and potential therapeutic targets courtesy of increased experimentation.

Traits of Reptilian Immunity: Seasonal Variation and Sexual Dimorphism

Reptiles are ectotherms, and the association of environmental temperature and physiology is also projected in their immune responses. Seasonal variation and sexual dimorphism are noteworthy aspects of reptilian immunity. Dissolution in the boundary of splenic white pulp and reduction in T-cell population during winters

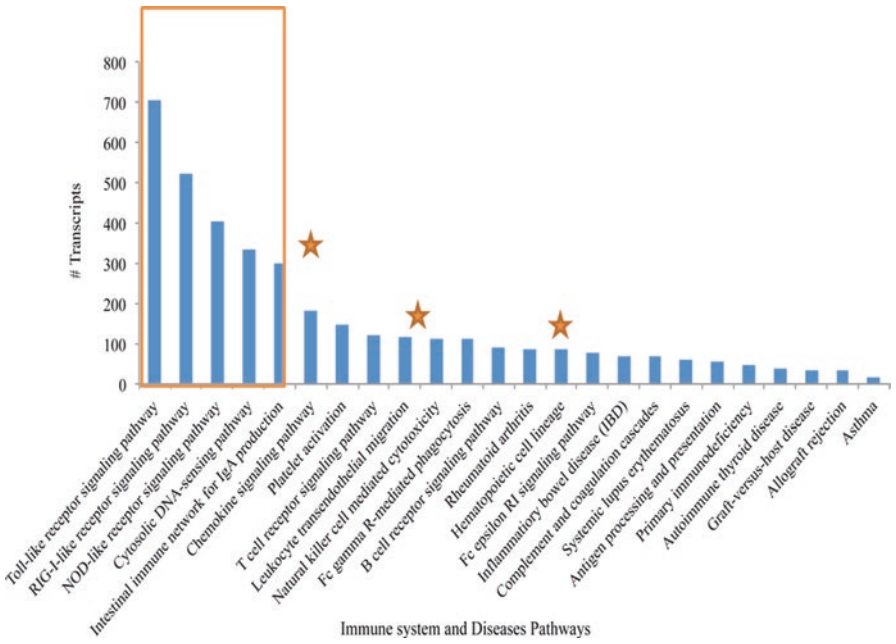

Fig. 4 Immune-relevant cluster assessed for immune system and immune diseases by frequency distribution of transcripts listed under KEGG categories. (Source: Priyam et al. 2016)

(Kanakambika and Muthukkaruppan 1972; El Ridi et al. 1981) are widely reported phenomena in squamates and turtles (Leceta and Zapata 1985). Complement proteins in alligator showed a considerable decrease in activity at temperatures below 15 °C and above 30 °C, suggesting the temperature dependency of immune components (Merchant et al. 2003, 2004). Basophil-mediated histamine release in turtle, *Chelydra serpentine*, was also immunocompromised at temperatures below or above 27 °C (Sypek et al. 1984). Experiments on wall lizard phagocytes in our laboratory have also shown a decline in IL-1 below or above 25 °C (Mondal and Rai 2001). Adaptive immune responses are also reported to exhibit season-dependent fluctuations in reptiles. Snake thymocytes showed a weaker proliferative response in a mixed leukocyte reaction and concanavalin A (Con A) stimulation in summer and winter as compared to spring and autumn (Saad and El Ridi 1984; El Ridi et al. 1987). Lymphocyte proliferation studies yielded similar results in turtle, *Mauremys caspica*, on stimulation with ConA and PHA (Muñoz and De la Fuente 2004). This dependency of immune response in reptiles is not just restricted to temperature but also to sex. Reptilian immunity exhibits sex-specific difference, wherein female striped sand snake has been reported to show higher proliferative capacity in response to ConA and PHA than their male counterparts (Saad 1989). However, contradictory reports have also been published in turtles (Muñoz and De la Fuente 2004; Keller et al. 2005a, b), where there were no sex-specific differences in lymphocyte proliferation in response to ConA or PHA. Sexual dimorphism in reptile

immunity needs to be explored in greater detail to gain insight into the dependence of immune response on sex steroids.

Evolutionary Gaps in Reptilian Immunology

All organisms, including plants and animals, invest their resources in orchestrating defense mechanisms for protection and survival. The sophistication and organization of these immune responses vary depending on the individual's organismal complexity as well as the pathogen profile in their niche. Hence, both internal selections within an evolutionary unit (e.g., order/class/phylum) as well as coevolution with exogenous factors are responsible for shaping the immunity of an organism (Du Pasquier 1992). Phagocytic cells in annelids are known to engulf and destroy foreign tissues once grafted from higher animals up the evolutionary scale like the starfish; yet tissues from the same individual are readily accepted. Similar phagocytic cells are present in insects, where they can bind to foreign cells and cause clumping, or agglutination. This clearly indicates that cellular immunity exists even at the level of invertebrates. Between the two, innate immunity is more ancient and involves the activation of robust cellular and enzymatic pathways in response to a pathogenic infection across vertebrates and invertebrates. During vertebrate immune evolution, defense mechanisms have become more specialized and specific, and the divergence between innate and adaptive branches of immunity has become distinct. In contrast, adaptive immunity is restricted to vertebrates and is distinguished from the former by its hallmarks of memory and higher specificity and diversity of response. Comparative immunology, apart from enlightening us on the functioning of immune networks in a particular clade of organisms, also helps us build an evolutionary arc of emergence of defense mechanisms in the living world. However, this arc remains incomplete because of the gap in the area of reptilian immunology.

Summary

Organism immunity is shaped by both selections within an evolutionary unit (e.g., order/class/phylum) and coevolution with exogenous factors and the pathogens it encounters. Reptiles are now known to have both innate and adaptive immunity, but the various aspects of cell-mediated immunity still need to be understood. Reptilian cellular immunity shows seasonal and sexual variations in its immune functions. The cellular component mainly consists of cells like macrophages, NK cells, heterophils, eosinophils, and B and T lymphocytes and the way these help to bridge innate and acquired immune systems. The macrophages, heterophils, and eosinophils are well studied as cells of the innate immune system, but little is known about NK cells or B and T lymphocytes. B cells display phagocytosis, underscoring the fact that they originate from a phagocytic progenitor. IgM is the most common immunoglobulin, while IgD and IgA have been identified in few species. The

biological significance of IgY still needs to be elucidated in reptiles. NK-type cells, though, are found in reptiles, though their biological importance remains unknown. MHC gene organization in reptiles reveals a structure between that of eutherians and birds. A large number of hybridizing bands in MHC loci are expressed in squamates and geckos. Tuataras, ancient reptiles, have a large polymorphic MHC region with a high repeat content and fewer genes, indicating ancient radiation among reptiles. Reptiles are also known to possess thymus, and functional T cells are found in squamates, sphenodons, crocodiles, and testudines. T cells also demonstrate a MHC-dependent response similar to that of mammals. It is now clear that a pathogen that may be lethal for higher vertebrates is easily dealt with in reptiles, even though their adaptive immunity is known to be primitive. Although reptiles possess a robust innate and a nascent adaptive immunity, still not much is known or has been studied in the reptilian immune system. Therefore, insights into the evolution of the immune system in reptiles will help us fill many gaps in our knowledge of vertebrate immunology. The immediate effect of studying reptilian immunology from the perspective of genomic, molecular, and proteomic resources would be to help generate enough data to analyze the comparative aspects of immune effector mechanisms. The ecoimmunology of reptiles can be investigated using this approach, and measures can be taken to develop preventive reptile medicine. This will lead to a better understanding of the epidemiology of diseases, especially those of zoonotic origin. Additionally, it can facilitate an improvised approach to vaccine development against pathogens.

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Aves: Immunological Characteristics of Fowls and Ostriches

Ke Mei Peng

The Immune System of Fowls

Immune Organs

The immune organs of fowls are divided into central (primary) and peripheral (secondary) immune organs, which are similar to those of mammals.

Central Immune Organs

The central immune organs of fowls include the thymus, bursa of Fabricius, and bone marrow, and they function to regulate the generation and differentiation of lymphocytes. These organs originate early in the embryonic stage and can induce the differentiation of hematopoietic stem cells from bone marrow to be immunoactive cells without any stimulation of antigens. Experiments have proven that removal of the central immune organ in early bird development can lead to the decline, and even loss, of immune function (Cui 2015; Tizard 2012; Godfrey 2004).

Thymus

Morphological Structure

The thymus of fowls is located in the hypodermis, bilaterally under the posterior segment of the neck. It has a long chain shape, with five to seven lobes on each side in chickens, four to six lobes on each side in ducks, and four to five lobes on each side in geese (Fig. 1). African ostriches have fewer thymic lobes: two to five on each side (detailed in section "Immunological Characteristics of African Ostrich"). The morphological structure of the fowl thymus is similar to that of mammals (Peng 2016a, b; Andrew 2012). The surface capsule of the thymus comprises a thin layer

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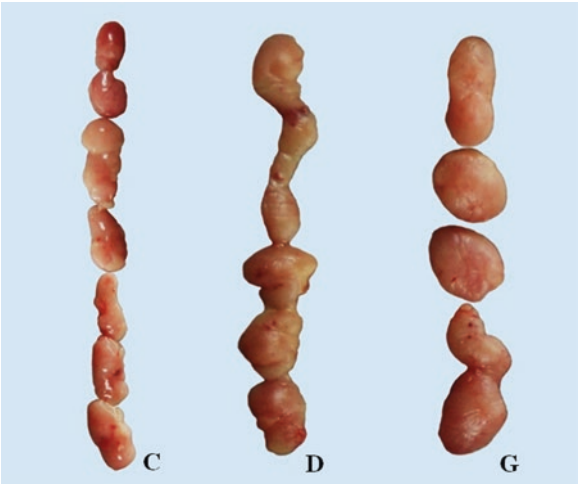

Fig. 1 Form of avian thymus. C chicken, D duck, G goose

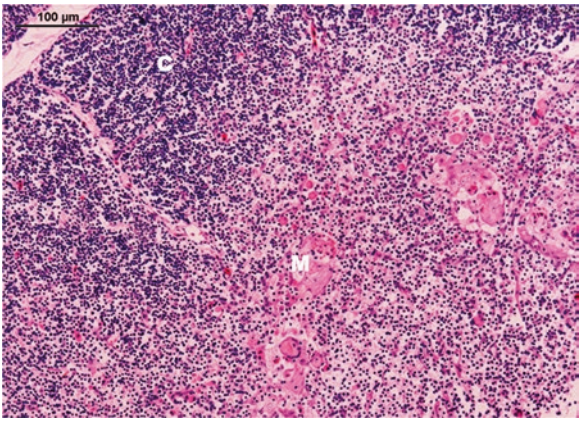

Fig. 2 Microstructure of chicken thymus. C cortex, M medulla

of connective tissue, which penetrates deeply into the thymus to form the interlobular septum and divides the parenchyma into various sizes of incomplete lobules (Fig. 2). Each lobule is divided into two parts: the cortex and the medulla. The cortex is located peripherally and stained darker because of the dense accumulation of small lymphocytes and small amounts of medium lymphocytes and epithelial reticulocytes. The size of the epithelial reticulocytes is larger and the cytoplasm is light-stained with a round or oval nucleus. The medullary region is in the center of the lobule. There are more lightly stained epithelial reticulocytes with a relatively reduced number of lymphocytes. The medullae of adjacent lobules are confluent with each other and house the Hassall's corpuscles (Fig. 3).

Immune Functions

The function of the avian thymus is similar to that of domestic animals, which mainly produces T lymphocytes associated with cellular immune activity. These

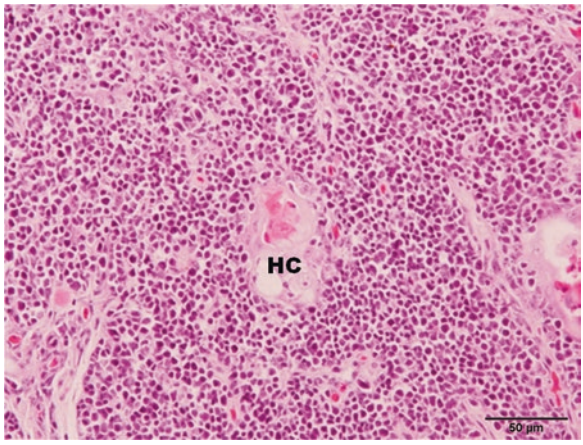

Fig. 3 Microstructure of chicken thymus, showing the Hassall corpuscle (HC)

cells are slow to proliferate, have a long life span (they can survive for months to years), and mainly complete cellular immune function. A variety of soluble substances can be obtained from thymic tissue, including mainly thymosin, thymopoietin, and serum thymus factors. Thymosin is a small polypeptide mixture that acts on the precursor cell of bone marrow and induces its maturation in order to become functional cells with certain T-cell characteristics. Thymopoietin comprises two polypeptides, which can cause the differentiation of T-cell precursors and enhance the function of T cells by reducing the level of cyclic adenylic acid (Andrew 2012). The serum thymus factor is a peptide secreted by thymic epithelial cells, which can partially recover the function of T cells after the removal of the thymus. Therefore, these substances play a significant role in inducing the maturity and differentiation of T cells (Tizard 2012).

Bursa of Fabricius

The bursa of Fabricius, also known as cloacal bursa, is a unique lymphatic organ of fowls and is a place where B cells differentiate and mature. It is located at the superior back of the dorsal cloaca and is communicated to the proctodaeum. The bursa of Fabricius is ball-shaped in chickens and turkeys, and those of ducks, geese, and most waterfowl are oval-shaped. The anterior part of the bursa of Fabricius is a blind end, and the posterior part forms the apical wall of the cloaca. The intramural surface of the bursa comprises more than ten strips of plicae of unequal size, with densely distributed papillary projections on the surfaces. The bursa of Fabricius develops gradually with age until sexual maturation and then gradually becomes atrophic and even disappears. The bursa of Fabricius of chickens and most pheasants disappears when they are about 10 months old, and that of ducks, geese, and other waterfowl disappears at about 12 months old. The bursa in African ostriches disappear much later (Keli et al. 2015; Song 2007).

Morphological Structure

The bursa of Fabricius originates from the cloaca, and the wall retains a four-layer structure similar to that of the digestive tract (Fig. 4). The innermost luminal surface

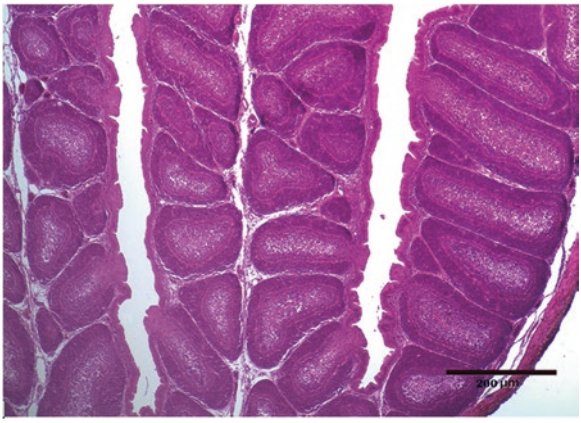

Fig. 4 Microstructure of the bursa of Fabricius

is covered with mucosa and has longitudinal folds full of lymphoid nodules. The folds comprise the mucosa and the submucosa. Different fowls have varied numbers of folds; a well-developed chicken has 12–14 folds but a duck has only two folds. Sometimes many small folds exist between large longitudinal folds (Peng 2016a, b; Song 2007).

The mucosa is divided into the epithelium and lamina propria. Most of the epithelium is pseudostratified ciliated columnar epithelium with some being simple columnar epithelium or simple cuboidal epithelium. Microvilli are found on the surface of the columnar epithelium. The lamina propria/submucosa is the thickest layer of the bursa of Fabricius and houses a large number of nodules in loose connective tissue (lymphoid nodules). A fold can sometimes have as many as 40–50 nodules, which are polygon-shaped because of the tight arrangement of polygons in the sections. Its structure is divided into cortex, middle layer, and medulla, which is different from the common lymphoid nodule.

1. *Cortex*: The cortex is darkly stained and comprises dense medium and small lymphocytes, macrophages, and epithelial reticulocytic stents. Lymphocytes divide and differentiate continuously in the mesh, and most have membrane antibodies of B cells, which are the more mature B lymphocytes. The cortex has a small number of capillaries, which are important channels for lymphocytes to be transferred from the medulla (Figs. 5 and 6).
2. *Middle Layer*: The middle layer is located at the junction of the cortex and medulla. The cells are undifferentiated epithelial reticulocytes with square or columnar shapes and eosinophilic cytoplasm. The cells are arranged in order, with a complete basement membrane. The basement membrane is close to near the cortex and incompletely surrounds the medulla. This layer is continuous with the mucosal epithelium and basement membrane of the bursa of Fabricius, and is not easy to see observe by hematoxylin and eosin (HE) staining.
3. *Medulla*: The medulla is stained lighter by HE and comprises large and medium lymphocytes, macrophages, and epithelial reticulocytic stents. Lymphocytes

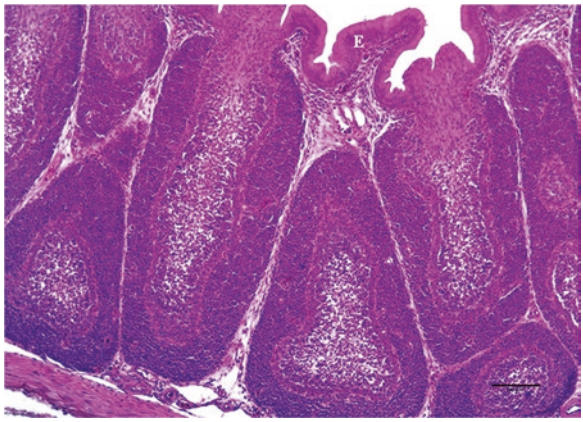

Fig. 5 Microstructure of the bursa of Fabricius. E epithelium

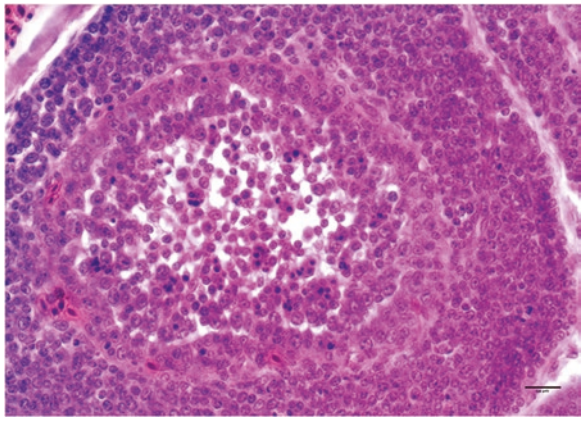

Fig. 6 Bursa nodules

arranged loosely divide and differentiate, and some of them possess membrane antibodies of B cells, which are immature lymphocytes.

4. *Submucosa*: The submucosa is composed of loose connective tissue and has no obvious boundary with lamina propria. It participates in forming trabeculae in mucosal folds.
5. *Muscular layer*: The muscular layer generally comprises the inner circular and the outer longitudinal layers of smooth muscle.
6. *Tunica adventitia*: The tunica adventitia is the thin layer of connective tissue on the outer side of the bursa.

Immune Functions

The bursa of Fabricius is the organ for B-lymphocyte differentiation. Stem cells enter the medullary region of the bursa of Fabricius through the blood circulation during embryonic development and divide and differentiate into various B lymphocytes. B lymphocytes then migrate to capillaries of the cortex and transfer to thymus-independent areas of lymphatic tissues and organs throughout the body. If they are

stimulated by antigens, B lymphocytes rapidly proliferate and differentiate into plasma cells and produce antibodies, namely those involving the humoral immune response.

In 1986, bursin was found in extracts of the bursa of Fabricius. Bursin is an active factor that plays a major role, which has important immune-regulation and physiological activities to lymphocytes, with the structure Lys–His–Gly–NH2. It can promote differentiation and proliferation of B-cell precursors of fowls as well as synthesis of protein in B cells, which then enhances the ability of B cells to produce and secrete antibodies. Bursin is mainly distributed in the basement membrane of surface epithelium between epithelial cells at the cortical margin and medulla in nodules of the bursa of Fabricius. It is also between the dendritic epithelial cells and nodules in the surface epithelial basement membrane. Bursin is the essential factor for the differentiation of embryonic immunoglobulin IgM+ cells in individual development of the chicken bursa of Fabricius. If the bursa of Fabricius is removed during the embryonic period, the hatched chicks lack B cells, which affects the humoral immune response. When they are infected, chicks often die due to low resistance to disease.

Bone Marrow

Morphological Structure

Bone marrow of fowls is located in the bone marrow cavity. It is the largest hematopoietic organ, which consists of reticular tissue that forms stents, and there are many kinds of cells in the mesh. Bone marrow in the nestling phase is distributed in the bone marrow cavity throughout the body. After entering adulthood, saccules expand into the bone and replace part of the bone marrow. Thus, only the epiphyseal end of tubular long bone always maintains the bone marrow (Peng 2016a, b). Bone marrow is a hematopoietic tissue full of blood cells and a small amount of hematopoietic stem cells, macrophages, fat cells, and mesenchymal cells of different development stages in the mesh of the reticular tissue stents. Sinusoid is composed of capillary branches, and the wall of porous endothelial cells, an incomplete base membrane, and pericytes, which allow mature blood cells to move into the bloodstream. The sinusoid is full of hematopoietic tissue. Macrophages around the sinusoidal wall and in the sinusoidal cavity help to phagocytose and eliminate foreign bodies, bacteria, and aging and dead blood cells (Fig. 7).

Hematopoietic stem cells are progenitor cells that generate various blood cells, originating in the blood island of the yolk sac during the embryonic period, which can be separated from the blood or bone marrow. Under certain environmental conditions, they differentiate into various committed stem cells; therefore, they are also known as multipotent stem cells. Their basic characteristic is the ability to self-sustain and self-renew. Stem cells continuously generate numerous committed stem cells by asymmetric mitosis. Committed stem cells further proliferate and differentiate to complement and maintain peripheral blood cells. The hematopoietic stem cells are differentiated from totipotent mesenchymal cells of the embryonic yolk sac. They are mainly located in the bone marrow, accounting for about 0.05% of the adult bone marrow cells, followed by the lymph nodes and spleen. Committed

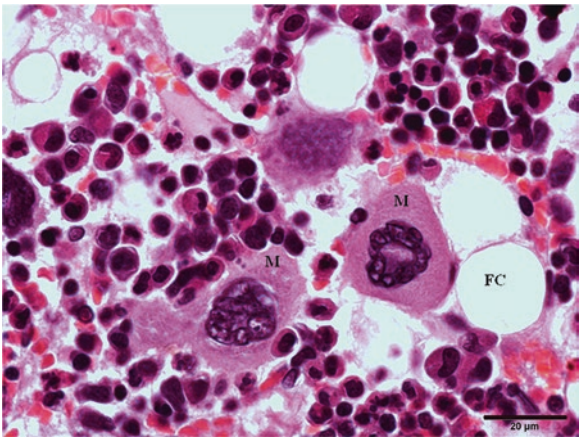

Fig. 7 Avian bone marrow. F fat cell, M macrophage

stem cells possess high proliferative capacity. According to their path of differentiation, they are divided into myeloid multidirectional hematopoietic stem cells: erythroid stem cells, granulocytic and macrophagic stem cells, megakaryocytic stem cells, and lymphoid stem cells.

Immune Functions

Bone marrow possesses an important immunological function. Although bone marrow is not a lymphatic tissue, it contains multipotent stem cells with powerful potential to differentiate into medullary stem cells and lymphoid stem cells. Medullary stem cells further differentiate into erythrocytic, monocytic, granulocytic, and megakaryocytic cell lines. Lymphoid stem cells develop into all sorts of lymphocyte precursors. The precursor cells of T cells, after reaching the thymus through the bloodstream, are induced and differentiated into mature T cells that are involved in cell immunity. With the blood flow into the bursa of Fabricius, the precursor cells of B cells develop into mature B lymphocytes, also known as bursa-dependent lymphocytes, and participate in humoral immunity. Abnormal proliferation and differentiation of hematopoietic cells can cause malignant proliferative diseases such as leukemia (Peng 2016a, b).

Bone marrow dysfunction not only seriously damages hematopoietic function but also causes immunodeficiency. Following extensive exposure to x-rays, the bone marrow of adult domestic fowls is destroyed, with consequent malfunction of the immune system. Increased radiation doses can completely destroy bone marrow function and eliminate the hematopoietic and immune functions of the body. If fowls with deficient bone marrow are injected with homogeneous compatible bone marrow, the destroyed lymphatic tissue can be reconstructed and the immune function can be recovered (Peng 2016a, b).

Peripheral Immune Organs

Peripheral immune organs are sites where lymphocytes settle, proliferate, and respond to antigens in the later phase. These kinds of lymphatic organs originate

Fig. 8 Form of the fowls' spleen. C chicken, D duck, G goose

from the embryonic mesoderm, develop in the later embryonic phase, and exist in avian bodies for life. They can respond to the stimulation of antigens. Thus removal of these kinds of lymphatic organs will not completely destroy the body's immune function (Peng 2016a, b).

Spleen

Morphological Structure

The spleen of fowls, which is a brownish-red or purplish-red color, is located at the right upper juncture of the glandular and the muscular stomachs. The spleen of the male is slightly larger. The spleen is ball-shaped in chickens, a flat ovoid in ducks, a triangle in geese (Fig. 8), and a long ellipse in pigeons. The ostrich has a long, kidney-shaped spleen that is a dark-red color and has a concave dorsal part and convex ventral part (detailed in section "Immunological Characteristics of African Ostrich").

The capsule of the fowls' spleen is relatively thin and extends to the splenic parenchyma, forming undeveloped trabeculae. The splenic parenchyma is composed of white pulp (WP) and red pulp (RP); there is no obvious boundary between them, and there is also no obvious marginal area. WP is a diffuse lymphatic tissue, and the periarterial lymphoid sheath is thin. The splenic nodule is located on one side of the periarterial lymphoid sheath (PALS); the germinal center within the splenic nodule is not obvious. Lymphocytes are densely distributed and are deeper staining. Numerous ellipsoids are often scattered or clustered in the marginal area at the juncture of the periarterial lymphoid sheath and the RP. Ellipsoids are generally 25.5–44.5 μm and composed of two to four layers of cells. The nucleus is larger, round or ovoid, light-stained, and located on one side of the cell. With Feulgen–methylene blue staining, the nucleus is purple-red and the cytoplasm is green (Fig. 9). There are homogenous eosinophilic substances between the ellipsoid and the peripheral lymphatic tissue, which are specially stained red using an improved Weigert method. The vascular cavity of the sheath capillary is small, and the endothelial cells are closely aligned. There are often erythrocytes in the cavity and ellipsoid. The RP fills the space between the WP and is made up of the splenic cord and splenic sinus. The splenic cord is a cord-like branch, which is interwoven

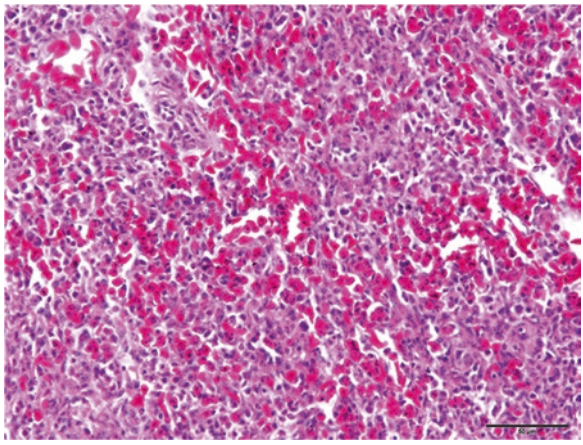

Fig. 9 Microstructure of the avian spleen

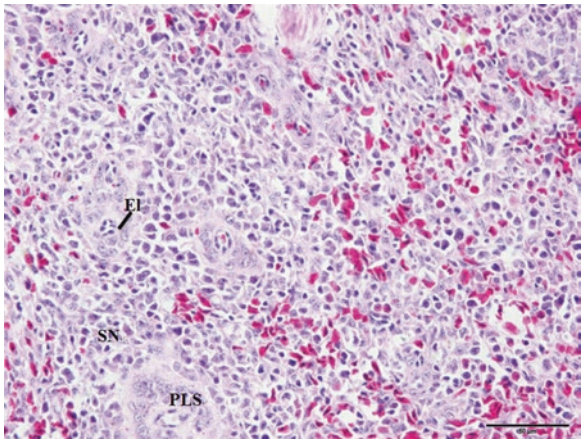

Fig. 10 Microstructure of the avian spleen, showing white pulp. EI ellipsoids, PLS periaterial lymphatic sheath, SN splenic nodule

into a web, and filled with lymphocytes, macrophages, and plasma cells. The splenic sinus is more developed and is distributed under the capsule, alongside the trabecula and between the splenic cords. The sinus cavity is larger under the capsule and around the trabeculae, and a large number of blood cells are dispersed in the cavity (Figs. 9 and 10).

Immune Functions

The major functions of the avian spleen are hematopoiesis, blood filtration, and immune response. Unlike the mammalian spleen, the avian spleen is not involved in blood storage and regulation of blood volume. Unlike lymph nodes, immune functions of the spleen are mainly to process the hemogenic antigen, while that of the lymph nodes is to process antigens in the lymph fluid. In addition, the spleen is the main "filter" for the blood. Macrophages in the RP can remove foreign bodies and aged and dead red blood cells, even when lacking a specific immune state. The main hematopoietic activity of the spleen of adult birds is to generate lymphocytes.

The spleen is also the main organ for the synthesis of the macrophage-enhancing hormone, and it can synthesize immune molecules, such as interferon (IFN), complement, cytokines, and other bioactive factors (Härtle 2013).

Lymph Nodes and Lymphoid Tissue

The lymphoid tissue in fowls is widely distributed in many substantial organs such as the lamina propria and submucosa of the digestive tract, respiratory tract, and reproductive tract. The caecum tonsil is a unique lymphoid tissue in the digestive tract of fowls. The diffuse lymphoid tissue or lymph nodules are also located in the liver, pancreas, lung, kidney, heart, bone marrow, skin, Harder's glands, and endocrine glands. Only a few species of fowls have lymph nodes.

Lymph Nodes

① *Morphological structure*: Only the duck, goose, lanruo, and other waterfowl have lymph nodes. There are two major pairs of lymph nodes: the pair of cervicothoracic lymph nodes are spindle-shaped, 15–30 mm long, 2–5 mm wide, and are located at the back neck in the angle of the jugular vein and the vertebral vein, often close to the jugular vein; the other pair are lumbar lymph nodes that are long, strip-shaped, about 25 mm long and 5 mm wide, and located at both sides of the aorta between the kidney and the lumbar sacrum, near the beginning of the thoracic duct. There is no hilum in the lymph nodes of fowls. The capsule is formed by connective tissue on the surface, and penetrates into the parenchyma to form the trabeculae. It is not common to distinguish between the cortex and medulla in the lymph nodes of fowls. Parenchyma is composed of the central sinus, lymphoid nodules, diffuse lymphoid tissue, lymphocytic cords, and peripheral lymphoid sinuses. The central sinus is located in the central part of the parenchyma and has irregular shape with branches that are communicated with peripheral lymphoid sinuses. Diffuse lymphoid tissue contains abundant capillaries and post-capillary venules. This area is correspondent to the paracortex of the lymph node in mammals. The light-stained germinal centers of lymph modules mainly contain B cells, and the diffuse lymphoid tissue mainly contains T cells. The lymphocytic cord and lymphoid sinus are distributed between the lymph nodules. There is no reticular tissue in the lymphoid sinuses of fowls, which contains only a small number of lymphocytes, macrophages, and plasma cells (Min et al. 2013).

② *Immune functions*: The lymph node of fowls exerts a weak filtration effect, but it is an important capture system in adult fowls, and it is the macrophages in lymph that perform this capture function. When lymph nodes contact antigens for the first time, most of them are engulfed by macrophages. Antigen-loaded macrophages migrate to lymphoid nodules and stimulate proliferation of correspondent lymphocytes to form germinal centers. In the germinal center, small lymphocytes are transformed into immunoblasts and immature plasma cells, which have less karyoplasm and therefore are stained light. The immature plasma cells migrate to the lymphatic cords and become mature plasma cells with antibodies a few days later. Certain plasma

cells can then be released into the lymph fluids, and they then enter other lymph nodes or lymphoid tissue to produce antibodies (Peng 2016a, Min et al. 2013).

Lymphoid Tissue

1. *Ileum lymphatic aggregate nodules*: The ileum lymphatic aggregate nodules are widely distributed in the posterior segment of the ileum in fowls, in parallel with which the central cecum has visible diffuse lymphoid tissue about 1 cm in diameter, similar to mammalian Peyer's patches, which play an important role in local immunity (Peng 2016a; Wang et al. 2010, 2011).
2. *Caecum tonsil*: The caecum tonsil is located in the mucosal lamina propria and the submucosa of the cecum at the junction with the ileum as well as in the rectum. It is well-developed and can be seen with the naked eye if slightly swollen. Cells in diffuse lymphoid tissue are divided into small lymphocytes and mature and immature plasma cells. The caecum tonsil has many larger germinal centers, which are an important source of antibodies, and plays a role in local immunity to intestinal bacteria and other antigenic substances.
3. *Intraocular lymphoid tissue*: The Harder's gland is also known as the accessory lacrimal gland or glandula membrana nictitans. It is relatively well-developed, usually light red to brown in color, ribbon-shaped, and is located in the orbit of the ventral side and internal posterior side of the eyeball, attached to the outer orbital fascia. The average size of the Harder's gland of an adult chicken is 17.3 × 7.4 × 2.2 mm and the average weight is 82.4 mg. The Harder's gland is a compound tubuloacinar gland, composed of acini and ducts. The capsule of connective tissue is inserted into the parenchyma, which divides the parenchyma into many lobules of varying sizes. The paranasal and paraocular lymphoid tissues that mainly contain Harder's glands are unique immune organs at the ocular fundi of chickens and play a major role in local immunity. It is suggested that they are the location of the differentiation and reproduction of bursa of Fabricius-independent B cells in the fowl's' body.
4. *Conjunctiva-associated lymphoid tissue*: Conjunctiva-associated lymphoid tissue is mainly distributed in the conjunctival fornix at the proximal end of the palpebra, and it approaches its peak development 25 days after birth. Its surface has many parallel bank-shaped folds and cracks. The squamous epithelium cells covering the surface of the lymphoid tissue are polygonal, are larger, and connect tightly to each other. The microvilli on the cell surface are stubby and arranged irregularly, with scattered orifices of goblet cells. High endothelial venules are located in the diffuse lymphoid tissue under the basement membrane of the epithelium and the germinal centers in the lymph. The lymphatic epithelial cell in the conjunctiva-associated lymphoid tissue has the function of uptake and delivery of antigens, mainly through pinocytosis and the transport system of cytoplasmic vesicles. It is the main portal for antigens into the lymphoid tissue of the ocular region, in which macrophages are involved in uptake and delivery of antigens.
5. *Lymphoid tissue in other organs*: Lymphoid tissue of fowls is scattered in many organs and tissues of the body, such as paraocular organs, paranasal organs, skin,

heart, liver, pancreas, larynx, trachea, lung, kidney, endocrine glands, and peripheral nerves. They are usually diffuse lymphoid tissues without capsules, or are infiltrated by surrounding cells, with germinal centers visible locally.

6. *Intramural lymphoid nodules of lymphatic ducts*: The intramural lymphoid nodules of lymphatic ducts are also a diffuse lymphatic tissue, first found in ducks and later in chickens and other fowls. The intramural lymphoid nodules are round, oval, or elongated oval brown bodies without capsules, which have obvious boundaries or are diffuse with a diameter of 0.1–2.5 mm, most commonly 0.3–0.5 mm, which are visible to the naked eye. In adult chickens, the intramural lymphoid nodules are scattered along lymphatic ducts at 12.5 mm intervals, and mainly contain small lymphocytes with three to four germinal centers. The function of intramural lymphoid nodules of lymphatic ducts is unknown, and the lack of communicating lymphatic sinuses is insufficient to support the suggestion that they lack a filtration function (Peng 2016a).

Immune Cells

Avian immune cells are similar to those of mammals, including immunocompetent cells and immunity accessory cells. The main purpose is to introduce several important immune cells in fowls (Cui 2015; Dan 2007; Ema et al. 2005; Banchereau et al. 2000).

T and B Cells

T and B cells originate from the multipotent stem cells of bone marrow (yolk sac and liver at embryonic period), differentiate and mature in the thymus (T cells) and bursa of Fabricius (B cells), and then settle down and reach the whole body to function after they migrate to the corresponding dependent area of other peripheral immune organs (Cui 2015). T cells live longer than B cells and can be recycled in the lymphatic system. The main differences between T and B cells are in the structure or composition of the membrane surface (namely the surface marker) (Table 1). T cells have many subclasses, such as helper T cells (Th cells), suppressive T cells (Ts cells), and cytotoxic T cells (Tc cells). They work together to perform the immune functions of T cells, namely cell immunity and immune regulation.

Table 1 Components of surface markers of fowls' T and B cells

Surface markers	T cells	B cells
Surface antigens	Chickens' T cell surface mainly includes CD3, CD4, CD8, etc.; the structure and function is similar to that of mammals	Mainly includes Ia antigen, CD antigen, etc.
Surface receptors	Mainly include antigen receptors, Fc receptors, and mitogen receptors	Mainly include antigen receptors, Fc receptors, complement receptors, Epstein-Barr virus receptors (CD21), etc.

Fowls' B cells mainly participate in humoral immunity, by producing specific antibodies. There are fewer studies on B cell subsets and the classification is not unified. According to whether the B cells need the assistance of T cells when producing antibody, they can be divided into two subsets: B1 and B2 cells. B1 cells are T cell independent and B2 cells are T cell dependent (Koskela et al. 2004; Piccirillo and Shevach 2004; Rothenberg 2000).

Heterophilic Granulocytes

Another important component of the avian non-specific immune system is polymorphonuclear cells, one of the most representative being heterophilic granulocytes, which exist in the bone marrow, blood, and connective tissue of fowls and are the main avian leukocyte. In vitro tests show that the bactericidal action of heterophilic granulocytes is not dependent on an opsonin. Phagocytosis of heterophilic granulocytes is more independent than macrophages and monocytes. Its action is similar to mammalian neutrophils, and it has an effect of swallowing and killing invading pathogens in order to protect the body from invasion. However, the avian heterophilic granulocytes are different from mammalian neutrophils in morphology, structure, and immune effect, and the heterophilic granulocyte of fowls cannot be called neutrophils. For example, heterophilic granulocytes are mainly dependent on the oxygen-independent bactericidal action rather than respiratory burst and glucose oxidation reaction (Cui 2015).

When an acute inflammatory reaction occurs, heterophilic granulocytes interact with vascular endothelial cells at a receptor level and migrate to the inflammatory site. There are also lymphocytes and basophils in the early inflammatory exudate, but their numbers are significantly less than heterophilic granulocytes. Because there are no stationed pulmonary macrophages in the respiratory tract of fowls, the heterophilic granulocyte flow of the inflammatory exudate becomes the first line of cell defense. Unlike neutrophils in mammals, heterophilic granulocytes that congregate at the site of inflammation can be concentrated in the form of caseous scabs which are not dissolved and absorbed. This mode of isolating pathogens and external stimuli is effective, but formation of caseous granulomas can interfere with certain organ functions in more severe inflammatory responses (Peng 2016a; Cui 2015).

Macrophages

Mononuclear macrophages, after a brief stay in the blood circulation, reach multiple tissues of the whole body and mature into macrophages. Macrophages are a class of scavenger cells that act as the body's first line of defense against infection. Activated macrophages play an important role in fighting against invading specific and non-specific immunity and mediating and activating other responses. Likewise, macrophages can be the target cells of infectious bursal disease virus (IBDV), infectious laryngotracheitis virus (ILTV), Newcastle disease virus (NDV), Marek's disease virus (MDV), and other pathogens to provide places for their reproduction and transmission. In addition, the occurrence of some specific autoimmune diseases is related to macrophages (Cui 2015).

Erythrocytes

Erythrocytes are also an important component of the animal immune system, involved in specific and non-specific immune responses and immune regulation. Erythrocytes have many immunity-related substances, such as complement receptor (CR) 1/CR3, lymphocyte function antigen 3 (LFA-3), degradation accelerating factor (DAF), superoxide dismutase (SOD), natural killer (NK) cells enhancement factor (NKEF), CD58, CD59. Erythrocytes also have a β endorphin receptor and β epinephrine receptor. The erythrocyte has the following functions: (1) adheres to immune complex in the circulatory system of the body; (2) promotes macrophage phagocytosis and eliminates immune complex; (3) identification, storage, and delivery of antigens; (4) promotes the proliferation and differentiation of lymphocytes; (5) enhances the antineoplastic activity of NK cells; (6) improves the activity of lymphokine-activated killer (LAK) cells; and (7) functions as an effector cell (Peng 2016a; Cui 2015).

Natural Killer Cells

NK cells are found in the peripheral blood and spleen, less so in the lymph nodes and bone marrow, and not in the thymus. NK cells are lymphocytes that are neither dependent on antibodies nor antigen stimulation and sensitization to kill target cells, and thus they are natural killer cells. The surface of NK cells has a receptor that identifies the surface molecule on the target cell, by which the receptor combines with the target cell to perform its killing function. NK cells have receptors for IFNs and interleukin (IL)-2 on the surface. IFN acts on NK cells to increase the ability to recognize the target cells and enhance the activity of dissolving and killing. IL-2 can stimulate NK cells to proliferate and produce IFN, which induces a greater role in killing. The Fc receptor of IgG is also found on the surface of NK cells. The target cells bound with IgG can be recognized and dissolved by NK cells through binding of its Fc receptor. Thus, NK cells also exert an antibody-dependent cell-mediated cytotoxicity (ADCC) effect (Peng 2016a; Cui 2015). The main biological functions of NK cells are non-specific killing of tumor cells, resisting multiple microbial infections, and excluding the transplantation of bone marrow cells. NK cells are effective in killing bone marrow cells and B cells, demonstrating their immunoregulatory ability. The killing effect of NK cells on tumor cells is broad spectrum. Thus, they may be an important part of the body's immune surveillance apparatus (Peng 2016a, b; Cui 2015).

Dendritic Cells

Dendritic cells are present in most immature tissues, but they are the most powerful antigen-presenting cells (APCs) responsible for activating immature T cells. Under such a state, they are able to capture and process antigens on their cell surface. After that, they mature and migrate to lymphoid tissues, where they activate specific T cells (Cui 2015). Lymphoid nodules are present in various tissues of the avian body and are re-formed at sites of infection. In these lymphoid nodules, T cells encounter the cell-bound antigen and initiate the acquired immune response. Mature dendritic cells are also able to interact with macrophages to release cytokines and can interact

with B cells to induce the formation of antibodies. Total lymphocyte activation and cloning amplification requires 4–5 days. During this period, the natural immune system continues to play a role in limiting growth of pathogens along with increasing immunity from acquired immune responses (Cui 2015; Liu 2001).

Immune Molecules

Immunoglobulin

The immunoglobulin of fowls mainly includes IgG, IgM, IgA, and IgD. The physicochemical properties and biological functions of each immunoglobulin of fowls are introduced in the following sections (Cui 2015; Shun et al. 2014).

IgG

The IgG of chickens has certain properties similar to those of mammals, and has also been named IgY by some researchers. IgG not only exists in serum, but also in external secretions such as egg yolk, tears, semen, intestinal juice, saliva, and bile. IgG is the most abundant and main immunoglobulin in serum. In the serum of adult chickens its volume is 5.29 ± 1.35 mg/ml. IgG has high neutralizing and complement-binding ability, but IgG of the chicken cannot activate guinea pig complements. Chickens produce a large amount of sedimentary IgG. Some of IgGs are not able to agglutinate erythrocytes, but only generate precipitation in a high concentrated salt solution, which is called an incomplete antibody (Cui 2015).

The molecular weight of avian IgG is greater than that of mammals, and the molecular weight of chicken IgG is 180 kDa with a sedimentation coefficient of 7S. Unlike mammalian IgG, avian IgG is easy to break down. Chicken IgG can be divided into IgG1, IgG2, and IgG3 subsets, but only IgG1 and IgG2 are present in yolk. The half-life of IgG in serum is 4.5 days. There is a lack of common antigens for avian IgG and mammalian IgG, while the IgG of chickens, turkeys, and pheasants shares a common antigenicity. Avian IgG plays very important roles in humoral immunity for resisting bacteria, viruses, and exotoxins. In antineoplastic immunity, IgG can bind to lymphocytes or macrophages to kill tumor cells. Furthermore, IgG may also be associated with the production of tumor-blocking factor in the body (Shun et al. 2014; Cui 2015).

There are four different regions between avian IgG and mammalian IgG. Avian IgG contains five regions (V, Cl–C4) and no hinge region, but it has an elastic finite variable region at the junction of υ l C– C υ 2 and C υ 3–4 C υ, which performs a unique biological function. The main difference between the IgG of chickens and that of mammals is that the H chain of chicken IgG is a gamma shape and longer. The IgG of the duck has two subtypes: the larger one is similar to that of chickens, which is also known as "7.8S IgG"; and the smaller IgG has only three H chain regions (V, C1, and C2), and the two constant regions are similar to the structure and antigenicity of the F(ab′)2 of normal IgGs, which can be combined with the Fc segment of the antibody.

IgM

IgM is the largest immunoglobulin in molecular weight, with a weight of 900 kDa and sedimentation coefficient of 19S. It is a wreath-like polymer structure by the polymerization of five 7S monomers and has a J chain, which predominates in the primary immune response. IgM of 7S can be detected in the amniotic fluid of young chicks that are 1 day old and in eggs. It is part of the oviduct secretion of hens, or an IgD counterpart of chickens. IgM is the immunoglobulin that appears earliest in the primary immune response. It mainly exists in serum, and is also found in bile, tears, intestinal juice, and saliva, but not in yolk. Generally, avian IgM has a slightly larger proportion than in mammals and the volume in the serum of adult chickens is 2.50 ± 1.25 mg/ml. IgM accounts for about 4% of the total Ig when young fowls are hatched, and its volume in serum is 0.71 ± 0.18 mg/ml, which is lower than that of mammals. The half-life of adult chicken IgM in serum is 1.7 days.

The properties and also the antigenicity of avian IgM are similar to those of mammals. The IgM of chickens, pheasants, and quail has common antigens with humans, monkeys, sheep, and other mammals. In the quantitative complement combining avian IgM and anti-human IgM serum, more than 50% cross-react (Cui 2015). Although the amount of IgM in fowls is relatively small, it has many functions such as precipitation, agglutination, and complement activation. The IgM monomer is closely associated with B cells and T cells. It is believed that it acts as a receptor in B cells, which can bind with antigens and thus regulate antibody production by plasma cells. In addition, IgM is a cytotoxic antibody which can destroy tumor cells with complement participation and plays an important role in antineoplastic immunity (Cui 2015).

IgA

IgA is also present in the secretory products of chickens, and, similar to mammalian IgA, it tends to form polymers. The major form of IgA in serum is the monomer or dimeric form, with a molecular weight of 160–170 kDa and volume of 30–60 mg/100 ml. The monomer does not play an important immune function in serum. IgA in saliva, tears, nasal and bronchial secretions, and other exocrine fluids is mostly in the form of a dimer with two monomers connected by a J chain. The molecular weight of a dimer is 370–390 kDa. IgA binds with the segments of the secretary proteins when crossing the epithelial cells. The secretory segments have no immune activity, but help IgA to pass through the mucosal surface of the gland and protect IgA against the decomposition of protease. The number of dimers in exocrine fluids is six to eight times higher than in serum, which is an important factor for defense against infection in the body mucosa (Cui 2015).

IgD

IgD was originally isolated as an abnormal myeloma protein, which exists in avian serum but in an extremely low concentration. Serum IgD is a monomeric immunoglobulin with a molecular weight of 170 kDa, and its immune function is not clear. However, IgD is also found on the surface membrane of lymphocytes in

normal peripheral blood, which has physicochemical properties generally similar to those in serum. Its biological characteristics mainly include the following: (1) most lymphocytes have both IgD and IgM on the surface membrane, and during B cell differentiation the membrane IgD appears later than the membrane IgM; (2) the appearance of IgD on the B cell surface seems not to be influenced by antigens and T cells; (3) membrane IgD is the main surface immunoglobulin of peripheral lymphocytes; and (4) membrane IgD is the antigen receptor synthesized by its B cells, and its function is to initiate and regulate further differentiation of B cells.

Major Histocompatibility Complex (MHC)

A cluster of tightly linked genes on chromosomes that are responsible for encoding the major histocompatibility systems (MHS) is known as the major histocompatibility complex (MHC). The blood types in chickens were first discovered in the 1950s, having been determined by a polymorphic erythrocyte antigen. Thus, the avian MHC was also named B complex. According to different encoded proteins, there are three functional zones of the avian MHC genes, known as *B-F* gene, *B-L* gene, and *B-G* gene. *B-F* and *B-L* genes are equivalent to class I and class II genes of mammals, respectively. The *B-G* gene is unique to fowls and is known as a class III gene (Cui 2015; Schneider et al. 2004).

Structure of MHC

The MHC of chickens is located on minute chromosome 16, consisting of a group of highly polymorphic and tightly linked gene clusters. These gene clusters are composed of a genetically independent B complex and Y complex plus a nucleolar organizer region (NOR).

B-F Gene

The *BF2* gene site is easier to express than the *BF1* gene site; therefore, the former is the primary factor, known as *B-FIV*, primary *B-F*, and *Bfa1*, and the latter is a secondary factor, known as *B-FI*, secondary *b-f*, and *Bfa2*. The exon 2 and 3 sequences of typical *B-FI* and *B-FIV* genes encode α1 and α2 domains. These two domains form the fissures that provide class I antigens, and the eight antiparallel β double strands that support two parallel α monocycles, which form the tertiary structure of this antigen.

B-L Gene

The avian *DB* gene loci contain two sites located in IIβ chain genes: *BLB1* and *BLB2* of crenarchaeal chromatin protein 1. The former can be expressed in the primary factor, which, therefore, is named *B-LB*, *B-LBa*, and *B-LβII*. The latter, as the secondary factor, is not easy to express, and is therefore named *B-LB*, *B-LBb*, and *B-LBI*. The gene exchange rate of the avian class II B gene internal site is higher than that of mammals. Some genes are rich in G + C, and the promotors of the *class IIα*, *class Iiβ*, and *β2m* genes near the beginning point of the transcription are all rich in the G + C regions (in mammals the TATA regions). The promotors of the *class Iα* and *β2m* genes also have IFN reaction elements.

B-G Gene

B-G has a blood type polymorphism, which is a huge polymorphic immunoglobulin superfamily (IgSF) gene family similar to that of mammalian MHC butyrophilin, myelin oligodendroglia glycoprotein, and the shared sequence of the terminus of the *TRIM* gene. The *B-G* of the chicken and quail has an independent gene locus, which is sometimes located 100 kb outside of CC1. *B-G* has typical characteristics of an adhesion molecule, including an extracellular domain interacting with other cells or an extracellular matrix and cytoplasmic tail that interacts with the cytoskeleton. This fits the relationship between the *B-G* sequence and myelin oligonucleotides.

Functions of MHC

The significant function of chicken MHC is to regulate the immune response. MHC cell surface protein plays a key role in distinguishing autoimmunity from non-autoimmunity. Therefore, MHC can maintain its own integrity at the same time that the immune system becomes active to foreign antigens. The process of interaction between APCs and T cells in the anti-immunity, as well as the important effect of BF/B-L antigens in interaction phenomena of all cells, are modulated by MHC. Other processes modulated by MHC include the actions of T cytotoxin, viral infection, and mutant chicken strains.

B-F (class I) antigens are distributed in almost all cell membranes of nucleated cells, among which the large number of expressions on the red blood cell membranes is unique to fowls. This kind of antigen plays an important role in graft rejection and restricts the Tc cell recognition. T cells can only be activated when they recognize not only the foreign epitope but also the class I antigens.

B-L (class II) antigens are only expressed on the surface of some immune cells (B cells, activated T cells, mononuclear macrophages, etc.). Class II antigens are associated with the immune response and regulation. Not only the *Ir* gene encodes antigens on them; T cells, B cells, and macrophages also interact with each other to recognize the same class II antigen.

B-G (class III) antigens exist on the surface of the erythrocytes or erythrocytoblasts in fowls, and mammals have no such substance.

Cytokines

Cytokines are soluble proteins with biological activity that are secreted by various immunocompetent cells and play an important role in the immune response process by regulating cell growth and activation. To date, the knowledge and application about avian cytokines is far less than that of mammals (Cui 2015; Shizuru 2005).

Basic Characteristics

1. There are many types of immunocompetent cells that produce cytokines, but they can only secrete cytokines after being activated by specific or non-specific stimuli. Specific stimuli mainly refer to antigens and certain specialized

cell-activating substances. Non-specific stimuli include phytohemagglutinin (PHA), concanavaline (ConA), and bacterial lipopolysaccharide (LPS).

2. The avian cytokines are mainly a set of secretory glycoproteins of immunocompetence with low molecular weight, but are different from immunoglobulins. The main differences are that there are many kinds of cytokines with different structural and physicochemical properties; the molecular weight is small, with an average of 4–60 kDa; the secretion amount is limited, and the separation and purification is difficult; the exertion of biological activity is not dependent on the presence of antigens, but they directly act on their own target cells; and they play an important role in local immunity.
3. Most cytokines can be induced in vivo or in vitro using artificial methods. However, because of the lack of cross-reactivity as well as the physiological characteristics of fowls themselves, the method used to quantify mammalian cell factors generally cannot be used directly for fowls.
4. The biological activity of cytokines is strong, and it can be detected easily in the range of 10^{-10}–10^{-5} mol. Its active form is mostly non-specific, and it is not limited by MHC.
5. There is a slight difference in the in vitro induction time and half-life of various cytokines of fowls, which can be detected in the culture liquid generally after 6–8 h of induction, and the yield reaches its peak in 20–2 h.

Basic Functions

Interleukin

IL is the cytokine produced by lymphocytes, monocytes, and other non-monocytes that play an important role in the process of immune regulation. IL-1, IL-2, IL-4, IL-5, IL-6, IL -7, and IL-12 can all stimulate the activation, proliferation, and differentiation of T and B cells, which facilitates the synthesis and secretion of antibodies or the production of sensitized lymphocytes to provoke humoral or cellular immune responses. By contrast, IL-4 and IL-l0 inhibit cell immune function by inhibiting the activation of macrophages and inhibiting Th1 cells from producing IL-2, IFN-r, and TNF-β. Chicken IL (ChIL)-1β (ChIL-1β) was discovered in 1982. ChIL-1β protein is composed of 267 amino acids. When the calmodulin-dependent kinase and protein kinase coexist, the chicken macrophage HD11 can secrete large amounts of IL-1β. Increasing the cyclic adenosine monophosphate (cAMP) level can also significantly increase the secretion of IL-1β. IL-2 is a class of important lymphatic factors mainly produced by activated T cells. In 1997, a gene was cloned from the complementary DNA (cDNA) library of an activated splenic lymphocyte. Its expression product can promote T cell proliferation, and therefore the gene was identified as ChIL-2. The biological activity of ChIL-2 suggests that it may act as an immune adjuvant to enhance cellular immune responses when it is inoculated together with the vaccine into animals.

IL-4 is a cytokine found in 1982 that has a variety of biological functions. Its molecular weight is approximately 18 kDa. Its main source is $CD4^+$ T cells (Th2),

mast cells, monocytes, and basophils, and IL-4 is more species-specific than other cytokines. To date, few studies have focused on IL-4 in domestic fowls and their biological characteristics. It was first reported in 1986 that ChIL-6 has the effect of hepatocellular-stimulating factor in the acute-phase reaction. IL-8 was found in 1995. With a molecular weight of 18.3 kDa, it had strong ability to induce IFN-r and plays an important role in the immune regulation. ChIL-18 was discovered in 2000 and was cloned from the chicken's macrophage HD-11, while the presence of ChIL-18 mRNA in other tissues and cells still lacks detailed study. Existing studies have shown that chicken IL-1β, IL-2, IL-15, IL-18, IFN-α, and IFN-r have potential to be used as therapeutic agents and vaccine adjuvants. The combinational use of cytokines has been increasingly valued in recent years. Production of IFN-r by combined use of IL-18 and IL-12 is much higher than when they are used individually, suggesting the two have synergistic effect. Compared with chickens vaccinated with tetanus toxoid alone, vaccination of tetanus toxoid together with chicken IL-1 β can improve the level of antibody response. In addition, vaccination of IL-1β, IFN-α, and IFN-r together has a cumulative effect on the tetanus toxoid-induced antibody response.

Interferon

In 1957, a soluble substance with the effect of interfering with the reproduction of viruses was found in chick chorioallantoic membrane and was named IFN. Its molecular weight is 20–34 kDa. The chicken type I IFN gene is similar to that of mammals. There are at least two types of sera type I IFN in chicken, which are named ChIFN1 and ChIFN2. Type I IFN mainly includes IFN-α, IFN-β, IFN-ω, and IFN-T, which can tolerate acid treatment of pH 2.0. They have relevant structures and share the same class of receptors. IFN-α, IFN-β, and IFN-ω are mainly produced in response to virus infection, and can induce the production of antiviral proteins (Ivan et al. 2001). Chicken type II IFN is also known as immune IFN, namely IFN-r (lymphocyte IFN), which is produced by activated T cells under the action of inducers, viruses, bacteria, and so on, and is sensitive to acid. It is mainly involved in induction of MHC expression and immunomodulation, its antiviral effect is weaker than type I IFN, and it is the main macrophage-activating factor of animals.

Other Cytokines

Transfer factor (TF) is an extract of splenic leukocyte (and can be extracted by dialysis). The delayed hypersensitivity reaction transferred is antigen specific. So far, the immune protection against *Eimeria tenella* has been successfully achieved by transferring TF from chickens (Cui 2015; Ivan et al. 2001). Macrophage migration inhibition factor (MIF) is produced after activation of the cells in the spleen, thymus, and bursa of Fabricius. The MIF in fowls is species specific, and cross-reaction between chickens, turkeys, and geese cannot occur. MIF can inhibit the random movement of macrophages, which is beneficial in allowing cells to stay and accumulate in the area of inflammation and enhances the function of phagocytosis and sterilization. Similar factors that can affect the movement of avian immune cells also include eosinophilic migration factor (EMF), monocyte chemotactic factor

(MCF), lymphocyte migration inhibition factor (LMIF), bone marrow monocyte growth factor (MGF), transforming growth factor (TGF)-β, IL-6, tumor necrosis factor (TNF)-α, and thrombocyte inhibition factor (TIF).

Summary

Each component of the avian cytokines is closely related through mutual inducement, mutual regulation of receptor expression, mutual functional restriction, as well as mutual synergic action, constituting a special network system that plays a significant role in maintaining the balance of body's immune system and exertion of normal function (Cui 2015; Ivan et al. 2001).

Immunological Characteristics of the African Ostrich

Native to the desert, the African ostrich belongs to the class Aves, order Struthioniformes, and family Struthionidae (Figs. 11 and 12). In animal evolution, the African ostrich is closely related to the American rhea. Ostriches and elephant birds all belong to the most primitive and oldest ratite lineages, ranking them as having low evolutionary status (Hui et al. 2012). The ostrich is a large herbivorous animal, with strong fecundity, a high meat production rate, resistance to coarse feed, low cost of feeding, strong adaptability, and resistance to disease. The history of intensive farming of ostriches by humans is much shorter than that of other animals. Studying the ostrich is difficult because it is tall, has a wide range of activities, and

Fig. 11 Adult African ostriches in Xi An, China

Fig. 12 African ostrich chicks in Zhengzhou, China

a strong, wild character; therefore, current research on ostrich immunology requires intensive effort. This section describes the current knowledge on the morphological and immunological characteristics of ostriches.

The major immune organs of African ostriches are the thymus, bursa of Fabricius, and spleen. There are significant differences between African ostriches and other animals in the shape and structural characteristics of their immune organs. For example, in ostriches there are densely distributed papillary protrusions on the surface of folds of the bursa of Fabricius. There is also a fibrous structure between the ellipsoid in the spleen and the surrounding lymphoid tissue, which has not been reported in the spleen of other fowls (Hui et al. 2012).

Thymus

Morphological Structure

The thymus of the African ostrich is pale yellow in color and located on both sides of the back neck, along the lateral ventral part of the neck and extending to the front of the first rib. It is watercress-shaped without obvious lobes. On both sides of the thymus the size and the number of lobules are different. The volume reduces gradually from the front to the back. There are two to four lobes on the left side, weighing about 2.38 g; on the right side there are one to five lobes, weighing about 2.41 g. The back of the thymus is closely associated with the thyroid gland and the parathyroid gland and even penetrates into them (Fig. 13). The number of thymus lobes in African ostriches is less than that in other domestic fowls.

The surface of the thymus is covered with a connective tissue capsule which extends into the lobes to form small and obvious lobular septa and divides the lobes

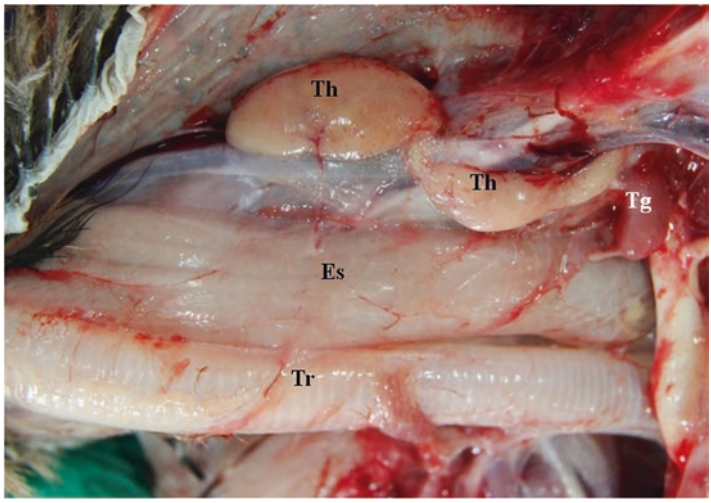

Fig. 13 Back neck dissection of an ostrich chick. Es esophagus, Tg thyroid gland, Th thymus, Tr trachea

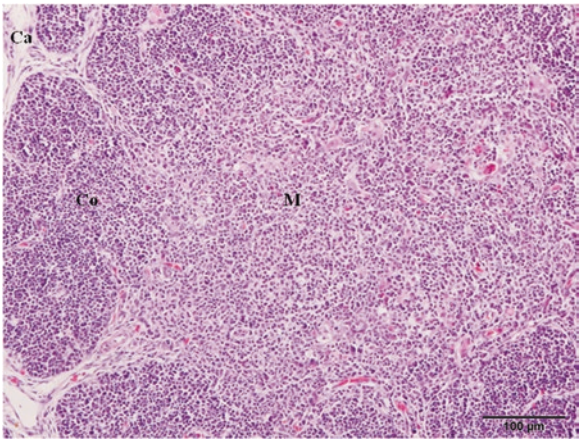

Fig. 14 Thymus microstructure of the ostrich chick. Ca capsule, Co cortex, M medulla

into several lobules of various sizes. Thymic lobules are formed by the stent of the epithelial reticular (ER) cells, and are filled with lymphocytes, macrophages, mast cells, and plasma cells. The lobule is divided into the cortex and medulla (Fig. 14). The cortex is located in the periphery with large number of lymphocytes and stains darker. ER cells are relatively fewer with a larger size. The cytoplasm of ER cells stains pale and the nucleus is round or oval (Fig. 15). The central portion is the medulla, which has more ER cells and reduced lymphocytes. The medullae of adjacent lobules are confluent with each other and stain much lighter. There are Hassall corpuscles in the medulla (Fig. 16).

Under the electron microscope, the nucleolus of the thymic lymphocyte is obvious. The cytoplasm contains rough endoplasm reticulum (RER), free ribosomes,

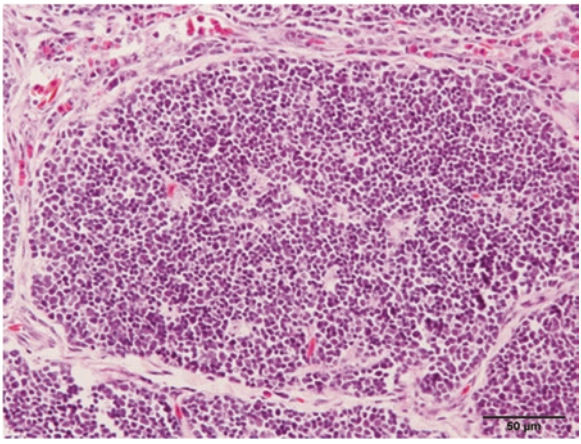

Fig. 15 Thymus lobule of the ostrich chick

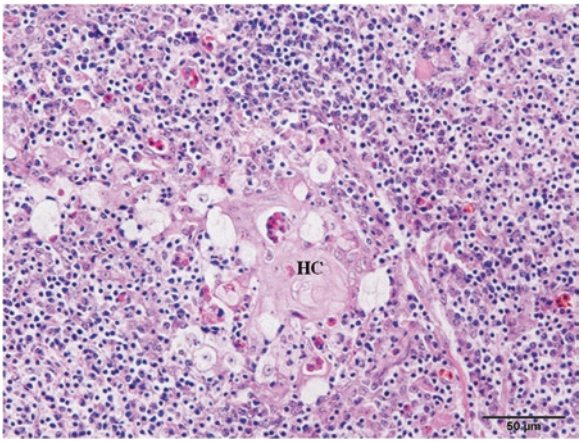

Fig. 16 Thymus medulla of the ostrich chick, showing Hassall corpuscles

and round or oval mitochondria. The nuclear membrane is bilayer with obvious nuclear pores. There are large numbers of autosomes within the nucleus, which belongs to a low electronic density zone. The heterochromatins are distributed along the nuclear membrane (Fig. 17).

Immune Functions

The thymus is a central (primary) lymphoid organ, and produces and nurtures T lymphocytes; it plays a central role in cellular immunity. In the thymus, T cells differentiate and develop when they move from the cortex to the medulla. T lymphocyte precursors run into the thymus, where they are differentiated and selected. In the process of differentiation, the thymocytes that can bind with the self-antigen or MHC antigens are incompatible thymocytes (approximately 95%) and are inactivated or eliminated. Only a few cells can continue to differentiate into mature T cells. Regarding the histological structure, in the sexual immaturity stage the thymus of the African ostrich has a larger proportion of cortex than of medulla, and this may ensure that there are more T lymphocytes for thymus selection. Thymic

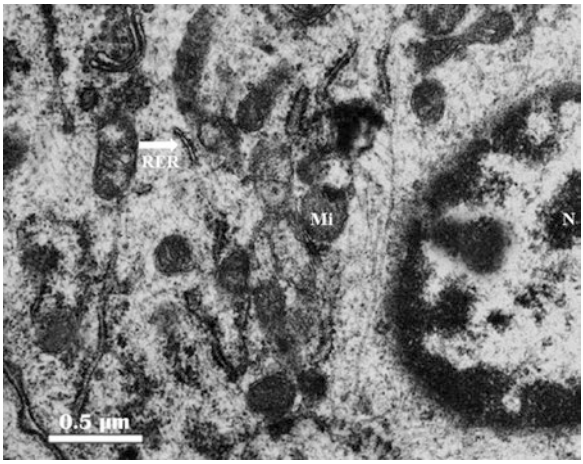

Fig. 17 Lymphocyte altrastructure of ostrich thymus. Mi mitochondria, N nucleus, RER rough endoplasm reticulum

corpuscles are found in the thymus of many animals. It is generally believed that thymic corpuscles play an important role in the selection of T lymphocytes in thymus development and have the function of localizing antigens and dissolving dead lymphocytes and cell debris. So, thymus that lacks corpuscles cannot perform normal primary functions. The major type of thymic corpuscle in the African ostrich thymus is cystic thymic corpuscles, but concentric round thymic corpuscles can also be observed. The presence of thymic corpuscles indicates that ostriches have a more significant development in structure and function of the thymus than other birds (Min et al. 2014).

Effect of Boron on Gene Expression Related to Growth and Development of Ostrich Chick Thymus

Foxn1 is necessary for the development of thymic epithelial cells, and *BMP2* and *BMP4* are associated with regulation of T cell growth and development. All of these genes are important for maintaining the thymus homeostasis. The relationship between boron and thymus development, and effects of boron on the expression of *Foxn1*, *BMP2*, and *BMP4* were investigated in a recent study (Ke et al. 2015). The histological changes in thymus were observed by HE staining. Ostrich *Foxn1* was sequenced using the rapid amplification of cDNA ends (RACE)–polymerase chain reaction (PCR) method. The expression of *Foxn1* was analyzed using immune-histochemistry and western blot, and the expression of *BMP2* and *BMP4* were analyzed using an immunofluorescence technique. The TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) technique was used to label the apoptotic cells in ostrich thymus. The expression of cleaved caspase-3 was detected by immunohistochemistry (IHC). The mRNA levels of *Foxn1*, *BMP2*, and *BMP4* were detected using quantitative real-time PCR (qRT-PCR). The results were as follows:

1. The effect of boron on the morphological structure of the ostrich chick thymus was detected using HE staining. As compared with a control group, thymic

cortex was histologically characterized by karyorrhexis of lymphocytes with active phagocytosis by macrophages, which created a prominent "starry-sky" appearance of the thymic cortex in boric acid (BA)-supplemented groups. Lymphocytes inside the thymus lobules were reduced and exhausted, and thymus lobules were indistinct, especially in the boric acid concentrations of B320 and B640 are 320 mg/L and 640 mg/L; The boric acid concentrations in groups III and IV are 80 mg/L and 160 mg/L. The structure of the thymus was changed, as the numbers of thymic epithelial cells decreased and the cortical and medullary compartments were disrupted in the B640 group.

2. To elucidate the effect of boron on cell apoptosis in thymus, apoptosis was detected using the TUNEL assay and IHC. TUNEL-positive cells were mainly localized in the thymic medulla and faintly in the thymic cortex. The control, B80, and B160 groups had low levels of TUNEL-positive cells, while in higher dosages of groups there was increased TUNEL staining, confirming more cell apoptosis with increasing dosage of boron.
3. The cleaved caspase-3 positive signals were markedly increased in the B320 and B640 groups, indicating that a high dose of boron could activate the cleaved caspase-3 and induce apoptosis in ostrich thymuses.
4. The sequence of the conservative middle segment of ostrich *Foxn1* was 1477 bp using the degenerate primer, and the sequences of the 5′ RACE and 3′ RACE of ostrich *Foxn1* were 384 bp and 1050 bp, respectively, by RACE-PCR analysis. The full-length sequence and encoded protein of ostrich *Foxn1* were 2736 bp and 654 amino acids, respectively. Ostrich *Foxn1* is highly conserved compared with other species, sharing a 92.1%, 91.1%, 90.8%, 89.6%, 88.1%, and 83.5% identity with the peregrine falcon, saker falcon, budgerigar, rock pigeon, mallard, and chicken, respectively.
5. *Foxn1*-positive cells were mainly distributed in the thymic medulla, and much less in the thymic cortex. *Foxn1*-positive signals were increased markedly in the B80 group, whereas *Foxn1*-positive signals were significantly decreased in the B640 group.
6. There was a dose-dependent effect of boron on the mRNA levels of *BMP2* and *BMP4* in ostrich thymuses. The mRNA levels were significantly increased in the B80 group, whereas the mRNA levels were decreased markedly in the B640 group.

These results demonstrated that an appropriate dose of boron played a protective role in thymus development, while a high dose of boron could damage the organ or even produce a toxic effect (Hui et al. 2012; Xin et al. 2014; Ke et al. 2015; Ke 2016). A high dose of boron induced thymus structural disruption, thymic cell apoptosis, enhanced the expression of cleaved caspase-3, and, finally, inhibited the growth and development of ostrich thymus. An 80 mg/l dose of boron moderately increased the mRNA and protein levels of *BMP2*, *BMP4*, and *Foxn1* in the ostrich chick thymus. These factors could promote the differentiation and development of thymic epithelial cells, and are involved in T cell growth and development, which enhance the ostrich chick body immunity. However, a high dose of boron not only

significantly inhibited the mRNA and protein levels of *BMP2*, *BMP4*, and *Foxn1*, but also resulted in the destruction and degeneration of the ostrich thymus histological structure, as well as disruption in the cortical and medullary compartments, ultimately leading to the inhibition of the growth and development of thymic epithelial cells (Ke et al. 2015; Wang 2014).

RNA Sequencing Analysis on the Ostrich Chick Thymus Response to Boron

A study was conducted with the objective of constructing a RNA sequencing (RNA-Seq) tag profile to identify genes and pathways potentially related to immunity in the ostrich chick. Boron exposure induces an immune response in ostrich, but the underlying mechanism is not completely clear yet (Haibo et al. 2015). Thus, transcriptomic data for ostriches are needed as an important resource to identify genes and gain insights into the function of boron on the immune response of thymus (Ke et al. 2015). RNA-Seq analysis was performed using the Illumina technique to investigate differentially expressed genes (DEGs) in ostrich thymuses treated with different concentrations of BA (0, 80, and 640 mg/l). The results of this study are as follows:

1. The raw reads in each library (control, B80, and B640) were 3.22, 3.43, and 3.20 billion. After filtering the low-quality tags, the total number of clean reads in each library was 3.175, 3.380, and 3.158 billion, which corresponded to 98.61%, 98.60%, and 98.65% of raw reads.
2. In the control, B80, and B640 libraries, 11.93, 12.85, and 11.41 million reads were uniquely mapped to the ostrich genome (corresponding to 75.14%, 76.04%, and 72.28%).
3. 72%, 71%, and 69% of the expressed genes exhibited a sequencing coverage of 90–100% in the control, B80, and B640 libraries.
4. The variations in gene expression were analyzed by comparing the control with the B640 group, the B80 with the B640 group, and the control with the B80 group. A total of 2044 genes, including 1816 downregulated and 228 upregulated genes, were identified in the B640 group compared with the control group. The total number of DEGs was 1085, of which 863 were downregulated and 222 were upregulated in the B80 group when compared with the B640 group. There were 902 genes, of which 593 were downregulated and 309 were upregulated in the B80 group compared with the control group.
5. We classified 6260 DEGs into seven profiles, of which 3807 were clustered into three profiles ($p < 0.05$). Profiles 0, 1, and 3 exhibited significant clustering trends. Profile 0 represented genes for which the expression consistently decreased with increasing boron concentrations. Profile 1 represented the genes for which the expression initially decreased and then maintained invariability as the boron concentrations increased. Profile 3 represented genes for which expression was initially stable but then decreased. Profiles 0, 1, and 3 contained 1290, 1030, and 1487 DEGs.

6. The KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis revealed that the significantly enriched inflammation- and immunity-related pathways associated with the DEGs were significantly enriched in "cytokine–cytokine receptor interaction", "pathways in cancer", "calcium signaling pathway", "MAPK (mitogen-activated protein kinase) signaling pathway", and "regulation of actin cytoskeleton".
7. The KEGG enrichment analysis between the paired samples (control–B80, B80–B640, control–B640) showed that boron could also regulate the Toll-like receptor (TLR) signaling pathway, B/T cell receptor signaling pathways, and apoptosis pathway (Ke et al. 2015; Min et al. 2014).

Results from this study provide comprehensive gene expression information at the transcriptional level that enhanced our understanding of the molecular mechanisms of boron on the ostrich immune system. These results demonstrated that boron could regulate the ostrich thymus immunity mainly through the MAPK signaling pathway, calcium signaling pathway, B/T cell receptor signaling pathways, apoptosis pathway, and pathway in cancer.

Regulatory Effect of Boron on Immunity, Inflammation, and Growth Development in the Ostrich Chick

MAPK Signaling Pathway

In the B80 group, the expression of specific genes was increased, whereas others remained unchanged compared with the control group. Of the 27 DEGs, 24 exhibited downregulated trends, of which three exhibited upregulated trends in the B640 group. Boron could regulate the Ras/ERK (extracellular signal-regulated kinase), JNK (c-JUN N-terminal kinase), and p38MAPK signaling pathway; however, we did not observe any genes that were differentially expressed in the ERK5 signaling pathway. The western blot results showed that the ostrich thymuses in the 80 mg/l of BA group exhibited increased expression levels of p-ERK, p-JNK, and p-p38 compared with the control group, and the expression levels of p-ERK, p-JNK, and p-p38 were gradually attenuated in a boron dose-dependent manner after 80 mg/l, reaching the lowest levels at 640 mg/l. The immune response to boron is mediated by the MAPK signaling pathway in the ostrich thymus.

Calcium Signaling Pathway

There was an enrichment in 11 DEGs in the calcium signaling pathway. Boron mainly regulated activity of calcineurin (CaN) and calcium/calmodulin-dependent protein kinase (CaMK). Boron regulated two subunits (PPP3CA, PPP3R1) of CaN, but mainly regulated activity of PPP3R1. In addition, boron could regulate downstream transcription factors NFAT (nuclear factor of activated T cells) and MEF2C and was involved in calcium–calcineurin–NAFT signaling pathway.

B/T Cell Receptor Signaling Pathway

There was an enrichment in seven DEGs in the B cell receptor signaling pathway, and 11 DEGs in the T cell receptor signaling pathway. B/T cell receptor signaling

pathways could mediate activity of regulation of the actin cytoskeleton pathway, MAPK signaling pathway, CaN-NFAT signaling pathway, and PI3K-AKT signaling pathway. All of these pathways interact with each other to regulate ostrich immunity. According to the KEGG enrichment and real-time PCR (RT-PCR) results, boron could regulate activity of the PI3K kinase and was involved in PI3K-AKT signaling pathway. Boron regulated activity of B/T cell receptor signaling pathways that are closely related with the activity of MAPK, calcium and PI3K-AKT signaling pathways

Toll-Like Receptor Signaling Pathway

There were 11 DEGs enriched in the TLR signaling pathway. Boron not only regulated the myeloid differentiation primary response gene 88 (MyD88)-dependent signaling pathway but also regulated the MyD88-independent signaling pathway (TLR3 signaling pathway). Moreover, high-dose boron negatively regulated the TLR signaling pathway, which could inhibit the activity of TLRs and induce the ostrich immune function.

Heat Shock Proteins (Hsp)

Heat shock proteins (Hsp) have a close relationship with apoptosis, as they are anti-apoptosis proteins. Hsp70 is the typical member in the Hsp family, and Hsp40 is a co-chaperone of Hsp70. High-dose boron significantly inhibited the protein levels of Hsp70 and Hsp40 in ostrich chick thymuses. Low levels of Hsp70 and Hsp40 could inhibit the anti-apoptosis effect and the immune response ability in ostrich.

Apoptosis and Cancer Pathways

Boron mainly regulated the TNF-related apoptosis-inducing ligand (TRAIL)-induced extrinsic apoptosis signaling pathway, and regulated the activity of the anti-apoptosis proteins FLIP (Fas-associated via death domain-like IL-1β-converting enzyme-inhibitory protein) and IAP (inhibitor of apoptosis protein). Boron had little effect on the caspase family; however, it could regulate the pathway in cancer, mainly by regulating the Wnt, PI3K-AKT, MAPK, and cytokine–cytokine receptor interaction signaling pathways in the cancer pathway. Regulation of these signaling pathways by boron is an elaborate and complex process—all of these pathways were likely to be involved in cell proliferation, differentiation, and apoptosis in ostrich chicks (Ke 2016).

Bursa of Fabricius

Morphological Structure

The bursa of Fabricius in the African ostrich is located above the cloaca, and its shape and structure are quite different to that of other birds (Hui et al. 2012; Peng 2016a, b). The bursa of Fabricius in ostrich chicks is cystic, but does not form an independent sac, with a blind end in the front (Keli et al. 2015). The posterior part forms a cloaca roof and the dorsal part is covered with the rectal coccygeal muscle (Fig. 18). The inner wall surface of the bursa of Fabricius has 15–19 folds of

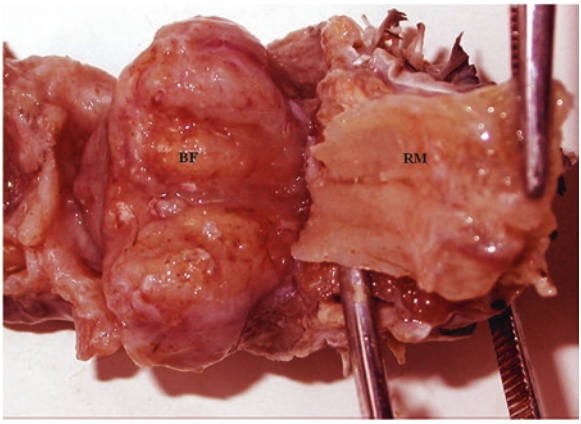

Fig. 18 Dorsal view of ostrich bursa of Fabricius. BF bursa of Fabricius, RM rectococcygeal muscle

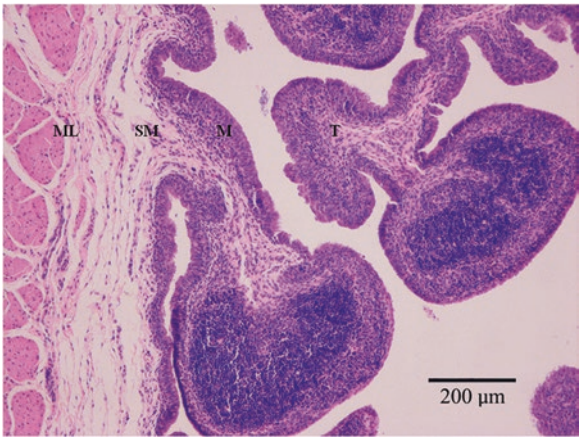

Fig. 19 Microstructure of the ostrich bursa of Fabricius. M mucosa, ML muscle layer, SM submucosa, T tuber

different sizes, showing the valve lobules. The surface of the folds has dense papillae (Fig. 19).

The wall of the bursa of Fabricius consists of the mucosa, submucosa, muscular layer, and outer membrane. The mucosal layer is comprised of the mucosal epithelium and lamina propria, and the mucosal epithelium is composed of columnar cells. In the base of the bursa of Fabricius is a pseudostratified columnar epithelium, which migrates to the top and becomes a monolayer. There is a single bursa of Fabricius in the inner layer of the surface of each nipple. The bursa is composed of three regions: the outer part, or the cortex, is relatively thin with dense lymphocytes, a small number of ER cells, macrophages, and is darkly stained; the inner zone, or the medulla, is more developed and is the thickest zone, with a large number of lymphocytes and a small amount of reticular cells and macrophages—staining of this zone is also dark; and the middle area between the cortex and medulla is thick and is mainly composed of undifferentiated epithelial cells—cells are arranged loosely and stained lightly (Fig. 20). The submucosal layer is thin loose connective

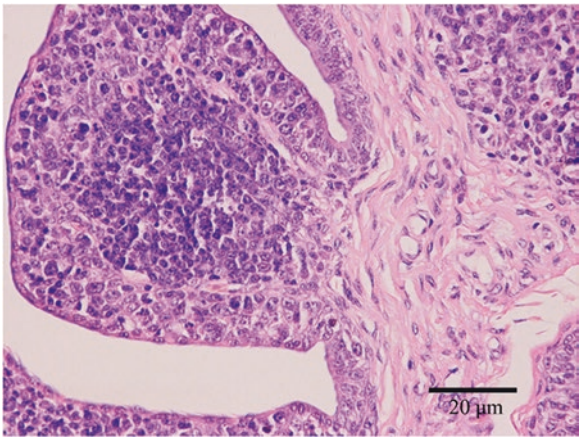

Fig. 20 Bursa of Fabricius nodule in the ostrich chick

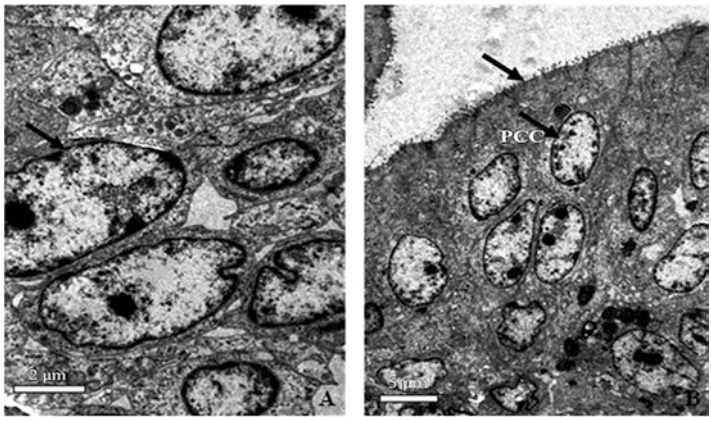

Fig. 21 Ultrastructure of the bursa of Fabricius: (**a**) mucosal epithelium; (**b**) microvilli. PCC ciliated columnar cell

tissue rich in blood vessels. The muscular layer is well-developed. The outer membrane is a fibrous membrane (Peng 2016a, b).

Under an electron microscope, lymphocytes of the bursa of Fabricius can be seen to be mostly immature cells with abundant chromatin in the nuclei. The endothelial reticulocyte within the nodule of the bursa of Fabricius is star-shaped and has protuberances. These endothelial reticulocytes are connected to each other by desmosomes between the cells or protuberances. The mucosal epithelial cells are columnar with microvilli on the surface, and there are lysosomes and mitochondria in the cytoplasm of the columnar cells (Fig. 21).

The nodules of the bursa of Fabricius in the African ostrich are well-developed. In chickens, the bursa of Fabricius is oval with 12–14 vertical folds; it is cylindrical in ducks and geese and spherical in pigeons. The bursa wall in ducks has only two

folds, while in oriental white storks it is composed of long, oval-shaped blind sacs. The bursa of Fabricius folds of chickens, ducks, geese, pigeons, oriental white storks, and other species have a smooth surface with no nipple distribution. Under a microscope, the ostrich's bursa of Fabricius nodules were found to be in a single distribution, located in the inherent layer of the nipple's fold surface. The bursa of Fabricius nodules of chickens, ducks, geese, pigeons, and other poultry are gathered in the inherent layer of the folds. The folds of the papillary surface in the ostrich bursa of Fabricius can greatly increase its surface area. The more folds the ostrich bursa of Fabricius has, the more nodules it contains. This is one of the reasons that young ostriches have stronger immunity than other birds (Peng 2016a).

Immune Functions of the African Ostrich Bursa of Fabricius

Differentiation and maturation of B cells occurs in the bursa of Fabricius. The pluripotent stem cells from the bone marrow move to the bursa of Fabricius via the blood circulation and differentiate into mature B cells in the microenvironment of the bursa of Fabricius in the presence of bursin. Mature B cells leave the bursa of Fabricius and circulate with the blood, and then settle in the specific parts of the spleen and lymph nodes and continue to proliferate. B cells can be induced in plasma cells to produce antibodies by antigen stimulation, and have a humoral immune effect. A similar selection process to that of the T cells in the thymus also occurs in B cell differentiation and maturation. Most B cells cannot be differentiated into mature B cells. Only a small number of cells survive as a result of appropriate gene recombination, and all B cells that can respond to their own antigen are eliminated (Peng 2016a; Cui 2015; Xiao et al. 2014).

Analysis of Gene Cloning and Gene Sequences in African Ostrich *BAFF*

In our study, primers were designed by consulting other B cell-activating factor (*BAFF*) gene sequences published in GenBank (Keli et al. 2015; Cui et al. 2012; Baek et al. 2012; Guan et al. 2007). Total RNA was extracted from the bursa of Fabricius of the African ostrich as a template and the *BAFF* gene of the African ostrich was obtained using RT-PCR. After sequencing, homology analysis and phylogeny tree analysis of *BAFF* were conducted using the MEGA version 5.05 program and Signal P 4.1 server software. Results showed that the nucleotide sequence and the encoded protein were 867 bp and 288 amino acids in length, which contains a predicted transmembrane domain (TMD) of 23 amino acids and a putative furin protease cleavage site. As determined using BLAST (Basic Local Alignment Search Tool), *OsBAFF* shows 52% to 49% amino acid sequence identities with BAFFs from chickens (*chBAFF*), quail (*qsBAFF*), ducks (*dBAFF*), geese (*gBAFF*), and doves (*doBAFF*). Phylogenetic analysis showed that the phylogenetic tree was divided into three different branches, with one containing all bird, one containing all mammalian, and the other all fish proteins, and *OsBAFF* was clustered with birds, being separated from other vertebrates. These results set reference values for the study of *BAFF* from the viewpoint of gene evolution and molecular biology in birds (Fu et al. 2009).

Prokaryotic Expression

Based on the complete *OsBAFF* sequence mentioned in section "Analysis of Gene Cloning and Gene Sequences in African Ostrich *BAFF*", two primers of Os-F and Os-R, which contain EcoR I and Hind III restriction sites, were designed to amplify the soluble part of *OsBAFF*. After being digested via EcoR I and Hind III, the PCR product was cloned into the pET-28a vector, forming a sequence encoding a fusion protein composed of *OsBAFF*. The pET-28a–OsBAFF plasmid was transformed into *Escherichia coli* Rosset (DE3) expression strain, and soluble *OsBAFF* was optimally expressed and purified by Ni Sepharose®. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting showed a single band of recombinant OsBAFF which appeared to have a molecular weight of approximately 32.2 kDa (Genovese et al. 2013; Vincent 2012; Mackay and Schneider 2009).

Tissue Expression

mRNA expression of the African ostrich *BAFF* gene in 18 types of tissues—muscle, esophagus, stomach, small intestine, large intestine, pancreas, trachea, lung, testis, ovary, bone marrow, bursa of Fabricius, spleen, thymus, liver, kidney, heart, and brain—was analyzed using RT-PCR. The results showed that OsBAFF mRNA was widely expressed in most of the tested tissues except heart and brain, and its expression profiles varied among different tissues. High levels of OsBAFF expression were detected in the bursa of Fabricius, thymus, spleen, and bone marrow; moderate levels in liver, lung, kidney, trachea, stomach, and intestine; and low levels in the other tested tissues. Different expression levels of the African ostrich *BAFF* gene indicate that its main functions in these tissues were different (Xiao et al. 2014; Genovese et al. 2013; Mackay and Schneider 2009).

Biological Activity

BAFF in mammals and birds is a survival factor for B cells. To test whether this also holds true for the African ostrich system, bursal lymphocyte cells from the African ostrich were isolated and treated with OsBAFF. Freshly isolated lymphocytes from African ostrich bursa were cultured with purified OsBAFF or OsBAFF + 2 μg/ml phorbol myristate acetate (PMA) for 24 h.WST-8 assay revealed that the numbers of living cells were higher in OsBAFF-treated cultures than in the control. A dose-dependent response to OsBAFF treatment was observed. A dose-dependent response to OsBAFF or OsBAFF +2 μg/ml PMA treatment was clearly observed, and saturation was reached if used at 12 μg/ml OsBAFF or 16 μg/ml OsBAFF + 2 μg/ml PMA. The negative control phosphate buffered saline (PBS) and Bovine serum albumin (BSA) had no survival effect on bursal B cells. Results from mouse B cells showed that OsBAFF could stimulate the survival/proliferation of mouse B cells in vitro and its effects under different doses were similar to the results in African ostrich bursal lymphocytes. The results following LPS stimulation showed that the expression levels of OsBAFF were higher in African ostrich bursal lymphocyte cells after 6, 12, 24, and 48 h LPS treatment. mRNA expression increased from 6 h and upregulated sharply to a maximum at 24 h, and remained at a high level until 48 h

($p < 0.01$) compared with the control group (Keli et al. 2015; Genovese et al. 2013; Vincent 2012).

Effects of Boron in Drinking Water

Effects of boron on the tissue expression of the *BAFF* gene in the African ostrich were tested in a recent study. Forty-eight 1-day-old African ostrich chicks were randomly divided into six groups and fed with different concentrations of BA added into their drinking water (0 [control group], 40, 80, 160, 320, and 640 mg/l), which was supplied uninterrupted for 90 days. Tissues such as bone marrow, thymus, bursa of Fabricius, spleen, lung, kidney, liver, and lung were collected at 1, 45, and 90 days after addition of BA. mRNA expression of the *BAFF* gene in African ostrich tissues was analyzed using RT-PCR. The results showed that the tissue expression of *BAFF* gene in African ostriches following addition of boron in drinking water is dose dependent. When the dose of boron was low, expression of the *BAFF* gene in the eight detected tissues increased along with the increased dose of boron. However, when the dose of boron reached a certain level, expression of the *BAFF* gene in the tissues decreased along with the increased dose of boron. For 45-day-old African ostriches, *BAFF* gene expression levels were highest in bone marrow, thymus, spleen, and lung when fed with 160 mg/l of boron, and were highest in bone marrow, thymus, spleen, lung, bursa of Fabricius, liver, and kidney when fed with 80 mg/l of boron. They were all significantly higher than the levels in the control group ($p < 0.05$). The highest level of *BAFF* gene expression in stomach was from the 80 mg/l boron group, and there was no significant difference compared with the control group. Following 90 days of treatment, the highest level of *BAFF* gene expression in bone marrow, bursa of Fabricius, liver, spleen, thymus, and kidney was in the group fed with 80 mg/l of boron, and was highest in lung from the 160 mg/l group. They were all significantly higher than the levels in the control group ($p < 0.05$). *BAFF* gene expression was highest in the stomach when fed with 160 mg/l boron, and there was no significant difference compared with the control group (Keli et al. 2015; Genovese et al. 2013).

Spleen

Morphological Structure of the Spleen

The spleen of the African ostrich is located in the triangular area constituted by the right kidney and the glandular stomach. It is long, bean-shaped, and dark red in color. The ventral side is slightly promontory, and back side concaves to form the hilum where blood vessels, lymphatic vessels, and nerves go in and out. Ostrich spleen at 3 months old is about 48 mm long and 15 mm in cross section. It weighs approximately 7.75 g in females and 7.31 g in males, which is about 0.064–0.122% of the total body weight. Some ostriches have a deputy spleen (Figs. 22 and 23) (Hui et al. 2012).

Using a light microscope, the African ostrich spleen surface is covered with a thin film of capsule formed by the connective tissue and surface serosa. It is approximately 25.0–41.5 μm in thickness and contains several layers of smooth muscle fibers. The connective tissue extends into the spleen to form trabeculae. The primary

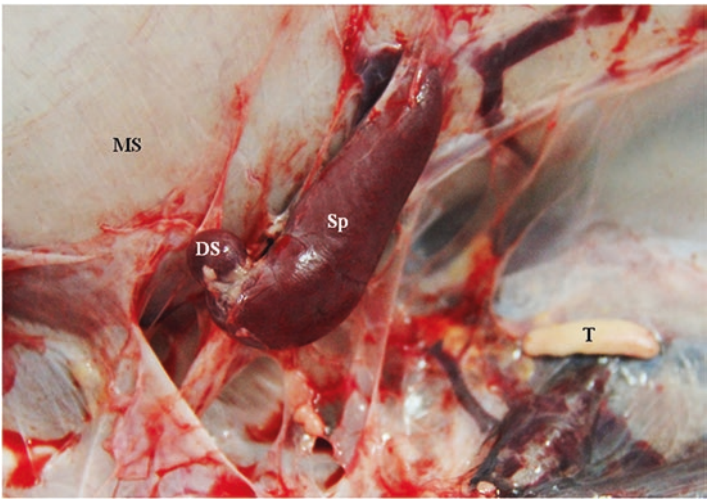

Fig. 22 Location of the ostrich spleen. DS deputy spleen, MS muscular stomach, Sp spleen, T testis

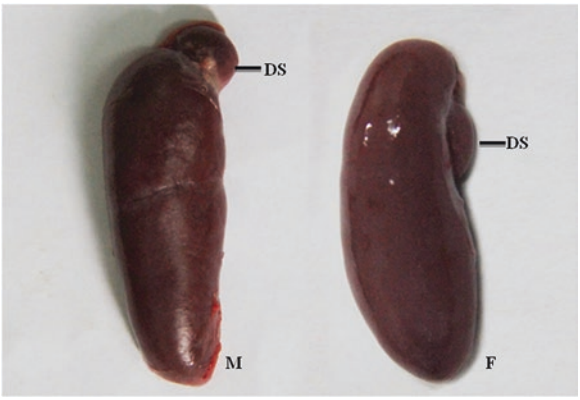

Fig. 23 Shape of the ostrich spleen. DS deputy spleen, F female, M male

trabeculae directly from the capsule are well-developed, rich in smooth muscle and trabecular artery, and continue to branch out as the secondary trabecular. The parenchyma of the spleen consists of mixed WP and RP without a clear boundary distinction between them. WP is composed of the periarterial lymphatic sheath (PALS) and spleen nodules. Lymphatic sheathes around the central arteries are each about 12.5 μm thick, and there are a relatively small number of them. There are few splenic nodules, which are darkly stained and mainly composed of dense accumulated small lymphocytes with a cell diameter of approximately 5.0 μm. There are numerous ellipsoids with a diameter of about 25–45 μm. In the spleen, most are scattered and a few are gathered together. The ellipsoid is also surrounded with lymphoid tissue, named the perielipeoidal lymphatic sheath. This perielipeoidal lymphatic sheath is thick in the African ostrich, approximately 27.5 μm, and there are a greater number

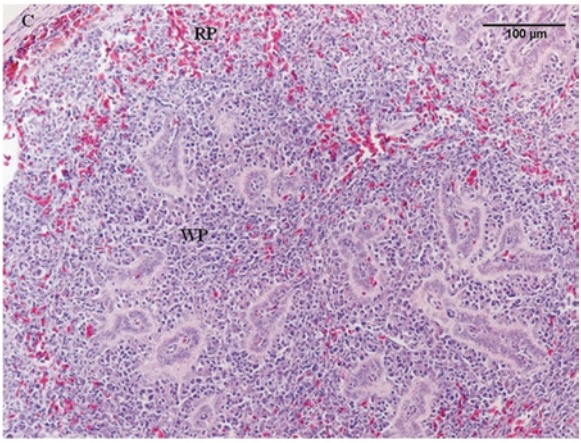

Fig. 24 Microstructure of ostrich spleen. C capsule, RP red pulp, WP white pulp

of them than there are PALS. In the center of the ellipsoid is the capillary with a small lumen. The endothelial cells of the capillary are closely aligned and there are two to four layers of lymphoid cells around the capillary wall. The nucleus of the cells is large, round or oval, and light stained. It is common to observe red blood cells in the lumen of the ellipsoid. There is a collagen fiber sac between the ellipsoid capillary and the surrounding lymphoid tissue. It is approximately 2–3 μm thick, strong eosinophilic stained homogeneously, and is the clear boundary between the ellipsoid capillary and the surrounding lymphoid tissue. This structure has not been reported in the spleen of other animals (Fig. 24) (Song 2007).

The RP of the spleen fills the space between the WPs and is composed of the splenic cord and the spleen sinus. The splenic cords intertwine into a net formed by the clearly visible reticular cells and a sparse number of lymphocytes, which is made up of plasma cells, large and small lymph cells, and many macrophages. The splenic sinus is distributed under the capsule, between the trabeculae and the splenic cord. Sinuses along the capsule and the trabeculae are large, and the cavity is scattered with a large number of blood cells (Fig. 25).

Under transmission electron microscopy, the nucleolus of the lymphocyte is obvious, and the cytoplasm contains many RER and free ribosomes and a small number of round or oval mitochondria. The nuclear membrane is double-layered with obvious pores. The peripheral lymphocytes of the PALS are mostly naive and rich in cytoplasm. The nucleus of these cells is often rich in chromatin (Fig. 26). The nucleus of the capillary endothelial cells is slightly stained, with obvious nucleolus and intramembrane pleats. There are interdigitated protrusions between the adjacent cells, which have scattered mitochondria, RER, free ribosomes, and a small number of vesicles in the cytoplasm. The cells in the outer layer have rich chromatin in the nucleus, and rich RER, free ribosomes, and mitochondria in the cytoplasm. Strong homogeneous eosinophilic material between the ellipsoid capillary and peripheral lymphoid tissues is composed mainly of collagen fibers. The cytoplasm of the reticular cells in the splenic cord mainly contains mitochondria, RER, and some vacuoles. The nucleus of the plasma cell is biased, with a double layer of nuclear

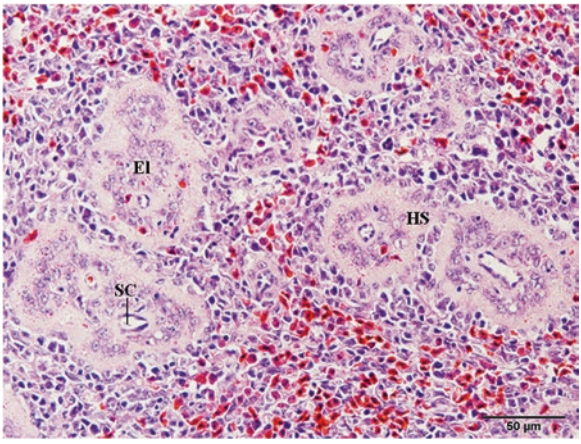

Fig. 25 High magnification of ostrich spleen. El ellipsoid, HS homogeneous structure, SC sheathed capillary

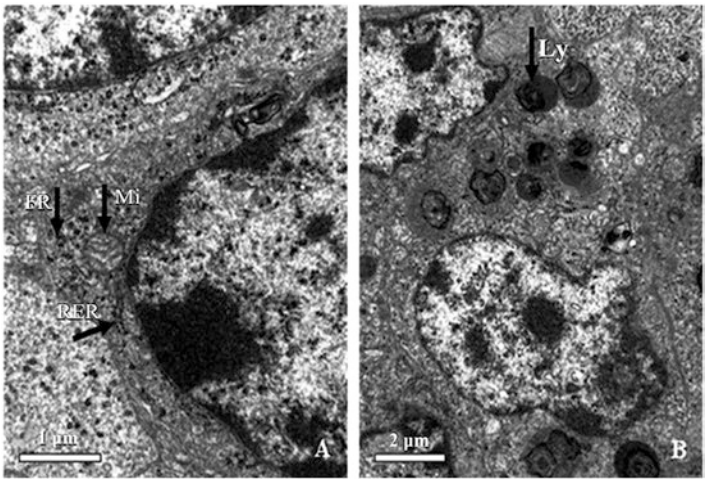

Fig. 26 Ultrastructure of the ostrich spleen: (**a**) lymphocytes; and (**b**) macrophages. FR free ribosome, FP foreign particles, Ly lysosomes, Mi mitochondria, RER rough endoplasmic reticulum

membrane, and the cytoplasm is rich in RER, ribosomes, and a small number of mitochondria. Macrophages are large cells with irregular shapes and surface wrinkles. The nucleus is branched or protruding. The cytoplasm is rich in lysosomes, free ribosomes and RER, and fewer mitochondria. Vacuoles and swallowed foreign bodies are commonly seen in these cells (Fig. 26).

Immune Functions of the Spleen

The ostrich spleen is the largest immune organ of the body. The functions of the spleen include hematopoiesis, blood transfusion, blood storage, immune response, and other important physiological functions. It is the place where the lymphocytes

proliferate and differentiate following antigen stimulation. The morphological structure of the ostrich spleen is different from other fowls. For example, the shape of the ostrich spleen is elliptical, while it is spherical in chickens and triangular in ducks. The African ostrich spleen has a thin capsule and undeveloped trabeculae, with fewer smooth muscles in them. The size and number of ellipsoids varies largely among different animal species. The ellipsoid of the African ostrich spleen is small in size and large in number, and is randomly scattered in the spleen. This feature demonstrates a positive correlation between the amount of smooth muscle cells and the size of the ellipsoids. It also shows a weak ability to store and regulate blood in the African ostrich spleen. The lymphocytes in the ellipsoid stain lightly. The nucleus of the cells is rich in euchromatin and has less heterochromatin. It is mostly located on one side of the cell. The cytoplasm is rich in rough endoplasmic reticulum. The nucleus is purple and the cytoplasm is green when stained with Feulgen–methylene blue. These cells are mainly plasma cells and lymphoblasts, indicating a relatively active stage of differentiation. In addition, there are homogeneous eosinophilic substances between the ostrich ellipsoid capillary and the surrounding lymphoid tissue which are confirmed as collagen fibers using the modified Weigert staining method and electron microscopy. This dense structure is a strong filtering barrier and can effectively filter bacteria, foreign bodies, and antigen and aging blood cells in blood, indicating a stronger immune function in ostrich spleen than in other avian species (Peng 2016a, b; Hui et al. 2012).

Effects of Boron Supplementation

The effect of boron on splenic development and Hsp70 expression levels in ostrich spleen was examined in our study. Thirty healthy ostrich chicks were randomly assigned to six groups, groups I, II, III, IV, V, and VI, and supplemented with BA 0, 40, 80, 160, 320, and 640 mg/l, respectively, in drinking water. The histological structure in spleen was tested using HE staining. The expression level of Hsp70 was analyzed by IHC and western blotting. mRNA expression of Hsp70 was investigated using qPCR. Apoptosis was analyzed using a dUTP–biotin nick end labeling (TUNEL) reaction. The results of this research are described in the following sections.

Spleen Structure

Ostrich spleen is divided into two functionally and morphologically distinct compartments: WP and RP. WP is further composed of two major parts, the PALS and marginal zone (MZ), while RP has blood-filled sinusoids and splenic cords. Histological analysis by HE staining revealed that there was a normal structure of ostrich spleen in group I, with less WP and thick PALS. The boundary between WP and MZ was apparent. There was significant splenic sinusoid with a lot of red cells. Not many differences were observed in group II compared with the findings of group I, although the average area of the WP was slightly increased, with thickened PALS. There was a thinner MZ; however, the boundary between the WP and MZ region could be observed. The number and the volume of WP increased in group III and group IV as compared with group I. The PALS was notably thickened, and

lymphocytes were intensive in the sheath. The WP and MZ were separated with a clear boundary. Overall, the histological structure in these two groups was well-developed compared to group I. Histopathological changes in the experiment groups V and VI were obvious; both the volume and the number of WP were reduced. Particularly in group VI, the splenic structure was atrophied. The WP became fewer and smaller. The number of lymphocytes in WP was reduced, and PALS was thinner. The lymphocytes condensed significantly, and some lymphocytes were sparsely arranged, and the MZ became thinner than group I. The MZ and WP boundary was not clear or missed (Haseeb et al. 2017).

Hsp70 in Chick Spleen

Hsp70 protein expression in ostrich spleen was evaluated using IHC techniques. Hsp70 mostly localized diffusely in the spleen. The expression of Hsp70 in group I and group II did not show many differences in term of localization; however, the distribution pattern of Hsp70 was obvious in group III, and especially in group IV with BA 160 mg/l, when compared with group I. On the other hand, localization of the Hsp70-positive signal started to decline in high-dose boron-treated groups, mainly in group VI (BA 640 mg/l) as compared with group I. The integral optical density (IOD) of positive products revealed a slight enhancement in group II (BA 40 mg/l) but this was not significantly different ($p > 0.05$). The IOD values of Hsp70-positive signals were enhanced in groups III (BA 80 mg/l) and IV (BA 160 mg/l), which were highly significant ($p < 0.01$). However, the IOD of positive products in group V (BA 320 mg/l) revealed an insignificant decrease ($p > 0.05$), but there was a highly significant decrease ($p < 0.01$) in group VI (BA 640 mg/l) compared with group I. These data show that the expression level of Hsp70 changes as the BA concentration changes, indicating a correlation between Hsp70 and BA. The trend of Hsp70 expression as assessed by western blot analysis was similar to that of IHC. As the concentration of boron increased, expression of Hsp70 increased and reached a peak in group IV (BA 160 mg/l). However, as the concentration amount of boron went beyond 160 mg/l, Hsp70 expression levels decreased dynamically, with the least expression in group VI (BA 640 mg/l) (Haseeb et al. 2017; Hui et al. 2012).

mRNA Expression of Hsp70

The mRNA levels of Hsp70 in group II (BA 40 mg/l) were higher than those in group I. The mRNA levels in the group III were significantly higher ($p < 0.01$) and reached a peak in group IV (BA 160 mg/l) ($p < 0.01$). The 160 mg/l concentration of BA can be considered the optimal dose of boron supplementation in drinking water. After that concentration the mRNA level of Hsp70 started to decline and the lowest level of mRNA expression was seen in group VI (BA 640 mg/l) as compared with group I (BA 0 mg/l) ($p < 0.01$). This decline in the expression of the Hsp70 mRNA level indicated that the expression of Hsp70 is dose dependent: the expression is first stimulated at lower doses and then inhibited at higher doses (Haseeb et al. 2017).

Cell Apoptosis

HE staining revealed that the spleen structure atrophied in the ostrich spleen in groups V and VI. Furthermore, IHC revealed that the expression level of Hsp70 decreased dynamically in those groups, with the least expression in group VI. This reduction in expression at the higher dosage of boron may be associated with apoptosis of cells in the ostrich spleen. In order to check this hypothesis, we evaluated the cell apoptosis using the TUNEL method. TUNEL-positive products were basically granule cells of brown color. In groups II (BA 40 mg/l), III (BA 80 mg/l), and IV (BA 160 mg/l), TUNEL positive signals were weaker than in group I, but enhanced in group V (BA 320 mg/l), and especially in group VI (BA 640 mg/l). The TUNEL positive signals IOD, investigated using Image Pro Plus (IPP), also indicated similar and intense differences among the groups. The IOD analysis of groups II–IV showed a lower expression level of Hsp70, with the lowest level in group IV ($p < 0.01$), while the level of IOD was significantly higher in groups V ($p < 0.05$) and VI ($p < 0.01$) than in group I. These results indicated a bi-physic effect of boron on cell apoptosis: reduced apoptosis was associated with the lower concentrations of boron, while apoptosis was boosted with a higher boron concentration (Haseeb et al. 2017; Hui et al. 2012).

When these data are taken together, it is suggested that proper dietary boron treatment might stimulate ostrich chick spleen development by promoting the Hsp70 expression level and inhibiting apoptosis, while a high amount of boron supplementation would impair the ostrich spleen structure by inhibiting the Hsp70 expression level and promoting cell apoptosis (Haseeb et al. 2017; Hui et al. 2012).

The Evolutionary Place and Phylogenetic Analysis of the African Ostrich

Avian Evolution

Birds are one of the strongest surviving animals in the world, and their numbers are widely distributed. Birds represent a very special group of vertebrates: they are related to reptiles but are homoiotherms, and they are not related to mammals that do not suckle their young (Zheng 2002).

Scientists believe that birds evolved from the lizard dragon of the Jurassic era, which had a beak without teeth and lungs and airbags to complete breathing. Its forelegs degenerated into wings for flying. Most of the bones of birds are hollow and they have no bladder, which makes the body lighter. In addition, the bird's chest muscle is highly developed; when it is contracted, the wings can fully fan down in order to obtain lift and thrust, which is conducive to flying (Darrent 2014; Sai 2008).

Like other birds, the ostrich is warm blooded and shows functional dichotomy of the immune system with the presence of the bursa of Fabricius. What is the difference between an ostrich and flying birds? A significant difference is that there is no end of the pectoral muscle fixed on the keel of the sternum. All of the flying birds have developed cariniform, while in African ostriches, American ostriches, Australia's and New Guinea's emu and cassowary, and New Zealand's kiwi birds the sternum

is not cariniform; they are also known as ratite, flat chest birds. The vast majority of birds have an open pelvis but the ostrich's pelvis is closed. In the case of the immune system, many waterfowl have lymph nodes, including ducks, geese, and lanruo, but ostriches have no lymph nodes. In addition, the ostrich has a deputy spleen (Darrent 2014; Song 2007).

Ostriches, reptiles, and the platypus—a monotreme mammal—have similarities in that they reproduce by laying eggs, and do not have an anus, urethra, or birth canal, but have a combined total discharge chamber; they are cloacal. Like most birds, the platypus is hatched by its mother but the hatchling, like other mammals, is raised by breast milk. Charles pointed out that 65 million years ago, after the extinction of dinosaurs, terrestrial ecosystems lost predators and the ground had a wealth of food sources. African ostriches, American ostriches, cassowary, emu, and other flightless birds ancestors would not have had to fly in the air or quickly escape a predators' attack. They would choose to live on the ground, with sufficient food sources to make their body bigger and eventually lose their ability to fly (Charles 2010; Zheng 2002).

Phylogenetic Tree Analysis

Animal evolution is manifested not only at the individual or group level, but also at the molecular level. The evolution of birds is history, and it is impossible to rebuild the history with absolute integrity. However, with the use of molecular biologic techniques to study the origin of bird differentiation, intra- and inter-species system relationships, and the genetic structure and classification of the population, system reconstruction of the birds' history is important (Zhang 2015; Sofia and Hans 2013; Prager 1976).

Translation of the nucleotide sequences into amino acids was performed using DNAMAN software and the nucleotide sequence of the ostrich *Foxn1* gene was compared with the corresponding reported sequences of other species (Ke et al. 2015). The results suggested that the nucleotide sequence of the *Foxn1* gene of the ostrich is highly conserved compared with those of other species reported. The ostrich *Foxn1* gene shares 92% identity with the peregrine falcon, saker falcon, and budgerigar, 91% with the rock pigeon, 88% with the mallard, 86% with the chicken, and so on (Fig. 27a). To characterize the phylogenetic relationships among *Foxn1* genes in these different species, a phylogenetic tree was constructed. The result showed that the ostrich and chicken were in the same group and the relationship between the African ostrich *Foxn1* and chicken *Foxn1* was close (Fig. 27b).

African Ostrich *BAFF* Gene

A phylogenetic tree was constructed using the neighbor-joining method based on the nucleotide sequences of the identified African ostrich *BAFF*, and other *BAFF* genes from chickens, mice, goats, ducks, geese, doves, quail, some fishes such as zebrafish and yellow grouper, and some mammals such as goats, cattle, and dogs (Keli et al. 2015). The data indicated that the African ostrich *BAFF* gene was more closely related to *BAFF* genes from chickens and quail followed by those from ducks, geese, and doves (Fig. 28a). Another phylogenetic tree was reconstructed

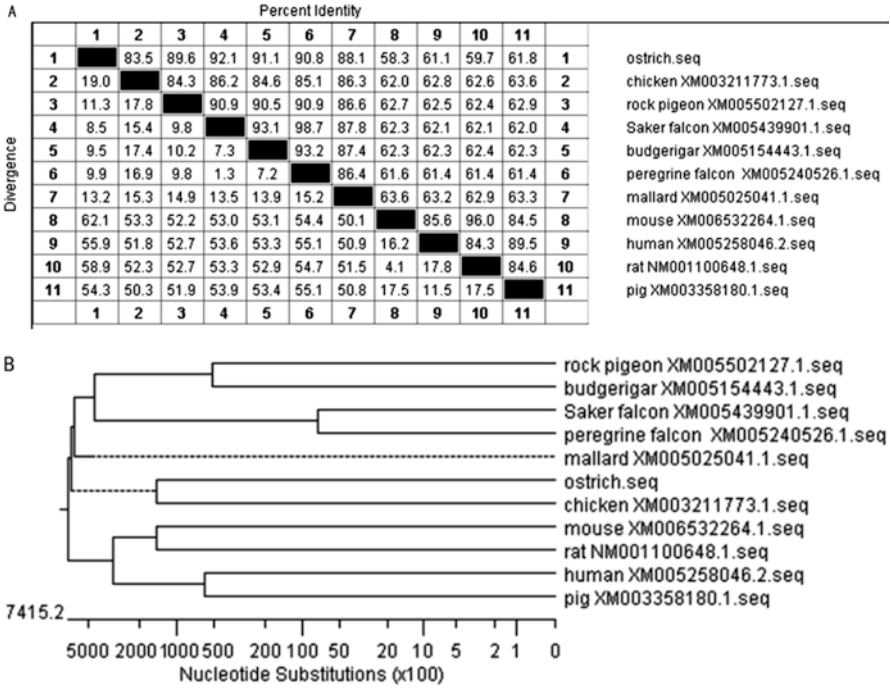

Fig. 27 (**a**) Construct of the homology tree, using MegAlign software. Sequence homologous identity comparison of the African ostrich and other species' *Foxn1*. The GeneBank accessions of *Foxn1* of the other species are listed on the right of the figure. (**b**) Phylogenetic tree constructed using DNAMAN software. Phylogenetic tree analysis of the African ostrich and other species' *Foxn1* based on the nucleotide sequences. The GenBank accessions of *Foxn1* of the other species are listed to the right of the species name

using the NJ is a method, Neighbor joining method and 18 representative vertebrate BAFF proteins. Phylogenetic analysis showed that the phylogenetic tree was divided into two different branches, with one containing all avian and the other all mammalian and fish proteins; OsBAFF was clustered with avian, being separated from other vertebrates (Fig. 28b).

The theory of evolution suggests that if the two sequences have sufficient similarity, it can be inferred that they may be derived from a common evolutionary ancestor, or may be homologous (Wang 2015). The protein sequence and primary structure are more highly conserved than the nucleic acid sequence, so if the similarity between the two protein sequences is greater than 30%, they are likely to be homologous. *BAFF* is highly conserved in evolution and highly homogeneous in birds and mammals. The gene homology of chicken *BAFF* with mouse BAFF was 49%, chicken *BAFF* with human BAFF was 51%, duck *BAFF* with mouse *BAFF* was 43%, and duck *BAFF* with human *BAFF* was 54% (Dan et al. 2008). In recent years, *BAFF* has been of interest to researchers because of its important role in humoral immunity. With the deepening of research, the *BAFF* gene of many

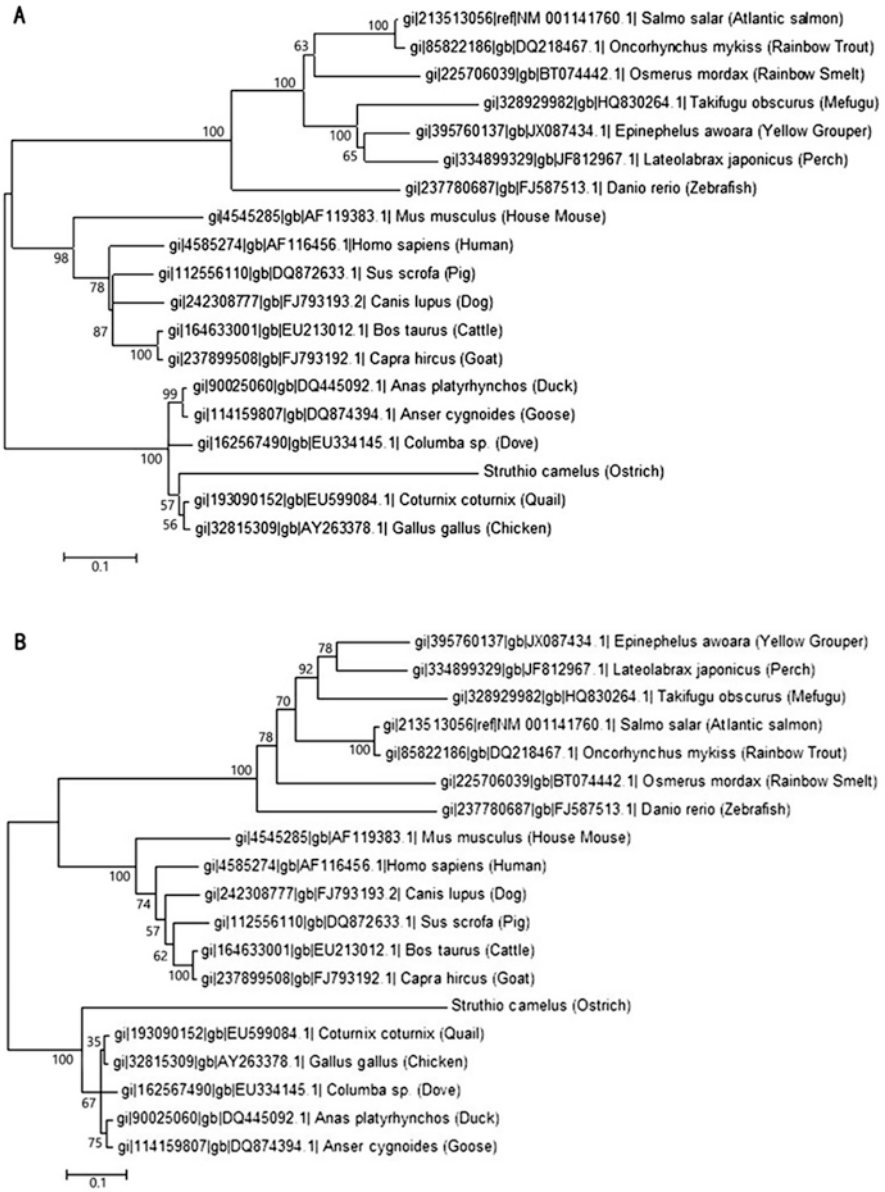

Fig. 28 Phylogenetic analyses of African ostrich and some other species based on nucleotide and amino acid sequences. (**a**) Phylogenetic tree of the African ostrich and other species' B cell-activating factor gene (*BAFF*) based on the nucleotide sequences. (**b**) The relationship between the OsBAFF amino acid sequence and those of the corresponding species as presented in (**a**). The GenBank accessions of *BAFF* from other animals are listed to the right of the species name

animals has been found, which has provided valuable data for the further study, development, and utilization of the *BAFF* gene. Sequence analysis showed that the African ostrich *BAFF* gene sequence was similar to that of other birds, and the gene sequence homology was higher. The phylogenetic tree analysis showed that the *BAFF* gene of the African ostrich was close to that of the chicken, duck, goose, quail, and pigeon *BAFF* genes, and it was far from the *BAFF* gene of mammals and fish (Keli et al. 2015). Among them, African ostrich *BAFF* was closest to the genetic relationship of the chicken and quail, which shown that the *BAFF* gene from the African ostrich and other birds was a common ancestor.

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Mammalia: Chiroptera: Immunology of Bats

Michelle L. Baker and Tony Schountz

Introduction

Bats (order Chiroptera) are a diverse group of nocturnal mammals comprising approximately 20% of all mammalian taxa and consisting of more than 1300 species across 21 families (Simmons 2005). Phylogenetic analysis places bats within the superorder Laurasiatheria, sister to carnivores (e.g., cats, dogs), ungulates (e.g., horses, cows), and cetaceans (e.g., dolphins) (Fig. 1) (Tsagkogeorga et al. 2013). Bats are believed to have diverged from other eutherian mammals approximately 88 million years ago (mya) (Lei and Dong 2016). The traditional classification system divided bats into two suborders: Microchiroptera (microbats) and Megachiroptera (megabats). Microbats are defined by their smaller size (4–16 cm), the use of echolocation, and the use of hibernation during the winter for many species. Megabats consist of the flying foxes (also called fruit bats) and are larger nonecholocating bats (up to 1.6 kg with wingspans of 1.7 m) belonging to the Pteropodidae family. However, more recent phylogenetic analyses based on molecular data have led to a reclassification of bats into the suborders Yinpterochiroptera and Yangochiroptera. The Yinpterochiroptera suborder includes the nonecholocating Pteropodidae family (flying foxes) and the echolocating Rhinolophoidea family, while the Yangochiroptera suborder consists of the remaining echolocating microbats (Teeling et al. 2005, 2016). The two suborders of bats are estimated to have diverged approximately 63 mya (Lei and Dong 2016). Although the new classification has strong statistical

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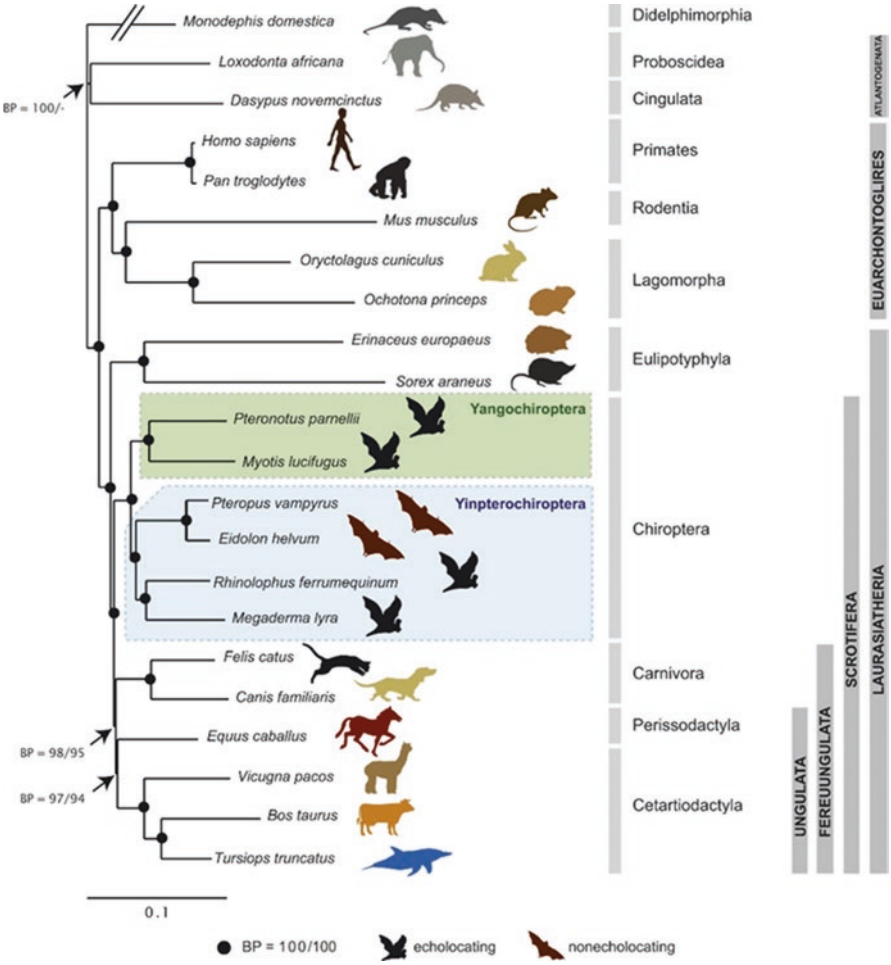

Fig. 1 Phylogenetic relationship of bats to other species. (From Tsagkogeorga et al. 2013 with permission)

support, it remains controversial as it suggests that laryngeal echolocation evolved twice in Chiroptera, once in Yangochiroptera and once in the rhinolophoids (Teeling et al. 2000).

Of all the mammals, bats are the most ecologically diverse. They are the only mammals that have evolved powered flight and have adapted to a variety of environments across all continents with the exception of the polar regions. Their diets are equally diverse, including fruits, pollen, insects, small vertebrates, and even blood, and they play important roles in the ecosystem through seed dispersal, pollination, and insect control. Bats have longer lifespans relative to other mammals, typically living 3.5 times longer than mammals of similar size (Austad 2010). Maternal investment is generally high, with most species giving birth to a single pup per year

and pups averaging approximately 23% of maternal body weight at birth (Barclay and Harder 2003). Curiously, despite their longer lifespans, there is anecdotal evidence that bats are resistant to tumors (Wang et al. 2011). The characteristic that has drawn the most attention in recent decades is their role as natural reservoirs for a variety of viruses that are highly pathogenic in other species yet rarely cause clinical disease in bats. This characteristic in particular has led to renewed interest in the immune systems of bats.

Bats are highly gregarious mammals, with most species living in high-density colonies, providing ideal environments for transmission and maintenance of pathogens within populations. Combined with their frequent movement between roosts, transmission of viruses, bacteria, parasites, and fungi could potentially occur readily between individuals and populations, resulting in a situation of constant pathogen exposure. Approximately 200 viruses have been detected across different bat species, and many of the viruses identified in bats are highly pathogenic in other species, including humans (Moratelli and Calisher 2015); however, they likely host many more (Anthony et al. 2013). Examples include high-profile viruses such as the severe acute respiratory syndrome coronavirus (SARS-CoV), Marburg virus, and Hendra and Nipah paramyxoviruses. These viruses occasionally spill over to other susceptible hosts, causing severe disease and mortality yet causing no disease in bats. The long coevolutionary history of bats and viruses has likely resulted in the establishment of a state of equilibrium, allowing both the viruses and their host to coexist in a disease-free state typical of natural reservoirs.

Bats also host a variety of other pathogens, including parasites, bacteria, and fungi. Unlike viral infections, there are examples of these pathogens causing disease among bats. The fungus that causes white nose syndrome (WNS), *Pseudogymnoascus destructans*, has resulted in mass mortalities among a number of North American microbat populations, with some species now threatened with extinction (Blehert et al. 2009). Evidence for lower fungal loads consistent with the development of resistance to the fungus have been observed in some bat populations, providing hope that selection on immune genes may lead to the development of resistance or tolerance mechanisms (Langwig et al. 2017). However, it is unlikely that this will occur rapidly enough for many affected populations. Several bacterial infections, including tick-borne spirochaete bacteria, *Borrelia* spp., and some enteric bacteria, have also been associated with pathology in bats (reviewed in Brook and Dobson, 2015). Brooks and Dobson (2015) presented evidence that bats may have evolved mechanisms to eliminate intracellular pathogens such as viruses at the expense of their ability to eliminate extracellular pathogens (bacteria, parasites, and fungi) and hypothesize that mitochondrial adaptations may play a role.

In light of the increasing emergence of infectious diseases and the impacts of pathogens such as WNS, deciphering the immune systems of bats has never been more critical and offers potential for identifying novel antiviral therapies and approaches to the conservation of bats threatened by diseases such as WNS. Fortunately, progress in the area of bat immunology is rapidly advancing as new groups enter the field and advances in technology provide opportunities for more rapid discovery. Several reviews that have appeared over the last 5 years have

described the various aspects of the immune systems of bats (Baker et al. 2013; Butler et al. 2014; Schountz 2014; Baker and Zhou 2015; Schountz et al. 2017). In this chapter we provide a broad overview, with a focus on recent highlights in bat immunology and areas for future research.

Immune Tissues and Cells

Although few studies have examined the histology of bat lymphoid tissues, from an anatomical perspective, bats appear to have the majority of primary and secondary lymphoid organs present in other mammals, including thymus, bone marrow, spleen, and lymph nodes (Papenfuss et al. 2012; Zhou et al. 2016b). Bone marrow has been isolated from long bones, including humerus and radius, and from the ribs but appears to be absent in the distal wing bones (Papadimitriou et al. 1996; Zhou et al. 2016b). Notably absent, at least in the species that have been examined to date, are Peyer's patches, which are generally located in the submucosa and lamina propria of the small intestine. No Peyer's patches were present in the horseshoe bat, *Rhinolophus hildebrandtii*, or the common pipistrelle bat, *Pipistrellus pipistrellus* (Strobel et al. 2015; Makanya and John 1994). The submucosa of the intestine of the horseshoe bat was devoid of lymphoid tissue, with the exception of a few aggregations of lymphoid nodules in the rectal submucosa (Makanya and John 1994).

A range of immune cell types also appear to be present in bats. Morphological characteristics have been used to identify lymphocytes, neutrophils, eosinophils, basophils, and macrophages in the Brazilian free-tailed bat, *Tadarida bra*silensis (Turmelle et al. 2010a). Macrophages and T- and B-cell populations have also been identified in the Indian flying fox, *Pteropus giganteus*, based on cellular adherence and scanning electron microscopy (Sarkar and Chakravarty 1991). More recently, the phenotype, morphology, and function of dendritic cells and macrophages have been characterized from bone marrow from the black flying fox, *Pteropus alecto* (Zhou et al. 2016b). Cells resembling follicular dendritic cells (FDCs) have also been described in the Indian flying fox (Sarkar and Chakravarty 1991). Unlike dendritic cells that originate in the bone marrow, FDCs are of mesenchymal origin and are found in primary and secondary lymphoid follicles in B-cell areas of lymphoid tissue. FDCs are essential for high-affinity antibody production and for the development of B-cell memory. They also have the ability to maintain intact antigen for extended periods (van Nierop and de Groot 2002; Heesters et al. 2014). Whether they play the same role in bats remains to be determined but presents an interesting possibility for the maintenance of persistent viral infections.

Genetics and Genomics of Immune System

The lack of species-specific reagents has often been a hindrance to comparative immunologists. However, bat immunology made a resurgence in an age of rapid advances in species-independent approaches such as next-generation sequencing,

proteomics, and gene editing technologies such as CRISPR/Cas9. RNAseq studies on tissues and cells from a variety of different species of bats have provided evidence that bats have nearly all of the major components of the immune system that are present in other mammals, including receptors and molecules associated with innate and adaptive immunity and microRNAs (Papenfuss et al. 2012; Shaw et al. 2012; Cowled et al. 2014). RNAseq data from virus-infected bat cells and WNS-infected bat tissues have also offered insights into the genes associated with host–pathogen responses (Wynne et al. 2014, 2017; Field et al. 2015).

Bat Genomes

To date, partial genome sequences of 14 bat species are available in the NCBI database, providing valuable insights into the evolution of immune genes and essential sequence information for the design of primers and the development of reagents essential for studies of the immune responses of bats. The Bat1K project, which aims to sequence the genomes of the approximately 1300 species of bats, will no doubt provide a valuable resource for comparing the immune repertoire of different species of bats (Teeling et al. 2018).

The genomes of bats are condensed compared to other mammals, ranging from 1.6–3.54 Gb. Smaller genome sizes in both bats and birds have been hypothesized to be associated with the metabolic requirements of flight (Kapusta et al. 2017).

Genomic Characterization of Immune Regions

A number of genomic regions associated with immunity have been analyzed in detail, in particular in the black flying fox (*P. alecto*), using a combination of whole-genome data and additional sequencing. These include regions associated with innate, for example, type I interferon (IFN), and adaptive immunity, for example, major histocompatibility class I (MHC-I) and MHC-II. Consistent with the smaller size of the genomes of bats, these regions are also condensed and contain fewer genes compared with the corresponding region from other mammals (Ng et al. 2016, 2017; Zhou et al. 2016a). For example, the type I IFN locus of the black flying fox is highly condensed and contains fewer IFN genes than any other species sequenced to date (Fig. 2).

The description of the genomes of two divergent bat species, the Australian black flying fox (*P. alecto*) and David's myotis (*Mytois davidii*), provided the first glimpse into unique genetic signatures within immune pathways of bats, lending support to the idea of inadvertent changes in the immune system associated with the evolution of flight (Zhang et al. 2013). These include changes in the genes associated with DNA response/DNA repair pathways that are tightly linked with innate immune pathways (Fig. 3). The DNA damage sensor, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which is also part of the cytoplasmic microbial nucleic acid sensing complex, was among the genes that have undergone selection in bats

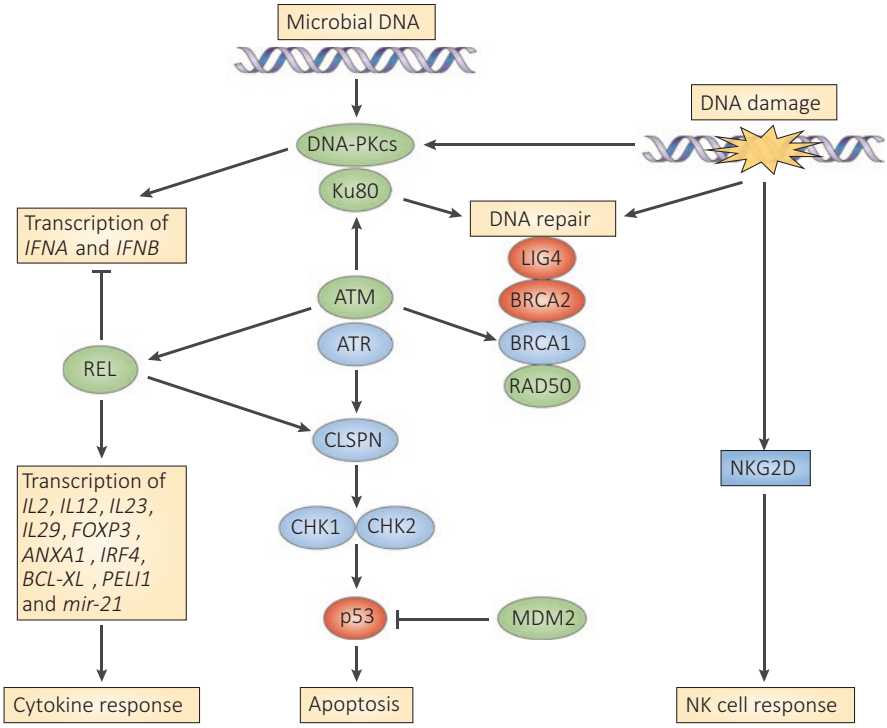

Fig. 2 DNA repair/immune pathway. Whole-genome analysis of two bat species (*Pteropus alecto* and *Myotis davidii*) showed that a high number of genes encoding components of these pathways are positively selected. Many of these genes are positively selected in both species (highlighted in green), whereas others have been positively selected in only one of the species (these encode proteins highlighted in red). (From Bean et al. 2013 with permission)

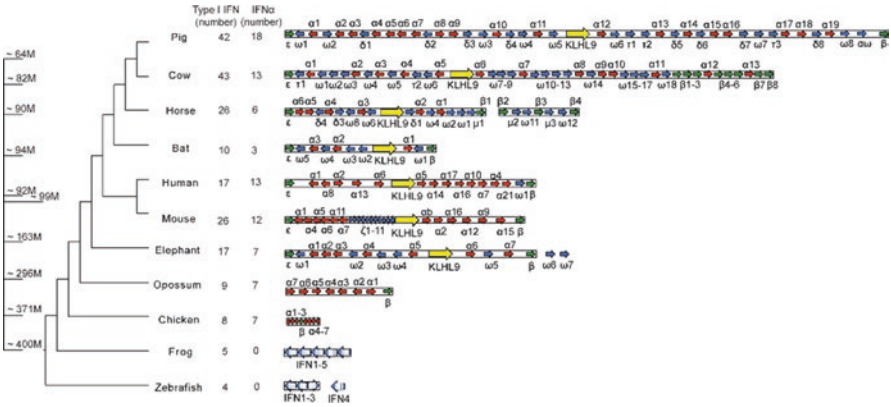

Fig. 3 The type I IFN locus of the black flying fox is highly contracted and contains fewer genes than other vertebrates. (From Zhou et al. 2016a with permission)

(Ferguson et al. 2012). Accelerated evolution of innate immune genes including nuclear factor-kB (NF-kB) family member REL, IFNAR1, Toll-like receptor 7 (TLR7), IFN stimulated gene 15 (ISG15), interleukin-18 (IL-18), and nucleotide-binding oligomerization domain-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) were also observed in the genomes of the two bats, an observation that may be a consequence of the coevolution of bats with viruses (Zhang et al. 2013).

Notably absent from the black flying fox and David's myotis genomes is the PYRIN and HIN domain (PYHIN) gene family, which are involved in the recognition of foreign DNA (Zhang et al. 2013). This finding was recently confirmed in eight additional bat species across both suborders (Ahn et al. 2016). The family member, absent in melanoma 2 (AIM2), is a cytosolic DNA sensor and also part of the inflammasome complex that results in the activation of inflammatory cytokines, including IL1β and IL18. A second component of the inflammasome, NLRP3, has undergone positive selection in the black flying fox and David's myotis, consistent with the possibility that the formation of inflammasomes is impaired in bats, which may in turn dampen the inflammatory response against pathogens (Zhang et al. 2013).

The absence of a number of natural killer (NK) cell receptors from bat RNAseq and genome data sets is also striking (Papenfuss et al. 2012; Shaw et al. 2012; Zhang et al. 2013). Genes that encode mammalian NK cell receptors are located within the leukocyte receptor complex (LRC) and the natural killer complex (NKC) of the genome. The two families have undergone convergent evolution to bind MHC-I molecules for the control of NK cell function. Genes within the LRC encode immunoglobulin (Ig)-like genes, including the killer cell Ig-like receptors (KIR), leukocyte Ig-like receptors (LILRs), and leukocyte-associated Ig-like receptors (LAIRs). Those within the NKC encode lectin-like receptors, including the Ly49 C-type lectin family. The composition of the LRC and NKC varies considerably among species. While most species have expanded either their LRC or NKC gene families, there are exceptions to this rule. In humans and nonhuman primates, the main NK cell receptors are encoded in the LRC and belong to the Ig superfamily. Rodents and horses have only expanded their Ly49 C-type lectin family of NK receptors (Kelley et al. 2005). In contrast, cattle appear to have diversified NK genes within both the NKC and LRC regions, whereas domestic dogs and four species of marine carnivores contain single copies of KIR and Ly49 genes (Hammond et al. 2009; Schwartz et al. 2017). In bats, KIRs and Ly49-like receptors appear to be absent from transcriptome and genome data sets from the black flying fox, and only a single pseudogene of Ly49 was identified in the genome of David's myotis bat (Papenfuss et al. 2012; Zhang et al. 2013). Two KIRs have been identified in the genome of the big brown bat, *Eptesicus fuscus*, but whether they are functional remains to be determined (Guethlein et al. 2015). Overall, evidence to date is consistent with the contraction of both KIR and Ly49 families of receptors in bats. Other NK cell coreceptors have been identified in bat genome and RNAseq data sets, hinting at some level of NK cell function in bats. These include the presence of CD94 and NKG2C, which form heterodimers to generate inhibitory signals. The

more divergent NKG2D, which binds MHC-I chain-related genes, MICA/B, and the UL16 binding proteins (ULBPs) in humans (Kelley et al. 2005), was also detected. Coreceptors, including CD16, CD56, and CD244, were also transcribed in the black flying fox (Papenfuss et al. 2012). The failure to identify a number of NK cell receptors in several bat species supports the hypothesis that bats may have atypical NK cell responses or use different subsets of receptors.

Characterization of Immune Genes

The availability of RNAseq and genomic data has also accelerated the characterization of a variety of immune genes and provided opportunities to examine transcription in various tissues and cells. Molecular information exists for a variety of mammalian cytokines that have been described in bats including interleukins (IL2, IL4, IL6, IL10, IL12), cytokines (TNFα, TGFβ), and IFNs (types I, II, and III) (Iha et al. 2009; He et al. 2010, 2014; Kepler et al. 2010; Zhou et al. 2011a, 2016a; Janardhana et al. 2012; Loria-Cervera et al. 2014). Detailed descriptions of pattern recognition receptors, TLRs, and RIG-I like helicases have also been reported (Iha et al. 2010; Cowled et al. 2011, 2012)

Although only a few studies have examined the nature of Ig genes in bats, a few unusual characteristics have already emerged that have been extensively reviewed elsewhere (Butler et al. 2014). The constant regions of bat Igs appear to correspond to the canonical structure and repertoire found in other eutherian mammals. Bats transcribe IgM, IgD, IgA, IgE, and multiple subclasses of IgG (Baker et al. 2010; Butler et al. 2011; Wynne et al. 2013), although some species do not have Ighδ genes and others have only a single Ighγ gene (Bratsch et al. 2011; Gerrard et al. 2017). Studies of the heavy chain variable (VH) region repertoires of black flying foxes and little brown bats (*Myotis lucifugus*) suggest bats may have the greatest number of VH gene segments among mammals (Baker et al. 2010; Bratsch et al. 2011). Furthermore, evidence from little brown bats indicates that bats may depend more on combinatorial diversity and less on somatic hypermutation (Bratsch et al. 2011). The antigen-binding region of black flying fox VH genes contains amino acids typically associated with lower antigen avidity but greater specificity (Baker et al. 2010). This, combined with the lack of evidence for somatic hypermutation, is consistent with the possibility that highly specific VH segments are encoded in the genomes of bats because of the long coevolutionary history of bats and viruses.

Functional Studies of Immune System of Bats

Innate Immune Activation of Bat Cells

The availability of cell lines from a range of different bat species has provided opportunities to study several aspects of the immune response of bat cells in vitro. This has been particularly useful for studying host–virus interactions. IFN responses

of bat cells and cell lines following stimulation with viruses and synthetic TLR ligands, including polyinosinic:polycytidylic acid (polyI:C) and bacterial lipopolysaccharide (LPS), have demonstrated that IFN production pathways are functional in bat cells and supernatant from stimulated cells has antiviral activity (Stewart et al. 1969; Omatsu et al. 2008; Crameri et al. 2009; Kepler et al. 2010; Zhou et al. 2011b). Significantly, IFNα and IFN signaling molecules, such as IFN regulatory factor 7 (IRF7), are constitutively expressed in unstimulated pteropid bat tissues and cells, consistent with the possibility that the innate immune systems of bats are at higher states of activation than other mammals, presumably allowing bats to rapidly respond to microbial infection (Zhou et al. 2014, 2016a). The constitutive expression of IFNα has been described in two species of pteropid bats (*P. alecto* and *Cynopterus brachyotis*) and is a first for any species. Curiously, fetal and kidney cell lines from a third pteropid bat species, the Egyptian rousette bat (*Rousettus aegyptiacus*), have low constitutive expression of IFNα, indicating that high baseline levels of IFNα may not be a feature of all bat species (Kuzmin et al. 2017).

The downstream signaling events triggered by IFN result in the induction of hundreds of IFN-stimulated genes (ISGs), which are responsible for the antiviral state induced by IFNs. The profile of ISGs in unstimulated bat cells and the kinetics of ISG induction following stimulation with IFN also differs from other species. Unstimulated cells from the black flying fox have higher levels of ISGs compared to human cells. The ISG profile of bat cells consists predominantly of a subset not associated with the acute inflammatory responses that often accompany elevated IFN activity (Cheon et al. 2013; Zhou et al. 2016a). Stimulation of cells from the black flying fox with IFNα also leads to the induction of novel subsets of ISGs, including ribonuclease L (RNaseL), that are not known to be induced by IFN to other species and the ISG response is elevated for a shorter period of time in bat compared to human cell lines (De La Cruz-Rivera et al. 2017; Zhang et al. 2017); RNaseL is also elevated in bats that die from experimental Tacaribe virus infection (Gerrard et al. 2017). Overall, these studies point to differences in the regulation and profile of bat ISGs as being central to the ability of bats to tolerate constitutive IFNα expression without pathology.

Consistent with the nature of the ISG response, additional evidence is also accumulating for differences in the activation of other components of the inflammatory immune response in bats. Comparison of the inflammatory cytokine production of polyI:C-stimulated cell lines from big brown bats (*E. fuscus*) and humans have demonstrated that the induction of high levels of proinflammatory cytokines, TNFα and IL8, occurs in human but not in bat cells (Banerjee et al. 2017). This result again demonstrates that bats may regulate their immune response more tightly compared to other species.

Innate Immune Responses of Bat Cells to Viruses

Experimental infections of bat cells and cell lines have also provided insight into the antiviral response of bats, revealing differences in the responses to different viruses

and between cell types. Infection of black flying fox splenocytes with the bat paramyxovirus, Tioman virus, resulted in the downregulation of type I IFNs and the upregulation of type III IFNs, indicating that type III IFNs may play an important role in the ability of bats to coexist with viruses (Zhou et al. 2011a). In contrast, henipavirus infection antagonizes type I and type III IFN production and signaling in black flying fox cells but only IFN production in human cells (Virtue et al. 2011a, b). The difference in the behavior of bat IFNs upon Tioman and henipavirus infection may reflect different IFN production mechanisms in splenocytes, which are professional immune cells, and cloned bat cells, which are predominantly fibroblast-like (Crameri et al. 2009). Infection of cells from the black flying fox with henipavirus and the Egyptian rousette bat with Ebola or Marburg results in the induction of IFNβ, but curiously no increase in IFNα has been observed, at least at the time points examined in these studies (Zhou et al. 2016a; Kuzmin et al. 2017). As described earlier, *P. alecto* has high constitutive IFNα, which may account for its low induction, but this does not appear to be the case for the rousette bat. Both Marburg and Ebola viruses, but particularly Marburg, induced a potent innate immune response in rousette cells, which was generally stronger than that in human cells. The timing of induction of IFNs and ISGs in Ebola-virus-infected cells was also delayed compared to cells infected with Marburg virus (Kuzmin et al. 2017). The natural reservoir for Marburg virus is known to be the rousette bat, but the reservoir for Ebola is unknown and believed to be another bat species. The differences in host response of rousette bat cells to the two filoviruses may therefore reflect adaptations associated with the role of this species as a natural reservoir for Marburg but not Ebola.

Although ISG responses have also been examined following viral infections in vitro, their ability to restrict viral replication has only been examined for a few ISGs (De La Cruz et al. 2017; Zhou et al. 2013). The best-characterized ISGs include Myxovirus resistance (Mx) genes and 20–50-oligoadenylate synthetase 1 (OAS1). Mx proteins are large GTPases that were initially described as inhibitors of influenza viruses and act by detecting viral replication and then trapping viral components. The OAS1 proteins are activated by dsRNA leading to the activation of Rnase L, which then degrades both cellular and viral RNA (Sadler and Williams 2008). Mx1 and OAS1 from the black flying fox have been demonstrated to be highly upregulated by pteropine orthoreovirus NB (PRV1NB) virus infection, an orthoreovirus carried by pteropid bats (Zhou et al. 2013). Furthermore, bat Mx1 proteins from Pteropidae, Phyllostomidae, and Vespertilionidae demonstrate antiviral activity against Ebola and bat influenza-like viruses. However, Thogoto virus, a tick-transmitted orthomyxovirus that is not known to infect bats, was not inhibited by bat Mx1 despite the ability of human Mx1 to inhibit Thogoto virus replication. Evidence for positive selection in two variable and surface-exposed regions of bat Mx1 genes were hypothesized to explain some of the species-specific antiviral activities of these proteins (Fuchs et al. 2017). However, antiviral activity of black flying fox RNaseL has been demonstrated against the yellow fever flavivirus, which is carried by mosquitoes, consistent with differences in specificity among different bat ISGs (De La Cruz et al. 2017).

Cell-Mediated Immunity In Vitro

Cell-mediated immune (CMI) responses are controlled by $CD8^+$ cytotoxic and $CD4^+$ helper T-lymphocyte populations and result in the killing of virus-infected cells or activation of the antibody and cytokine response. Fewer studies have examined CMI in bats. The single type II IFN, IFNγ, is produced by black flying fox bat cells stimulated with mitogens such as phytohaemagglutinin (PHA) and ConA, and recombinant bat IFNγ has antiviral activity against Semliki Forest virus and HeV in vitro (Janardhana et al. 2012). At least in vitro, IFNγ from the black flying fox appears to have activity similar to that of IFNγ from other mammals, consistent with its role in the CMI response. Curiously, in rousette bat cell lines, IFNγ is induced following infection with Marburg virus but not following infection with Ebola virus, indicating there may be differences in the CMI response induced by these two closely related viruses (Kuzmin et al. 2017). A number of earlier studies have described the in vitro responses of pteropid bats and microbats to T-cell mitogens and mixed lymphocyte responses in pteropid bats (McMurray and Thomas 1979; Chakraborty and Chakravarty 1983; Chakravarty and Paul 1987; Paul and Chakravarty 1987). Although these studies have been relatively crude due to the absence of specific reagents, they have all reported delayed responses compared with those of conventional laboratory animals. The presence of regulatory T cells was implicated in the delay in mitogenic responses of B cells in bats (Chakravarty and Paul 1987). Whether these cells are involved in the delay in T-cell-mediated immune responses observed in bats remains to be determined.

More recent studies have used proteomics to functionally characterize black flying fox MHC-I molecules and identify endogenous and viral peptide ligands. Peptides derived from bat MHC-I molecules display a relatively broad length distribution, consistent with earlier observations based on sequence information demonstrating relatively large peptide binding grooves in the bat class I molecules (Ng et al. 2016; Wynne et al. 2016). Furthermore, an unusual preference for a C-terminal proline residue was identified in endogenous and Hendra virus (HeV)-derived peptides presented by bat MHC-I molecules, consistent with the possibility that differences in antigen presentation or processing may exist in bats (Wynne et al. 2016).

Cell-Mediated Immune Responses of Bats In Vivo

Bats are capable of mounting antibody responses to viruses and model antigens, and the appearance of antibodies appears to follow the same succession as that of other mammals with the early appearance of IgM followed by IgG (Hatten et al. 1968, 1970; Chakraborty and Chakravarty 1983; Wellehan Jr et al. 2009). Although all of the Ig isotypes have been detected at the mRNA level in a variety of bat tissues, IgA protein appears to be present at surprisingly low levels in tissues and secretions from the black flying fox, which may have implications for its role in mucosal immunity in bats (Wynne et al. 2013). There are also differences in the time course, quantity, and duration of antibody responses, and questions exist over the protective

nature of antibodies in bats (Hatten et al. 1968; McMurray et al. 1982; Chakraborty and Chakravarty 1984; Davis et al. 2007; Wellehan Jr et al. 2009; Turmelle et al. 2010b). Responses to antigens such as ϕX174 bacteriophage and sheep red blood cells have demonstrated that the generation of neutralizing antibodies is delayed in the big brown bat, the pteropid bat, and the Indian flying fox (*Pteropus giganteus*) (Hatten et al. 1968; Chakraborty and Chakravarty 1984). Isotype switching from IgM to IgG also appears to be delayed in the big brown bat (Hatten et al. 1968). Despite genetic evidence for limited somatic hypermutation in the little brown bat, an increase in antibody affinity as measured by the ability of antibodies to dissociate from ϕX174 increased following secondary immunization in the big brown bat (Hatten et al. 1970).

Measures of CMI in bats have been crude relative to studies in other species and are limited to studies demonstrating T-cell-mediated inflammation to protein antigens such as purified protein derivative (PPD), PHA, and bovine serum albumin (BSA). Such skin sensitivity tests in two bat species, the common vampire bat (*Desmodus rotundus*) and Seba's short-tailed bat (*Carollia perspicillata*), immunized with PPD or BSA revealed delayed responses in both species compared to similar reactions in mice (McMurray and Thomas 1979). Lack of inflammatory responses have also been reported in most Indian flying foxes subjected to skin sensitivity tests using the contact allergen 2–4 dinitrofluorobenzene (Chakraborty and Chakravarty 1983).

Immune Responses of Bats to Experimental Viral Infections

Unlike conventional laboratory animals, few "clean" captive colonies of bats exist, and experimental infections often rely on the use of wild caught individuals, which represent a mixed population of unknown age, susceptibility, and prior viral exposure. Experimental infections have been performed on a number of species of bats using rabies virus, Australian bat lyssavirus (ABLV), Marburg, HeV, Nipah virus (NiV), Japanese B encephalitis (JE) virus, and Tacaribe virus (TCRV) (Williamson et al. 1998, 1999; Almeida et al. 2005; Davis et al. 2007; Middleton et al. 2007; Turmelle et al. 2010b; Halpin et al. 2011; Cogswell-Hawkinson et al. 2012; Paweska et al. 2012). Although the only immune parameter measured during these studies has been antibody responses, these experiments have provided valuable information on the kinetics of viral infection, the timing and duration of antibody responses and the nature of protective immunity following reinfection. With the exception of rabies virus, ABLV and TCRV, bats generally show no clinical signs of disease following infection. Neutralizing antibodies to a variety of viruses have been detected in wild caught bats, demonstrating they are capable of mounting an antibody response to viruses (Halpin et al. 2000; Lau et al. 2005; Leroy et al. 2005). The transfer of maternal antibody to pups occurs in bats, and the decline of maternal antibodies has been examined in captive black flying, variable flying foxes (*Pteropus hypomelanus*), and straw-colored fruit bats (*Eidolon helvum*) (Epstein et al. 2013; Baker et al. 2014). However, whether bats transfer maternal antibody both pre- and

postpartum and the isotypes involved is unknown. The interpretation of antibody responses in bats is extremely challenging, and, as described earlier, the nature of antibody responses in bats often differs both qualitatively and quantitatively compared to other species.

Experimental Infection of Bats with Rabies and ABLV

Rabies and ABLV are among the only viruses known to result in clinical disease in naturally infected and experimentally infected bats. However, not all bats develop disease, and the mechanisms responsible for differences in disease outcome between individuals are not understood. Evidence from experimental infections has demonstrated that even the development of neutralizing antibodies does not always provide protection from reexposure. For example, a group of wild caught bats (12 big brown bats, *E. fuscus*, and 12 Mexican free tailed bats, *Tadarida brasiliensis*) challenged by oral-nasal inoculation with rabies virus all developed antirabies neutralizing antibodies within 3 months. Rechallenge by intramuscular inoculation 6 months later resulted in an amnestic response in 21 animals, including 9 that developed clinical rabies (Davis et al. 2007). Low seroconversion rates have also been reported in big brown bats inoculated with rabies by intramuscular challenge with only 15 of 43 inoculated animals developing antibodies. This study also reported clinical disease following secondary or tertiary infections in bats that had seroconverted following primary inoculation (Turmelle et al. 2010b). Similarly, Almeida et al. (2005) described the intramuscular challenge of 40 vampire bats (*D. rotundus*) with rabies virus, of which 30 bats survived. Once again, there was no correlation between the level of neutralizing antibody and survival. Many bats that developed low or undetectable antibodies, as well as those with high antibody titers, survived infection. Infection of gray-headed flying foxes, *Pteropus poliocephalus*, with rabies or ABLV results in similar rates of mortality and seroconversion. McColl et al. (2002) reported clinical signs of disease in three of ten ABLV-infected and two of four rabies-infected gray-headed flying foxes, none of which seroconverted prior to euthanasia. Five of the ABLV-infected survivors seroconverted by 23 dpi, with titers waning by 50 dpi. One of the rabies-infected survivors also seroconverted, but not until 70 dpi (McColl et al. 2002). These studies indicate that antibodies may not provide protection and support a role for other components of the immune response in those animals that survive infection.

Experimental Infection of Bats with Other Bat-Borne Viruses

Unlike rabies and ABLV infections, clinical disease has not been reported in any bat species either naturally or experimentally infected with a variety of other bat-borne viruses, including HeV, NiV, Marburg, Ebola, and JE viruses. However, similar to rabies infection, the role of the antibody response in providing protection remains unclear, and many animals survive infection but fail to seroconvert. The

henipaviruses HeV and NiV are carried by pteropid bats. In Australia, HeV antibodies have been identified in all four species of Australian flying foxes (*P. alecto*, *P. poliocephalus*, *P. scapulatus*, and *P. conspicillatus*) (Field et al. 2001). NiV antibodies have been identified in bats from Southeast Asia and Africa. In Malaysia, two pteropid species, small flying foxes (*P. hypomelanus*) and Malayan flying foxes (*P. vampyrus*), are considered to be the reservoir hosts (Yob et al. 2001). A number of experimental infections of pteropid bat species have been performed to understand the nature of viral infection in the natural reservoir of these viruses. NiV infection of 11 gray-headed flying foxes by subcutaneous injection resulted in the production of neutralizing antibody in all individuals inoculated, but in a separate study, only 4 of 8 Malayan flying foxes that were infected by the oral-nasal route produced a neutralizing antibody response (Middleton et al. 2007; Halpin et al. 2011). Both subcutaneous and oral-nasal routes of infection have also been used for HeV inoculation of pteropid bats. Neutralizing antibody responses were detected in 10 of 20 black flying foxes inoculated oral-nasally with HeV (Halpin et al. 2011). Similarly, in gray flying foxes challenged with HeV, neutralizing antibodies were detected in two of four bats inoculated by subcutaneous injection and three of the four bats inoculated by the oral-nasal route, with none of the bats displaying clinical signs of disease (Williamson et al. 1998). A study of four gray-headed flying foxes in late gestation infected subcutaneously with HeV also described the presence of neutralizing antibodies in all four bats, and no abnormalities were observed in the fetuses or adults at necropsy (Williamson et al. 1999). In other mammals, pregnancy results in a bias in the immune response toward humoral immunity and away from CMI, which could be harmful to a fetus (Szekeres-Bartho 2002). Whether the nature of the maternal immune response facilitates greater production of antibody in infected bats during pregnancy remains to be investigated.

A natural reservoir of Marburg virus are the Egyptian rousette bats (*R. aegyptiacus*) (Towner et al. 2009), and a number of experiments have been performed to study the nature of viral transmission and infection in this species (Paweska et al. 2012; Schuh et al. 2017a, b). Marburg virus is capable of horizontal transmission between inoculated and naïve *R. aegyptiacus*. All inoculated bats seroconverted, with IgG antibodies peaking between 14–28 dpi. Marburg virus antibody titers in both inoculated and in contact bats declined within 1 month following attainment of peak levels and were undetectable after 2 months (Schuh et al. 2017a). A subsequent study revealed that bats rechallenged with Marburg virus 17–24 months following primary experimental infection developed virus-specific secondary antibody, indicative of the development of long-term protective immunity (Schuh et al. 2017b).

Clearly, additional work is needed to understand the antibody responses of bats and the nature of antibody-mediated protection against various viruses. Given what we have learned about innate immunity, particularly in pteropid bats, it is possible that innate immune mechanisms, such as IFN, reduce viral replication to low levels, delaying the generation and magnitude of an antibody response. Evidence for a highly diverse germline repertoire of antibodies and the absence of somatic hypermutation could indicate that bats have evolved a repertoire of antibodies that are

highly pathogen specific. Such antibodies may provide some level of early protection without reaching the higher titers observed in other species. Although no studies have examined the CMI responses of bats to viral infections, the generation of an IFNγ reagent for pteropid bats has been described and will assist in future studies to examine CMI in bats (Janardhana et al. 2012).

Immune Responses of Bats to Fungal Infections

Immunity to *P. destructans*

WNS is caused by a cold-loving (pyrophilic) and keratinophilic fungus (*P. destructans*) first identified in North American bats in 2006 that infects the epidermis and dermis of the muzzle, ears, and wings. Since its discovery, it has been detected in six species of North American bats, and infected populations have undergone a decline of up to 90%, with several species threatened with regional extinction within the next decade. *P. destructans* infects bats during hibernation, causing them to arouse early, leading to depletion of energy reserves and ultimately leading to a severe inflammatory response and resulting histopathology. The fungus is widely distributed in North America and Europe and has recently been found in Asia (Hoyt et al. 2016). Although naturally infected European bats also develop histopathological lesions in response to *P. destructans*, no mass mortality is observed in European or Asian bats (Zukal et al. 2016). Similar to the situation with viruses, the long coevolutionary relationship of European and Asian bats with *P. destructans* has presumably led to an equilibrium between the host and pathogen. In the longer term, this may also evolve in North American bats, and evidence of some level of resistance has been reported in some populations (Langwig et al. 2017). However, the rate of mortality among some bat species is too high to ignore. Understanding the host–pathogen relationship and the genes and pathways associated with disease tolerance and resistance will be important for identifying viable treatments and assessing the immune responses of bats to drugs or vaccines.

Earlier reports describing the immune response of bats during hibernation indicate that, like other hibernating mammals, their immune responses are suppressed during torpor when they are initially infected with *P. destructans*. For example, hibernating *E. fuscus* bats maintained at 8 °C fail to generate antibodies in response to infection with JE virus (Sulkin et al. 1966). In addition, activation plasma complement against bacteria (*Escherichia coli*, *Staphylococcus aureus*) and fungi (*Candida albicans*) is lower in hibernating little brown bats compared to nonhibernating bats (Moore et al. 2011).

Several studies have now begun to examine the host response of bats to *P. destructans* to determine the level of immune activation that occurs during torpor and after arousal. *P. destructans* begins to colonize bat skin during hibernation, yet visible signs of inflammation are characteristically absent in torpid animals, and neutrophils and macrophages are absent from sites of pathogen invasion in hibernating bats with WNS. In little brown bats, overt skin damage does not occur until

2–3 weeks after bats have emerged from hibernation with intense neutrophilic inflammation associated with invasive *P. destructans* infection (Meteyer et al. 2012). Studies of bats from WNS-affected and unaffected sites have also demonstrated significantly higher circulating leukocyte counts in WNS-affected bats with elevated body temperatures (above 20 °C). The latter is consistent with the mobilization of cells associated with arousal from torpor and euthermia (Moore et al. 2013). The absence of neutrophil and T-cell infiltration has been confirmed through RNAseq analysis of WNS-infected little brown bat wing tissues during hibernation (Field et al. 2015). Despite the absence of neutrophil invasion, increases in gene expression for inflammatory cytokines have been detected in wing tissues from hibernating WNS infected bats compared to hibernating bats not affected by WNS. These include IL1β, IL6, IL17C, IL20, IL23A, IL24, and G-CSF and chemokines, such as Ccl2 and Ccl20. Hibernating little brown bats exhibiting visible fungal infections elevated levels of transcripts for proinflammatory cytokines, IL23 and TNFα, the anti-inflammatory cytokine IL10, and the antimicrobial peptide cathelicidin in lung tissue compared to hibernating uninfected bats (Rapin et al. 2014). Overall, these studies are consistent with the induction of an innate antifungal response in WNS-infected bats prior to emergence from hibernation followed by infiltration of immune cells and, presumably, activation of adaptive immune responses following arousal. Overactivation of the immune response following arousal from torpor, combined with a depletion of energy reserves, appears to be the main cause of mortality.

Immunity to Other Fungal Pathogens

In contrast to *P. destructans*, bats are known to carry other fungal pathogens, such as *Histoplasma capsulatum*, without disease. *H. capsulatum* is a pathogenic fungus that causes pulmonary and systemic infections in humans. Bats are considered to be the main reservoir of this fungus, and it is commonly found in bat guano (Taylor et al. 2005). Although bats are susceptible to infection, mortality is rare in bats that are inoculated intranasally, which is the most likely route of natural infection. Higher mortality rates are observed in bats inoculated intraperitoneally (McMurray and Greer 1979; Greer and McMurray 1981). Great fruit eating bats (*Artibeus lituratus*) respond to infection with the generation of complement fixing antibodies by 3 weeks and precipitating antibodies by 5 weeks post infection (McMurray and Greer 1979). Natural infection rarely results in disease, indicating that, similarly to the situation with most viruses, bats have likely evolved mechanisms to control infection, at least under conditions where they are infected under nontorpid conditions.

Future Directions

The field of bat immunology is very much in its infancy, and significant opportunities exist for future research. Thanks to advances in technology, such as whole-genome sequencing and RNAseq, considerable progress has been made, in particular with regard to our understanding of the immune system of the black flying fox, *P. alecto*. However, as bats are a highly diverse group of mammals that have evolved independently for a long period of time, it is possible that different immune mechanisms exist between the two suborders and across species. There is likely much more to be learned from comparative studies across different bat species.

Comparative genomics of bats have provided important clues to the adaptations that may allow bats to coexist with viruses in the absence of disease. These include evidence for positive selection on a variety of immune genes and differences in the repertoires of NK cell receptors. Additional genomic data, including long read assemblies, will be required to resolve highly repetitive regions such as the LRC and NKC to confirm the absence of important receptor families and to resolve other repetitive regions of the bat immunome. A number of genomic regions also remain largely unexplored, partly owing to their repetitive nature. These include B- and T-cell receptor (BCR and TCR) regions. Examining the repertoire and diversity of these regions will provide opportunities to examine their functional activities and importance. For example, no information exists on the repertoire of TCRs in bats and the relative importance and roles of αβ and γδ T cells. Observations from genomic data sets pave the way to further addressing the role of different components of the immune system in the responses of bats to infection. The mechanisms involved in TCR and BCR diversification also remain unknown. The roles of terminal deoxynucleotidyl transferase (TdT), recombination activating gene (RAG), and activation-induced cytidine deaminase (AID) on recombination, somatic hypermutation, gene conversion, and class switching remain to be explored.

A number of important differences in the innate immune system have also been identified in bats that are at odds with the responses in humans and other species. In particular, the constitutive activation of IFNα in the black flying fox is striking. In other mammals, constitutive IFN expression can have implications for inflammation and autoimmunity. Identifying the mechanisms responsible for the ability of bats to tolerate high levels of IFN in the absence of inflammation has significant potential for identifying novel therapeutics to treat viral diseases in humans and other species. To this end, functional characterization of the different subsets of ISGs already identified in both unstimulated and stimulated cells would provide valuable insights into the mechanisms responsible for the control of viral infection in the absence of inflammation. Additionally, dissection of the signaling pathways responsible for the control of IFN response will contribute to our understanding of differences in the regulation of IFN in bats compared to other species.

As described earlier, a number of functional differences have been identified in the immune system of bats compared to other species. These include the nature of cell-mediated and antibody responses of bats. To advance our understanding of the nature of these responses, appropriate bat-specific reagents will be required. Some

commercially available human and mouse antibodies generated against highly conserved intracellular proteins are cross reactive with bat proteins and have already proven useful (Zhou et al. 2016b). A handful of bat-specific antibodies have also been generated (Janardhana et al. 2012; Wynne et al. 2013). Additional reagents will be necessary to advance the field, including monoclonal antibodies for use in flow cytometry, immunohistochemistry, and ELISAs, to dissect the roles of different cell types, including B and T cells, dendritic cells, and macrophages. Reagents will also be required to examine the responses of various cytokines to examine proinflammatory and anti-inflammatory pathways for comparison to other species and to answer specific questions, including the confirmation of cytokine expression at the protein level (e.g., IFNα to confirm its constitutive expression). Recombinant cytokines and growth factors will also be important for examining the responses of cells to cytokine stimulation and the expansion of specific subsets of antigen-specific lymphocytes. Lastly, the development of closed breeding colonies of bats will be essential in progressing research into immunity in bats, overcoming the issues associated with wild caught individuals of unknown age and history of infection.

Conclusions

Renewed interest in bat immunology emerged following the identification of bats as reservoirs for a number of viruses, including SARS-CoV and Ebola, that are highly pathogenic in other species. Prior to the emergence of these viruses, few studies had examined any aspect of bat immunology. A number of important observations have already been made through studies of the immune systems of bats, with evidence for adaptations not observed in any other species. Significant progress has now been made in the identification of genes and pathways associated with immunity, and one of the recurring themes that is emerging with regard to viral infections is the ability of bats to control inflammatory responses. Regulation of the immune system is likely an important mechanism for preventing pathology associated with infection. However, bats are an extraordinarily diverse group of mammals, and the adaptions identified to date may not apply across all bat species. In contrast to the apparent regulation of the immune response during viral infections, uncontrolled inflammatory responses due to infection with pathogens such as WNS clearly demonstrate that bats are capable of overactivating their immune system, causing immunopathology. As described earlier, there are still gaps in our understanding of the immune systems of bats, and significant opportunities exist. Studies of bat immunology provide opportunities to identify novel mechanisms that could be applied to redirecting the immune system of other species to prevent disease and to the conservation of bats affected by pathogens such as WNS.

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Mammalia: Proboscidea: Elephant Immune System

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This chapter reviews our current knowledge of the elephant immune system, followed by a discussion of how further elephant immunology research will benefit the health of elephants and potentially other species (Fig. 1). Unfortunately, we know very little about the elephant immune system compared to what we know about the mouse and human immune systems. This lack of knowledge does not stem from a lack of desire to know more but rather from a lack of funding opportunities to support more complete analysis of elephant immune system function as well as a lack of tools. To properly study the immune system of any species, a wide variety of species-specific reagents are required. The cost of a single reagent, like an antibody to recognize a specific type of immune cell in a specific species, is extremely expensive and, at times, cost prohibitive. The immune system consists of many different cell types and subtypes. To perform flow-cytometry-based studies similar to those performed to extensively characterize the mouse and human immune cell populations, many different types of elephant-specific antibodies would be required. While some of the currently available antibodies that recognize mouse or human immune

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Fig. 1 Asian elephants. (Photo courtesy of Stephanie Adams/Houston Zoo)

cell surface proteins may also recognize elephant immune cell surface proteins (due to epitope overlap), extensive validation work will be required to ensure the specificity of the antibodies. Without a good set of elephant-specific reagents, what we can learn about the elephant immune system is limited. However, a handful of elephant-immune-system-specific reagents have recently been developed by us and others, and in the future our elephant immunology toolkit is likely to grow. As our scientific tools grow, so will our knowledge of the elephant immune system. Increased understanding will lead to better treatment plans for elephants with infections and immune disorders.

Organs of the Immune System

Bone Marrow

Bone marrow contains hematopoietic stem cells, which give rise to all cells of the blood, including the white blood cells of the immune system (Birbrair and Frenette 2016). In addition, the bone marrow is the site of B lymphocyte development (Hardy and Hayakawa 2001). While bone marrow is often located in the long bones and ribs of mammals, elephant long bones and ribs lack open marrow cavities. In the elephant, these bones are dense, which is thought to be necessary to support heavy body weight (Nganvongpanit et al. 2017). Instead, bone

marrow can be found in the dorsal spinous process of the first lumbar vertebrae (Mikota 2006). A study of elephant long bones and ribs published in 2016 found an unexpected high level of iron in the humerus, radius, fibula, and ribs of Asian elephants. Because iron is associated with hematopoiesis, the authors suggest that these bones may play a role in hematopoiesis. The absence of an open medullary cavity in these bones and failure to detect any hematopoietic cells in these bones mean future studies will be required to define a role for these bones in the production of blood cells in the elephant and their relationship to iron stores (Nganvongpanit et al. 2017).

Thymus

The thymus is another important immune organ where T cells develop and are selected before being released into the periphery (Viret and Janeway Jr 1999). The elephant thymus is located in the anterior mediastinum/anterior pericardial sac. Similar to the thymus of other mammals, it is bilobed (Pearse 2006; Lowenstine 2006). Little is known about thymic development in elephants.

Spleen

As in other animals, the elephant spleen is located on the left side of the abdomen between the stomach and the diaphragm (Mikota 2006). Antigens enter the spleen through the blood vascular system, as the elephant spleen has only efferent, but not afferent, lymphatics (Mikota 2006; Valli and Jacobs 2000).

Elephants have both lymph nodes (associated with the lymphatics) and hemal nodes (associated with the blood vascular system). As seen in other ungulate species, hemal lymph nodes are reddish brown and distributed along the vessels of the anterior portion of the body (Lowenstine 2006). Lowenstine observed that elephants have a chain of cervical lymph nodes in the head and neck. In addition, she notes that they also have parotid lymph nodes, axillary lymph nodes, inguinal nodes, tracheobronchial nodes, jejunal lymph nodes, ileocecal lymph nodes, and colonic lymph nodes (Lowenstine 2006). Elephants are also reported to have parotid, mandibular, superficial cervical, and prescapular lymph nodes in locations similar to other species (Woodford et al. 2000). Tonsils, on either side of the pharynx, have also been reported in both Asian and African elephants (Lowenstine 2006; Cave and Aumonier 1962). Lymph node organization in elephants is similar to that found in other mammals (Lowenstine 2006; Cave and Aumonier 1962). Enlarged lymph nodes can be found in elephants with infections, which indicates immune cell expansion. However, due to the location of the lymph nodes and thickness of elephant skin, swollen lymph nodes are not usually detectable upon clinical examination (Lowenstine 2006).

Cells of the Immune System

Similar to other mammals, elephants have lymphocytes, monocytes, eosinophils, and basophils circulating in their blood (Harr and Blue 2010). Unlike many mammals, elephants have heterophils rather than neutrophils. Heterophils function similarly to neutrophils but can be distinguished by the contents of their cytoplasmic granules. The granules in heterophils look reddish in Leishman-stained blood smears (Silva and Kuruwita 1993). Heterophils, rather than neutrophils, are also found in manatees. Manatees and elephants are both members of the superorder Afrotheria and therefore are more closely related to each other than to other mammals (Stanhope et al. 1998; Foley et al. 1699). In addition, elephants also have bilobed and more rare trilobed immune cells that circulate through their blood, which are not found in most mammals. While these cells have been counted as both lymphocytes (Brown and White 1980; Simon 1961) and monocytes (Silva and Kuruwita 1993; Allen et al. 1985) in various publications, the current consensus is that they are monocytes because they have cytoplasmic granules that stain positive for peroxidase (Mikota 2006; Silva and Kuruwita 1993). Elephants have two populations of monocytes, typical monocytes with unsegmented nuclei and monocytes with segmented nuclei (Silva and Kuruwita 1993). The closest living relative to the elephant, the hyrax, also has these unique monocytes (Aroch et al. 2007). The function of segmented nuclei in monocytes is unknown. While most mammals lack multilobed monocytes, most mammals, including elephants, have multilobed neutrophils or heterophils. The nuclei of elephant heterophils are hyposegmented compared to human neutrophils. Other mammals, like hyenas and rabbits, have hypersegmented nuclei compared to humans (Hoffmann et al. 2007). These differences among species may hint at the function of these segmented nuclei, which is still under active investigation. It has been suggested that segmented nuclei help these cells rapidly migrate through tight spaces like the junctions of blood vessels (Campbell et al. 1995; Olins et al. 2008). The segmented nuclei of neutrophils are also associated with infections and may play an important role in the release of neutrophil extracellular traps (NETs), which capture and kill microbes (Lukasova et al. 2013). Perhaps the segmented nuclei of elephant monocytes are also important in early response to infection and have evolved the ability to rapidly migrate through tight spaces and release NETs.

The most common techniques for characterizing elephant immune cells have employed various histologic stains. Transmission electron microscopy has also been used (Harr and Blue 2010). Future studies of the surface proteins on immune system cells will help to characterize specific cell types and cell subtypes, track population shifts in response to infections, and identify beneficial versus suboptimal responses within these populations. Our knowledge of elephant cellular response in the context of infection is currently under active investigation, and researchers are working to characterize the T-cell response to several types of infection using a number of newly developed tools (Landolfi et al. 2014, 2015; Fuery et al. 2018). Understanding the role that different subsets of T cells and their mediators play in elephant infections and other inflammatory conditions is

an important step in characterizing the elephant immune system. A summary of what is currently known about the T-cell response to infection can be found in the following sections.

Other Immune Mediators

Antibodies are important mediators of the adaptive immune response. When the body is exposed to foreign antigens, B cells can generate antibodies that specifically bind regions of these antigens. Antibodies can provide protection against foreign invaders by blocking virus entry into cells or by coating bacteria and encouraging phagocytosis by cells that can digest the invaders. Due to their critical role in protective immunity, many vaccines stimulate antigen-specific immune responses. Elephants have at least five subclasses of IgG (Kelly et al. 1998), and they can also generate IgM and IgA antibodies (Humphreys et al. 2015). A study of humoral response to rabies vaccination in Asian elephants (n = 16; age range: 6-48 years of age) found that elephants generated neutralizing antibodies to rabies virus 35 days after vaccination with 1 or 2 doses of inactivated rabies virus vaccine. A booster vaccine was given 344 days after the initial vaccination, and the elephants again tested positive for rabies-virus-neutralizing antibody 40 days after the booster (Isaza et al. 2006). Rabies vaccination of African elephants also resulted in detectable antibody responses (Miller and Olea-Popelka 2009). In addition to generating antibody responses to rabies vaccination, Asian elephants have been shown to generate antibody responses to tetanus toxoid vaccine (Lindsay et al. 2010). This tetanus toxoid vaccine study found that older elephants mounted a stronger response to vaccination when compared to younger elephants (Lindsay et al. 2010), possibly indicating natural exposure throughout an elephant's lifespan. Other species, including horses (Fermaglich and Horohov 2002; Muirhead et al. 2008) and humans (Hainz et al. 2005), are reported to show a decrease in antibody responses to antigens with age.

Overall, these results suggest that elephant B cells and the antibodies they produce are important mediators of elephant immunity.

Major histocompatibility complex (MHC) molecules present antigens to T cells, which recognize specific antigens bound to specific MHC molecules (Janeway Jr et al. 2001). Every individual expresses multiple MHC genes, and the set of genes expressed from individual to individual varies. The DQA-like MHC locus from 30 African elephants and 3 Asian elephants was characterized by sequencing and analysis. Ten unique alleles were identified, six in African elephants and four in Asian elephants. Overall, the diversity of the DQA locus in elephants was similar to the diversity found in other mammals. One unusual finding compared to other mammals was that a single allele (LoafDQA*01) occurred in more than half of the individuals sequenced. Usually this low level of polymorphism is only observed in animals with two or fewer alleles. The authors of the study speculate that perhaps the LoafDQA*01 has been under selection in elephants because it offers an advantage in resisting a prevalent

disease. More individuals from various populations need to be sequenced to confirm the frequency of this allele and to characterize other MHC alleles (Archie et al. 2010).

Cytokines are mediators of immunity secreted by a variety of different cell types (Kelso 1998). These signaling molecules can either stimulate or suppress inflammatory responses. They play critical roles in immune response and control of infections, and when produced in excess, they can cause sepsis (Chaudhry et al. 2013). Assays to measure cytokine responses in elephants were developed by Landolfi et al. Using real time RT-PCR assays, they detected expression of TNF-α, TGF-β, IFN-γ, IL-2, IL-4, IL-10, and IL-12 in the blood of Asian elephants (Landolfi et al. 2009). Cytokine response to tuberculosis in elephants has been measured using these assays (Landolfi et al. 2010), and the results of those studies are described in a subsequent section on the immune response to pathogens.

Immune System During Pregnancy

Maternal Transfer of Immunity

There are two mechanisms by which mammalian infants can acquire immunity from their mothers (Fig. 2). The first is through placental transfer of antibody, and the second is through colostrum in breast milk. Asian and African elephants have endotheliochorial placentation (Cooper et al. 1964; Perry 1974), which is associated with modest maternal to fetal antibody transfer (5–10%) (Chucri et al. 2010; Heddle and Rowley 1975). In a study by Nofs et al. (2013), it was found that elephants acquired more antibody transplacentally than would be expected from animals exhibiting endotheliochorial placentation, where elephants more closely resemble humans and rodents who have hemochorial placentation (Nofs et al. 2013). In this study, calves at birth had levels of anti-tetanus and anti-rabies antibodies equal to or greater than their dams (who had been vaccinated for both rabies and tetanus during pregnancy) before the intake of colostrum. McGee et al. (2014) had similar findings in their study of tuberculosis-positive dams and calves, where they found that calves born to tuberculosis-positive Asian elephant dams had detectable anti-tuberculosis antibodies in day-of-birth or presuckling samples compared to samples from calves who were born from tuberculosis-negative mothers, which had no detectable antibodies (McGee et al. 2014).

Immune Tolerance to Fetus

The placenta of mammals acts to connect mother and fetus to allow for an exchange of biological materials. Because the fetus expresses antigens from both the mother and the father, cells of the fetus express non-maternal antigens and should be recognized by the maternal immune system as foreign. However, mechanisms of immune tolerance to the fetus are in place to ensure that the

Fig. 2 Baby Asian elephant with mother. (Photo courtesy of Stephanie Adams/ Houston Zoo)

fetus is not rejected by the mother's immune system (Guleria and Sayegh 2007). As mentioned earlier, elephants have endotheliochoral placentation. This type of placenta is less invasive compared to the hemochorial placenta of humans. Comparison of the transcriptome of elephant placenta compared to human placenta revealed the expression of a variety of immune-related genes in human placenta that were not expressed in elephant placenta. The authors of this study propose that the human immune system has undergone adaptations due to deep trophoblast invasion into the uterus that requires more immune system alterations to prevent rejection of the fetus when compared to the less invasive placenta of elephants (Hou et al. 2012). Despite this enrichment for immune-related gene expression in human placenta, mechanisms of immune tolerance in elephants are likely in place and some mechanisms are likely shared between humans and elephants, like changes in MHC expression at the maternal–fetal interface (Guleria and Sayegh 2007). One could also speculate that the extended gestation of 22 months in elephants somehow contributes to this different placental immune response in elephants.

Immune Response to Pathogens

A useful approach to characterizing the immune system is to look for changes in response to infection. A study by Stacy et al. reported changes in leukocyte morphology during various inflammatory conditions. They analyzed blood smears from 12 elephants with gastrointestinal disease, 2 with salmonellosis, 5 with chronic foot disorders, 4 with EEHV, 2 with dental issues, 1 with a retained fetus causing metritis, and 1 with metastatic ovarian adenocarcinoma. Blood smears were examined during disease and after recovery from disease for signs of morphological changes in leukocytes. An increased number of heterophil precursor cells (left-shifting), heterophil toxicity, reactive lymphocytes, and activated monocytes were the most common leukocyte changes in the context of these inflammatory conditions, none of which were present upon recovery from disease. In two cases of gastrointestinal disease, plasma cells were also observed (Stacy et al. 2017). These results, combined with the observation that all but three of the elephants recovered from disease, indicate that the cells of the elephant immune system are able to successfully respond to immune challenges. The responses observed by blood smear could not be grouped by disease type, gender, age, or species of elephant. It is possible that the differences in response reflect the dose of antigen or severity of disease, as the authors did note an increase in heterophil toxicity and left-shifting as disease progressed (in the three cases of elephants that died from disease) (Stacy et al. 2017). Further exploration of these changes in leukocytes and their role in responding to infections in elephants will help us better monitor disease progression. Much effort has been devoted to characterizing elephant-immune-response-specific pathogens, and this continuing work has contributed significantly to our understanding of the elephant immune system.

Elephants can be host to a number of pathogens that affect multiple species, including *Mycobacterium tuberculosis* (Mikota and Maslow 2011), *Clostridium tetani* (Burke 1975), *Salmonella typhimurium* (Janssen et al. 1984), and rabies (Wimalaratne and Kodikara 1999). Elephants can be infected by a strain of cowpox (*Orthopoxvirus bovis*), where a number of outbreaks have occurred (Meyer et al. 1999; Wisser et al. 2001), but cases have decreased now that cowpox vaccines are included in their routine care. Elephants themselves have coevolved as hosts to EEHV, where several species and sub-species of virus have been identified as described below.

Elephant Endotheliotropic Herpesvirus

Despite early reports of widespread herpesvirus-like lesions in Asian and African elephants (McCully et al. 1971; Jacobson et al. 1986; Pilaski et al. 1986), the details of an acute hemorrhagic disease caused by the same herpesvirus were first described in a young Asian elephant that died in 1983 (Ossent et al. 1990; Metzler et al. 1990). EEHV was then identified by Richman et al. (1999) after a number of deaths in North American and European zoos in the 1990s from a rapidly developing

hemorrhagic disease that seemed to affect mostly young elephants. At that point it was believed that perhaps this was a virus endemic to African elephants transmitted to Asian elephants housed in the same facility, but this theory has since been disproven, because separate species of virus are now known to be endemic to each species of elephant (Long et al. 2016). Since its identification, EEHV has accounted for the deaths of more than 100 Asian elephants (Long et al. 2016). EEHV can be divided into several species and subspecies: EEHV1A and 1B, EEHV4, EEHV5 (Asian elephants), EEHV2, EEHV3, EEHV6, and EEHV7 (African elephants). EEHV1A and EEHV1B, which affect Asian elephants, are responsible for most EEHV-related deaths. While EEHV is broadly classified as a betaherpesvirus, it has recently been proposed that the genetic differences between EEHV1A, 1B, and EEHV2 compared to other betaherpesviruses make them candidates for a potential new subfamily named deltaherpesviruses, which would form an intermediate branch between betaherpesviruses and gammaherpesviruses in a phylogenetic tree comparing herpesviruses (Richman et al. 2014). Like other herpesviruses, EEHV latently infects its hosts and goes largely unnoticed. However, with closer monitoring of herds, it can be detected in blood and trunk wash samples following reactivation.

The lethality of EEHV hemorrhagic disease is something not seen in other mammalian herpesviruses, which have coevolved with their hosts over thousands of years (Long et al. 2016). EEHV hemorrhagic disease itself is characterized by acute hemorrhaging, where it resembles the hemorrhagic fevers of humans caused by viruses such as Ebola, dengue, and Lassa virus. As such, pathological findings from necropsy samples show pericardial effusion, with extensive hemorrhaging, cyanosis of the tongue, hepatomegaly, and large oral, laryngeal, and intestinal ulcers (Richman et al. 2000). A major difference between EEHV and other herpesviruses is that most herpesviruses are epitheliotropic, whereas EEHV is endotheliotropic, as indicated by the intranuclear inclusion bodies in the endothelial cells of elephants that have died from EEHV (Richman et al. 1999). With more recent developments in technology, we have seen that elephants that die from EEHV infection have levels of EEHV in their blood within the range of 10–75 million viral genomes per milliliter of blood (Long et al. 2016), indicating that these elephants have been overwhelmed with virus.

Immune Responses to EEHV

Several reports have cited a marked lymphopenia, monocytopenia, and thrombocytopenia in association with EEHV viremia (Richman et al. 2000; Fuery et al. 2016a, b; Atkins et al. 2013). In the studies by Fuery et al., in which EEHV1B and EEHV4 viremia were detected in two 4-year-old elephants, levels of EEHV increased as platelet, monocyte, and lymphocyte levels decreased (Fuery et al. 2016a, b). As viremia diminished, it was accompanied by substantial rebounding in each of these populations. However, these two elephants survived, and evidence suggests that this rebounding does not occur in elephants that die from hemorrhagic disease. Hence, lymphocytosis and monocytosis as part of a normal immune response are essential

in recovery from viremia, but why some elephants can achieve recovery and others cannot remains unknown. To draw upon knowledge of responses to other hemorrhagic fevers, there is evidence that innate factors, including the interferon response and adaptive factors, particularly specific T cells, and to a lesser extent humoral responses, play a role in distinguishing between survivors and nonsurvivors of Ebola and Lassa virus infections (Prescott et al. 2017).

The decrease in platelets (thrombocytopenia) is a hallmark characteristic of the hemorrhagic fevers of humans. It is not clear whether it is the loss of platelets that results in acute hemorrhaging or whether the loss of platelets is a byproduct of multiple other immune response failures. Cytomegalovirus (CMV), another betaherpesvirus, can replicate in megakaryocytes, the precursors of platelets (Crapnell et al. 2000), possibly leading to the characteristic decline in platelets.

The cytokine storm associated with many severe diseases, including the hemorrhagic fevers, refers to an uncontrolled burst of immunological mediators known as cytokines. How this response is triggered and how it can be avoided are subjects of much research, as this out-of-control production of cytokines can be more detrimental than beneficial (Tisoncik et al. 2012). In the case of EEHV hemorrhagic disease, we have no evidence of a cytokine storm as such, but it is possible that cytokines still contribute as one of the mechanisms by which acute hemorrhagic disease ensues. Several researchers have developed strategies to detect elephant cytokines (Landolfi et al. 2009; Angkawanish et al. 2013), and these strategies could be useful in studying cytokine production in acute hemorrhagic disease when samples are available.

What makes some juveniles susceptible to hemorrhagic disease caused by EEHV remains unknown, but one hypothesis is that juveniles who succumb to EEHV hemorrhagic disease do not possess EEHV-primed T cells. Peak incidence of death from EEHV hemorrhagic disease occurs in 1- to 8-year-old elephants, making it apparent that newborn calves are probably protected to some extent by immunoglobulins obtained from their mothers, both from placental transfer of antibody and through breast milk, although the contribution of the latter is not clearly understood (Nofs et al. 2013). Both methods of transfer depend on a mother having some preexisting immunity to EEHV. The nature of EEHV as a virus makes a strong case for a cellular immune response, in preference to a humoral one, in providing protective responses. Indeed, T cells are critical for protection against related human herpesviruses, including CMV (Sester et al. 2001; Gerna et al. 2006), HHV-6 (Wang et al. 1999), and VZV (Weinberg and Levin 2010). If T cells are also critical for immunity to EEHV, it would be ideal if a calf had its first exposure to EEHV while still protected under the umbrella of maternal antibody, where their T cells could be primed adequately. Then when maternal antibody waned, the young elephants would be left with protective cellular immune responses in the form of primed memory T cells. Ongoing EEHV research is focused on studying T-cell responses to EEHV in latently infected Asian elephants in order to identify vaccine candidates for juveniles (Fuery et al. 2018). Fuery et al. (2018) studied nine potential T-cell-stimulating proteins of EEHV1A in a single herd of latently infected Asian elephants. They found that three of these proteins (glycoprotein B,

major capsid protein, and E40) elicited substantial T-cell responses, measured by their ability to secrete the cytokine IFN-γ in response to stimulation with these antigens. They also found that CD4+ T cells are largely responsible for this response, indicating that dominant epitopes, at least from these proteins, are MHC class II associated (Fuery et al. 2018). Similar studies are currently being carried out in other EEHV-infected elephant herds with the aim of increasing the significance of these findings. If primed memory T cells are indeed necessary for protection against EEHV hemorrhagic disease, it is hoped that delivery of these EEHV antigens to juvenile elephants will be enough to prime their T cells prior to their becoming susceptible to EEHV infection.

Anthrax

Anthrax is a bacterial disease caused by infection with *Bacillus anthracis*, a large Gram-positive bacterium that can produce heat-resistant spores. Disease is usually spread by contact with spores. Many types of animals are susceptible to anthrax, including all mammals and some bird, reptile, and amphibian species (Hanna 1998). The death of both African and Asian elephants has been associated with anthrax (Turnbull et al. 1991; Berry 1993; Encyclopedia 1995). A potential source of infection for elephants is drinking water. *B. anthracis* has been detected in waterholes frequented by elephants. Other sources of infection include soil and feces (Lindeque and Turnbull 1994). In addition to ingestion, anthrax infection can occur by spores entering through abrasions or inhalation (Hanna 1998).

Toxins are the source of most of the disease symptoms associated with anthrax. Edema toxin complex (EdTx) causes the fluid and edema seen in cutaneous anthrax infections, and lethal toxin complex (LeTx) causes shock and death from systemic anthrax (Hanna 1998). Interestingly, macrophages are cellular mediators of anthrax toxicity because they are targeted and killed by LeTx. When mice were depleted of macrophages, then challenged with a lethal dose of LeTx in normal mice, 100% of the macrophage-depleted mice survived. Coinjecting these macrophage-depleted mice with LeTx and cultured macrophages resulted in the death of the mice, indicating that macrophages are necessary for the shock and death associated with LeTx. Lysis of macrophages by LeTx leads to the release of reactive oxygen intermediates and cytokines, causing shock and death (Hanna 1998). Because macrophages are also important mediators of host immune response to bacteria, this lysis likely gives anthrax the upper hand. Because this disease is not spread from individual to individual, *B. anthracis* may be under less selective pressure to limit toxicity to keep the host alive.

While systemic anthrax is usually fatal, animals can survive cutaneous anthrax (Hanna 1998). Humoral immunity plays an important role in host response to anthrax infection. Antibodies against one of the anthrax toxins, called protective antigen (PA), can offer protection against disease (Cizauskas et al. 2014; Marcus et al. 2004; Reuveny et al. 2001). PA is a protein that is a part of both toxin complexes (EdTx and LeTx) responsible for anthrax disease symptoms (Hanna 1998). Cizauskas et al. measured anti-PA titer by ELISA in elephants and other animals in Etosha National Park in Namibia, where regular outbreaks of anthrax are known to

occur. Anti-PA antibodies were detected in elephants, which suggests that they can mount adaptive immune responses against anthrax. In addition, these results suggest that elephants can be infected with anthrax and survive infection under some circumstances (Cizauskas et al. 2014). Close monitoring of elephants with active anthrax infections will be challenging because, while cases of anthrax have been documented in a captive setting (Encyclopedia 1995), the disease more often occurs in wild elephants, which are difficult to monitor. However, if the opportunity arises, then it will be very interesting to document the contribution of macrophages to both disease recovery and disease progression.

Tuberculosis

Asian elephants are susceptible to infection with *M. tuberculosis* (TB), where in the period 1994–2013 there were 57 culture-confirmed cases in North America (Maslow and Mikota 2015). It has since been recognized in several other regions worldwide (Lewerin et al. 2005; Stephens et al. 2013; Angkawanish et al. 2010). As TB is an intracellular pathogen, immune responses to TB are likely to be from the cellular compartment, and many studies of TB infection in humans have demonstrated a cellular response (Jasenosky et al. 2015).

A number of studies by Landolfi et al. have investigated the expression of cytokines in TB-positive and TB-negative elephants to ascertain the nature of the immune response in elephants who succumbed to TB infection (Landolfi et al. 2010, 2015). An intracellular pathogen such as TB should trigger a predominantly T_H1 response in elephants as it does in other species; in support of this, Landolfi et al. (2010) found that the systemic mRNA levels of TNF-α and IFN-γ (both signature T_H1 cytokines) were significantly higher in TB-seropositive elephants. A more detailed study of lung specimens from both seropositive and seronegative elephants indicated that while poorly formed granulomas consisted predominantly of B cells and macrophages, well-formed granulomas had more T cells present in the local area, and that those T cells expressed either the cytokines TNF-α and IFN-γ together or IL-4 on its own (detected by in situ hybridization) (Landolfi et al. 2015). Given the lack of T cells in the lungs of elephants who were seronegative for TB (Landolfi et al. 2015), these findings indicate that T cells are necessary in controlling infection, but it is difficult to conclude whether a predominantly T_H1 or T_H2 response is required.

Autoimmunity

Little is known about autoimmunity in elephants. Cases of rheumatoid arthritis have been reported (Encyclopedia 1995; Clark et al. 1981) and a potential case of insulin-dependent (type 1) diabetes mellitus was reported in a 50-year-old Asian elephant. This diagnosis was made based on values of insulin, C-peptide, pancreas-specific amylase and fructosamine (van der Kolk et al. 2011). Because these are biomarkers of pancreatic beta cell destruction, it is possible that this case of diabetes was caused by an autoimmune attack on the pancreas. Hence, it

is likely that elephants are susceptible to a variety of autoimmune diseases, but cases have largely not been reported due to an inability to confirm the disease type.

What Can We Learn from Future Studies of the Elephant Immune System?

Better understanding of the elephant immune system may unlock key mysteries regarding the animal's susceptibility to pathogens. For instance, it is unknown why some Asian elephants succumb to EEHV hemorrhagic disease while others appear unaffected. Determining the significance of each arm (humoral and cellular) of the immune response to EEHV and how these vary among individual elephants will help in understanding susceptibility to this disease. If antibody is an important factor in protection, then perhaps unaffected elephants have better transfer of maternal antibodies compared to those that are susceptible. If T cells are a key factor in protection, then perhaps susceptible elephants do not possess EEHV-primed T cells. If this is the case, then an intervention such as a vaccine expressing EEHV antigens given to juveniles would be one way of arming these T cells prior to exposure to the virus itself. However, in making a vaccine that primes T cells, knowledge of the ideal type of T-cell response is necessary. We know from mouse immune studies that the predominance of a T_H2 response during a viral infection can increase mortality (Sin et al. 1999). Evidence from studies of *M. tuberculosis* infection in elephants points to an important role for T cells and T_H1 cytokines in response to this bacterium (Landolfi et al. 2015). In preliminary steps to identify an EEHV vaccine candidate, latently infected adult elephants were found to have significant T-helper-cell responses to several EEHV proteins. However, due to a lack of elephant-specific reagents, only one cytokine (IFN-γ) was studied, making it difficult to determine the nature of this T-helper response (Fuery et al. 2018). As we expand our tools for studying the elephant immune system while increasing the number of studies of response to specific diseases, we continue to gain a clearer understanding of the immune factors that make up a successful response to infection. EEHV hemorrhagic disease with high-level viremia still has a mortality rate of over 80% (Richman et al. 2014), and it is therefore essential to understand all factors that contribute to pathogenesis if we hope to save elephants suffering from this serious illness.

Lessons Learned About Cancer from Elephants

Studies of comparative biology have the potential to not only increase our understanding of how different species are able to cope with various threats to health, but also to teach us how to manipulate our own cellular responses to achieve improved outcomes to those same threats. For instance, a study of cancer across species revealed that elephants are less likely to die from cancer

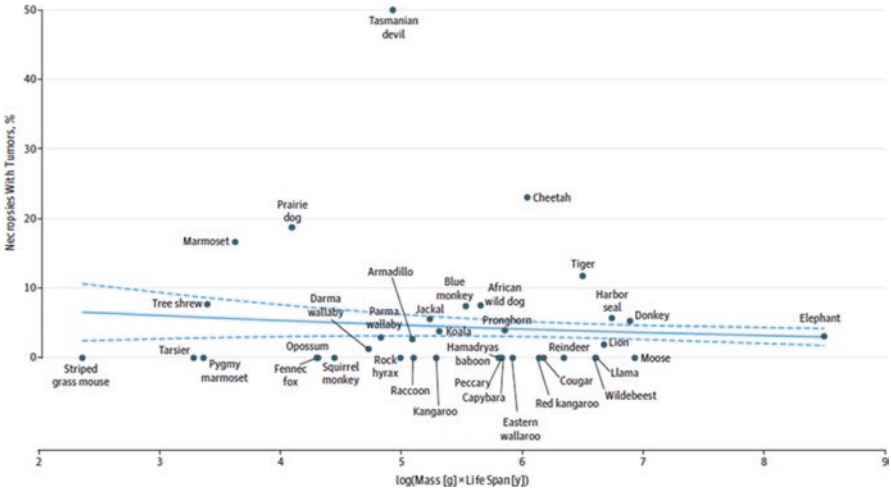

Fig. 3 Cancer incidence across species by body size and lifespan. Cancer incidence is not associated with mass and lifespan, as shown by the logistic regression (blue line: model fit; dashed lines: 95% confidence intervals). (Reproduced with permission from Abegglen et al. 2015) Copyright©(2015) American Medical Association. All rights reserved

compared to humans. Due to their large size and long life span, elephants would actually be predicted to develop high rates of cancer (a phenomenon known as Peto's Paradox) (Caulin and Maley 2011). However, analysis of cancer incidence across species revealed that cancer incidence was not associated with animal size or lifespan (Fig. 3) (Abegglen et al. 2015), and the elephant stood out as an example of a very large animal with a long lifespan and very little cancer. The genome of the African elephant was analyzed to look for genetic clues to explain this cancer resistance. Surprisingly, elephants were discovered to have additional copies of the *TP53* tumor suppressor gene. *TP53*, called the guardian of the genome, is a critical tumor suppressor gene mutated in 50% of all human cancers (Kastenhuber and Lowe 2017). Loss of one functional allele of *TP53* in germline DNA leads to a human cancer predisposition syndrome known as Li-Fraumeni syndrome with more than a 90% lifetime risk of developing cancer and multiple primary tumors (McBride et al. 2014). The elephant genome contains 20 *TP53* genes: 1 conventional gene with introns (*EP53*-ancestral) and 19 retrogenes that lack introns (*EP53*-retrogenes: 1–19) versus humans with 1 conventional *TP53* gene. Functional studies comparing p53 response in elephant cells versus human cells revealed that this *TP53* amplification was associated with increased p53-mediated, DNA damage induced apoptosis of elephant cells compared to human cells (Abegglen et al. 2015). Now that this mechanism of cancer resistance in elephants has been revealed, current work is under way to determine whether the human response to DNA damage

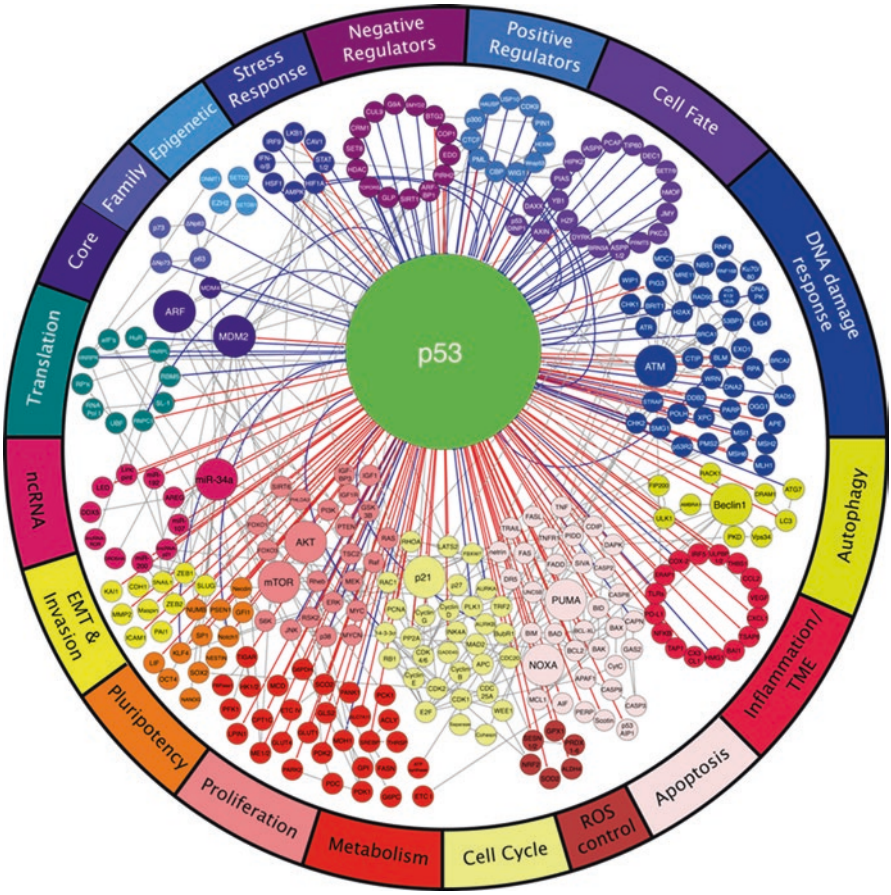

Fig. 4 p53 network. A wide variety of regulators govern the activity of p53 (top), which in turn controls many distinct biological processes (bottom). Each node represents a gene and each line an interaction. Blue lines: direct p53 inputs; red lines: direct p53 outputs. Noticeably, p53 controls effector processes by activating multiple target genes. Downstream pathways are highly interconnected (gray lines). Interactions are annotated as positive (arrow), negative (T-bar), or modifying (solid circle). (Reprinted from Kastenhuber and Lowe 2017, Putting p53 in Context, Copyright (2017), with permission from Elsevier)

can be altered in such a way as to mimic the *TP53* response that evolved in elephants. In parallel, work is under way to better understand the mechanism of action of elephant p53 retrogenes and how they affect key biological processes in the p53 network (Fig. 4) (Kastenhuber and Lowe 2017). p53 controls many biological processes related to cancer, including proliferation, cell cycle, invasion, apoptosis, autophagy, and even inflammation. Because the immune system is also intimately linked to cancer risk, it is likely that further study of elephant immunology and elephant p53 may also lead to further insight into the relatively low rates of cancer in elephants.

Comparative Immunology and Health

As we learn more about the elephant immune system and compare it to the human immune system (and that of other mammalian species), scenarios in which elephants respond more efficiently to certain types of infections might be revealed. Alternatively, we might learn that elephants can better regulate the control of their immune responses and perhaps make them resistant to immune-mediated disorders like autoimmunity and sepsis. Conversely, comparative immunology may also reveal that the immune systems of humans and other species work more efficiently in certain situations compared to elephants. Whatever knowledge we gain, we can then try to leverage that information to modulate the immune response of elephants to elephant-specific infections or even apply this to humans to trigger a more effective response where needed.

As described earlier, the role of the immune system in elephant cancer is unknown. Certainly, studies of human cancer have revealed that the immune system can both contribute to the development of cancer and be critical for the control of cancer (Janssen et al. 2017). The rapidly progressing field of immunotherapy is focused on manipulating the immune system in favor of cancer control (Farkona et al. 2016). Although malignant tumors in elephants are rare, they do still occur. In one reported case of metastatic fibrosarcoma in an Asian elephant, aggregates of lymphocytes were observed in the tumor (Liu et al. 2004). This case was associated with an earlier infection in the area where the primary tumor developed, and so the immune response, or lack thereof, may have played a role in this case of tumorigenesis. Close analysis of elephant tumors in the future will help us understand what contributes to cancer in elephants and the role, if any, of elephant immunology. It would be fascinating to discover that the elephant immune system contributes to the reportedly increased cancer resistance of elephants. Better understanding of efficient immune responses that control or eliminate cancer in any species may lead to the development of more effective cancer therapeutics that fight cancer in situations where ineffective immune responses allow tumors to grow out of control. As with all animals, the study of comparative immunology in the elephant has the potential to improve the health of all species.

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Comparative Phylogeny of the Nasopharynx-Associated Lymphoid Tissue

Ryan D. Heimroth and Irene Salinas

Introduction

Olfaction is one of the most conserved and ancient sensory systems in vertebrates (Niimura 2009a, b; Tacchi et al. 2014). The general neuronal and molecular mechanisms of vertebrate olfactory systems are considered to be highly conserved (Kermen et al. 2013; Saraiva et al. 2015). The olfactory system of vertebrates is a sensory neuroepithelium that is in direct contact with the environment. The main function of this sensory organ is to detect external chemical stimuli and rapidly convey this information to the central nervous system (CNS). Thus, olfaction is critical for the survival and success of every species.

Many pathogens have evolved neurotropic strategies to invade their hosts. As a consequence, the olfactory route is a common mode of invasion by neurotropic microorganisms, especially viruses. In addition to chemosensory functions, vertebrate olfactory organs are equipped to defend the host from any invading pathogen thanks to the presence of a network of immune cells and molecules known as the nasopharynx-associated lymphoid tissue (NALT). As with other mucosa-associated lymphoid tissues (MALTs), different vertebrate groups have evolved unique anatomies and immune strategies with respect to nasal immunity. The common themes and unique aspects of NALT and nasal immune responses in different vertebrate groups will be discussed in detail in this chapter.

Since 2014, great progress has been made toward understanding nasal immune systems in nonmammalian species. This knowledge has not only contributed to the basic biology of innate and adaptive immune responses at the nasal mucosa but has also resulted in novel nasal vaccines for use in veterinary medicine.

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One of the long-standing questions in the field of evolutionary immunology is why and when lymphocytes first began to form organized structures such as tonsils and Peyer's patches found in mammals. Recent studies on NALT in sarcopterygian fish have helped elucidate the origins of mucosal lymphocyte aggregates in vertebrates.

This chapter reviews the phylogeny of NALT in vertebrates and highlights the unique aspects of NALT compared to other vertebrate MALTs.

Comparative Anatomy of Olfactory Systems

Due to the intimate relationship between the immune cells that form NALT and olfactory sensory neurons (OSNs), we will first review the phylogeny of olfactory receptor (OR) genes as well as the anatomy of olfactory systems in different vertebrate groups.

Evolution of Olfactory Receptor Genes

The chemosensory process of detecting and differentiating tens of thousands of odorants is achieved via OR genes, the largest superfamily of genes within the vertebrate genome. OR genes belong to the G-protein-coupled receptor (GPCR) family with several transmembrane α-helical regions (Olender et al. 2008; Niimura 2009a, b). Interestingly, several immune receptors such as chemokine receptors also belong to this family. Analysis of insect genomes shows that these species contain OR genes that are GPCRs. Insect ORs are also responsible for the recognition of chemical signals from the external environment. Insect OR genes, however, do not share any sequence similarity with the OR genes of vertebrates. They are composed of several transmembrane α-helical regions but with inverted membrane topology. Thus, insect OR genes appear to have a different evolutionary origin than vertebrate OR genes (Nei et al. 2008; Nimura 2009a, b). Through evolution, OR gene expression was lost in Urochordates and reemerged in vertebrates where this gene family went through multiple divergences and expansions. All vertebrate OR genes have been characterized into two classes, fish class I genes and mammalian-like class II genes (Niimura and Nei 2003).

OR genes are expressed by OSNs in the vertebrate olfactory system. In those organisms where a vomeronasal organ (VNO) is present, vomeronasal neurons express vomeronasal receptor (VR) genes. In general, they follow the one gene, one cell dogma, although some exceptions have been reported. Signal transduction following the interaction between an odorant and a given OR/VR is very fast and is integrated by mitral cells in specialized glomeruli in the olfactory bulb (OB). Next, this information is sent to higher sensory areas of the brain. The molecular and anatomical organization of vertebrate olfactory organs are homologous from teleost to mammals, underscoring that these olfactory circuits are the optimal answer to the problem of chemical detection. Importantly, the fact that both aquatic and vertebrate

animals have similar olfactory systems indicates that both water-borne and air-borne odorants are detected by similar molecules, cells, and circuits.

Evolution of Olfactory Organs

Cephalochordates

The earliest circumstantial evidence for an olfactory system is in the lancelet (amphioxus) (Nimura 2009a, b). Although amphioxus lacks any type of identifiable olfactory organ or OB, dopaminergic neurons present in the anterior nerve cord resemble the vertebrate OB. These neurons provide the first glimpse of an olfactory system in evolution (Lacalli 2004; Satoh 2005).

Agnathans

Extant agnathans, hagfish and lamprey, possess a single nostril in comparison to gnathastomes, which are characterized by having two nostrils. In hagfish, this anterior nostril is situated above the mouth with sensory cells located on both sides of seven olfactory lamellae that are attached to the dorsal roof of the olfactory cavity. Sensory cells express ORs and have axons that terminate in the OB (Døving and Trotier 1998). Lampreys have a well-developed olfactory system with two separate olfactory epithelia (OEs), like tetrapods, that project to a medial OB (Chang et al. 2013). Currently, no immune cells (NALT) have been characterized in the primitive olfactory organs of agnathans.

Cartilaginous Fish

Sharks are known to have a uniquely sensitive sense of smell on the basis of the size of their OEs and OB (Niimura 2009a, b). The shark olfactory organ consists of numerous primary lamellae. These lamellae are covered by an OE populated with OSNs (Meredith and Kajiura 2010). OSNs have short, thin axons that extend directly into the OB, where in glomeruli they interact with mitral cells (Hamdani and Døving 2007; Yopak et al. 2015). No studies have reported the presence of NALT in cartilaginous fish.

Bony Fish

The teleost olfactory organ is composed of a paired anatomical structure, the olfactory rosette, containing both OSNs and neurons expressing VRs (Taniguchi and Taniguchi 2014). The two olfactory rosettes are located within two separate nasal cavities with openings to the external environment. Each rosette is made up of variable numbers of olfactory lamellae attached to a median raphe (Fig. 1a) (Atta 2013). Each lamella may include separate sensory and nonsensory regions in some species such as salmonids. The sensory component of the olfactory lamella contains OSNs including microvillus, ciliated, and crypt cells, while the nonsensory regions, considered the mucosal epithelium, have abundant goblet cells. The OE projects to a primitive OB (Fig. 1a), which is referred to as the fish-type main olfactory bulb (MOB). Interestingly, the recombination activation gene 1 (Rag1) is expressed in a

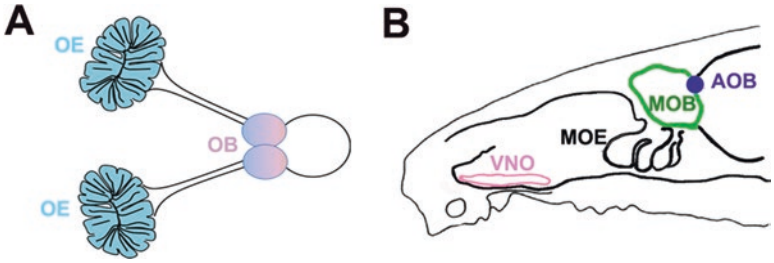

Fig. 1 (**a**) General anatomy of teleost olfactory system. OE: olfactory epithelium; OB: olfactory bulb. (**b**) General anatomy of rat olfactory system. MOE main olfactory epithelium, VNO vomeronasal organ, MOB main olfactory bulb, AOB accessory olfactory bulb

subset of zebrafish olfactory neurons (Feng 2005), but this observation has not been linked to any immune roles in these cells.

Sarcopterygian Fish

Lungfish are the closest extant relative to all tetrapods (Zardoya and Meyer 1996; Amemiya et al. 2013). Lungfish represent the transition to terrestrial life during vertebrate evolution and are exposed over their lifetime to both water-borne and airborne antigens (Tacchi et al. 2015; Sepahi and Salinas 2016). The olfactory organ of lungfish is composed of a series of suspended lamellae projecting from a midline groove with epithelial crypts between the lamellae. The lamellae of the nasal cavity harbor ciliated and microvillus OSNs. A primordial vomeronasal organ has been described in lungfish (Gonzalez et al. 2010; Nakamuta et al. 2012; Taniguchi and Taniguchi 2014). Unlike tetrapods, the lungfish VNO is not anatomically distinct but rather is scattered in epithelial crypts at the base of lamellae.

Amphibians and Reptiles

Amphibians are the first tetrapods to possess a mammal-type MOB, which is also present in reptiles, birds, and mammals. Anuran amphibian's nasal cavity is composed of three chambers, upper, middle, and lower. These chambers are lined with OEs, nonsensory epithelium, and vomeronasal sensory epithelium (VSE), respectively (Oikawa et al. 1998). The OE and VSE in anurans correspond to that of the mammal-type OE and a component of mammal-type VNO, respectively (Taniguchi and Taniguchi 2014).

The interaction between the nasal and oral cavities and the development of the VNO vary across reptilian species (Allison 1953). The VNO in lizards corresponds to a mammalian-type VNO. It is isolated from the nasal cavity and, via the vomeronasal duct, anteriorly opens into the oral cavity and ends blindly at the posterior end (Taniguchi and Taniguchi 2014). The nasal cavity is a single rounded chamber without a nasal concha and appears almost completely covered with a mammalian-like OE. The OE projects to a MOB that closely corresponds to the mammal-type MOB (Brykczynska et al. 2013).

Aves

It has been hypothesized that, owing to avian flight, the olfactory system is not well developed in birds. The avian olfactory system is composed of a MOE and MOB but lacks the accessory olfactory system of mammals. The avian nasal cavity originates at the end of the beak and ends in front of the eye pit, which is divided into three segments. The OE is on a single spiral turbinate inside of the nasal cavity (Gomez and Celii 2008; Kang et al. 2013). The OSNs are goblet-shaped cells with sensory cilia projecting from a terminal dendritic knob that is embedded in the OE. The cilia extend into the olfactory cavity and possess axons that project into glomeruli in the OB (Gomez and Celii 2008).

Mammals

The mammalian olfactory system is divided into two distinct systems: the main and accessory olfactory systems (Fig. 1b). The main olfactory system is composed of the MOE as the receptor of external chemical stimuli and the MOB. The accessory olfactory system is composed of the VNO and septal olfactory organ of Masera as receptors, with the accessory olfactory bulb (AOB) as the primary center (Taniguchi et al. 2010). The mammalian olfactory system has evolved to be the most complex olfactory system in vertebrate evolution, allowing for the successful survival and diversification of the mammalian lineage. However, it is worth mentioning that different mammalian groups have more or less developed olfactory systems. For instance, rodents rely heavily on olfaction, whereas humans have a less developed sense of smell. This is illustrated by the presence of over 1000 OR genes in the mouse genome compared to about 350 intact OR genes in the human genome (Malnic et al. 2003; Niimura and Nei 2003).

Evolutionary Origins of NALT

NALT (nasopharynx-associated lymphoid tissue), according to the Mucosal Immunology Society (MIS), is defined as the lymphoid tissue of Waldeyer's pharyngeal ring, unpaired nasopharyngeal tonsil, and the paired palatine tonsils (Perry and Whyte 1998; Bradtzaeg et al. 2008). Since the MIS does define NALT in non-mammalian hosts, the mammalian definition requires expansion to encompass other groups of species as well as the concept of diffuse NALT. Thus, from the perspective of comparative immunologists, NALT refers to the network of immune cells, regardless of their level of organization, that reside in the olfactory organ of an animal. Since the first bona fide olfactory organ is found in agnathans, we will review our knowledge on NALT from agnathans to mammals.

Agnathans

Agnathans possess an adaptive immune system based on leucine-rich repeats (LRRs) rather than immunoglobulins (Igs) and T-cell receptors used by

gnathostomes. These highly diverse LRR segments along with an invariant membrane-proximal stalk make up variable lymphocyte receptors (VLRs). Though morphologically different, VLRs and Igs provide a similar adaptive immune response such as delayed-type hypersensitivity, circulating agglutinins after immunization, and rejection of second set skin allografts at an increased rate (Pancer et al. 2004; Guo et al. 2009). Thus it is clear that agnathans have a robust adaptive immune system. To date, the presence of VLR^+ immune cells or any other immune cell type has not been investigated in the agnathan olfactory organ.

Cartilaginous Fish

Cartilaginous fish have an adaptive immune system based on B and T cells. As mentioned earlier, cartilaginous fish are highly sensitive to chemical stimuli in their environment due to their particularly acute olfactory system that they use to locate their prey (Niimura 2009a, b). The olfactory organ of cartilaginous fish possesses a large surface area that is subject to microbial invasion as well as parasites. Thus, it is likely that NALT also exists in this vertebrate group; however, we currently have no information regarding NALT in cartilaginous fish.

Bony Fish

Teleost fish are a large and diverse infraclass within the class Actinopterygii, which makes up the majority of extant vertebrate species (Volff 2005). NALT was first described in bony fish in 2014 as a diffuse network of lymphoid and myeloid cells within the epithelium as well as in the lamina propria of the OE. Similar to other teleost MALT, no organized lymphoid structures such as tonsils or adenoids are found in teleost NALT (Fig. 2) (Tacchi et al. 2014). Although detailed studies on

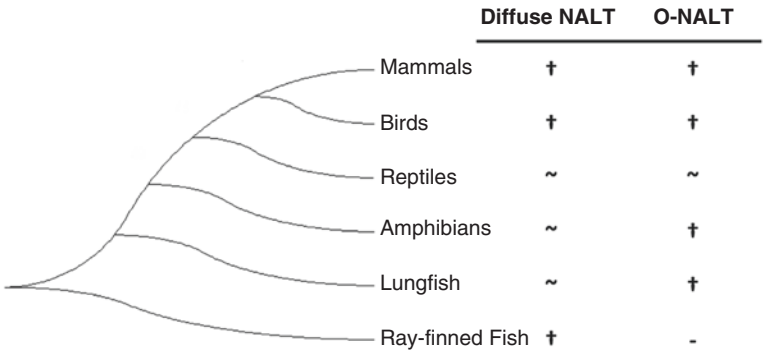

Fig. 2 Phylogenetic tree showing presence of NALT (diffuse and O-NALT) in different vertebrate groups. – indicates not present, ~ indicates that information is not available but feature is predicted to be present, † indicates presence

teleost NALT are restricted to trout, histological examination of the olfactory rosettes from four different teleost classes showed the presence of lymphocyte-like cells in all specimens, suggesting that NALT may be present in most teleosts. The two main regions of the trout olfactory organ (mucosal epithelium and sensory epithelium) appear to generate two unique microenvironments that compartmentalize the nasal immune system in this species. The mucosal regions of the trout olfactory organ express greater levels of key chemokines and adhesion molecules, creating an environment where $CD8^+$ T cells cluster as sentinels in the absence of antigenic stimulation (Sepahi et al. 2016). B cells and T cells are also present in the neuroepithelium among the OSNs, but they do not form groupings like those observed in the mucosal regions.

Sarcopterygian Fish

Lungfish have an organized NALT (O-NALT). Although diffuse NALT has not been described in sarcopterygian fishes, it is expected to be present (Fig. 2). Lungfish O-NALT structures have been proposed as the evolutionary forerunners of mammalian tonsils and Peyer's patches, and they are called lymphoid aggregates (LAs). Both encapsulated and unencapsulated LAs have been found in the nasopharyngeal mucosa of both *Protopterus dolloi* and *Protopterus annectens*. These LAs are embedded within the submucosa of the upper or lower mouth cavity or directly underneath the epithelium (Sepahi and Salinas 2016). The covering epithelium is modified with lower numbers of visible goblet cells. LAs are approximately 350 μm in diameter and are lymphocyte-rich structures. However, distinct T-cell or B-cell zones are absent, and no germinal centers can be seen in response to bacterial infection. Pathogenic bacteria delivered to the nasal cavity of *P. annectens* are transported to nasal LAs, and dendritic-like cells appear to be the main bacterial antigen-uptake cell type. African lungfish also form inducible LAs in response to nasal infection. These smaller LAs may be the equivalent of mammalian tertiary lymphoid structures (Tacchi et al. 2015). To date, NALT in coelacanths has not been described.

Amphibians and Reptiles

As with agnathans, amphibian and reptile NALT have not been well studied (Fig. 2). Anuran amphibians have been shown to possess semiorganized LAs in their lingual and sublingual mucosa (Goldstine et al. 1975; Tacchi et al. 2015). LAs were also observed in association with the OEs in *Lithobates catesbeianus* (American bullfrog) tadpoles and other anuran amphibians. Diffuse NALT has not been investigated in amphibians.

NALT has not been investigated in reptiles. It is known that reptile respiratory diseases are prevalent especially in captive animals (Schumacher 2003). These infections range from those by *Mycoplasma agassizii* in certain turtles to

adenovirus infestations in snakes (Marschang 2011; Jacobson et al. 2014). Thus, NALT (if present) should play an important role in reptiles during respiratory infection and should be investigated in detail in the future.

Aves

Avian NALT has been investigated primarily in chickens and ducks (Ohshima and Hiramatsu 2000; Kang et al. 2013, 2014). Chicken NALT consists of both diffuse and O-NALT (Fig. 2). Avian O-NALT includes an abundant amount of secondary lymphoid follicles in the lamina propria and underneath the epithelial wall of the nasal meatus in the nasal cavity. Avian O-NALT structures, similar to those of mammals, that possess a follicle-associated epithelium (FAE) that is composed mainly of nonciliated cells (Kang et al. 2013). NALTs in birds are more organized than in ectothermic vertebrates with germinal centers segregating B cells from T cells. Lacking lymph nodes, NALT plays a vital role in activation of immune responses in birds following nasal antigen exposure (Śmiałek et al. 2011; Kang et al. 2013). Thus, as discussed later, vaccines targeting NALT in chickens are used in the farming industry.

Mammals

NALT was first described in rats and was shown to form postnatally as an aggregation of lymphoid cells (Kiyono and Fukuyama 2004). NALT in rodents is characterized by paired lymphoid aggregates in the caudoventral portion of the nasal passages at the beginning of the nasopharyngeal duct (Debertin et al. 2003; Cesta 2006). There seem to be no distinguishable structural or functional differences for NALT among rodents (mice, rats, and hamsters). NALT in rodents is considered to be the equivalent of Waldeyer's ring and tonsil in humans. In humans, NALT includes adenoids along with collections of lymphoid follicles in the lateral and posterior walls of the nasopharynx around the opening of the Eustachian tube and the nasopharyngeal surface of the soft palate (Asanuma et al. 1997; Haley 2003). In all mammals, NALT is highly organized with germinal centers and T-cell zones of roughly equal size and antigen-presenting cells scattered throughout the tissue. Normal murine NALT is composed of dense aggregates of lymphocytes comprising 80–85% uncommitted B cells ($sIgM^+$) and low frequencies of IgA^+ and IgG^+ (3–4% and 0–1% respectively). Antigen-specific $CD4^+$ T cells produce T_H2-type cytokines for antigen-specific B-cell activation, proliferation, and differentiation. Mononuclear cells isolated from murine NALT are 30–40% $CD3^+$ T cells with a majority of that proportion coexpressing CD45RB, indicating these are resting T cells. These T cells are in a 3:1 ratio of CD4/CD8, providing plenty of helper T cells after antigen stimulation (Heritage et al. 1997). This evidence supports the notion that NALT exhibits characteristics of a

mucosal inductive site. In nonhuman primates, NALT is more extensive than in rodents, reaching the lateral surface of the nasal cavity (Haley 2003; Cesta 2006). NALT has thus become a target tissue for an induction site for nasal vaccination in both veterinary medicine and human medicine.

Nasal Immune Responses in Vertebrates

Nasal Immune Responses in Bony Fish

Given the body of work that exists on the topic, this section of the chapter will focus on nasal immune responses in bony fish, birds, and mammals. Nasal vaccines are well studied in these groups, shedding light on the cellular and molecular mechanisms of nasal immune responses. Investigation of the nasal immune responses in teleost fish is currently limited to rainbow trout and the nasal delivery of a live attenuated viral vaccine, infectious hematopoietic necrosis virus (IHNV). Future studies will expand our view of nasal immune responses in other fish hosts and in response to other pathogens such as bacteria and parasites. Viral antigen is taken up by both the mucosal region and the sensory region of the OE of trout. Moreover bath delivery of nanoparticles to zebrafish results in uptake by the olfactory epithelium. In rainbow trout nasally vaccinated with IHNV vaccine, the peak of the innate immune response occurs 4 days post vaccination as measured by oligomicroarray transcriptomic analysis (Tacchi et al. 2014). Gene expression responses were paralleled by histological observations showing enlargement of the lamina propria, angiogenesis, and infiltration of myeloid cells 4 days post vaccination. This inflammation decreases by day 14, showing a response similar to that of a lack of significantly modified proinflammatory cytokines in day 14 microarray analysis (Tacchi et al. 2014). The peak of the innate immune response also coincides with the last day that the viral antigen is detectable in the nasal mucosa (Larragoite et al. 2016). Innate immune responses were characterized by increased expression of proinflammatory cytokines, chemokines, antimicrobial peptides, Toll-like receptors, and antiviral immune genes. One of these significantly modified genes was C-C motif chemokine 19 (CCL19). Further studies found that CCL19 has diversified into six CCL19-like genes in salmonids. Of the six CCL19-like molecules in trout, CK12a appears to have specialized nasal antiviral immune responses and is able to orchestrate systemic immune responses following nasal vaccination in trout (Sepahi et al. 2017).

Teleost NALT includes resident populations of B and T cells (Tacchi et al. 2014; Sepahi et al. 2016; Sepahi and Salinas 2016). Similar to other MALTs, trout NALT B cells are 50% IgT^+ B cells and 50% IgM^+ B cells (Tacchi et al. 2014). $CD8^+$ T cells account for 8% of all lymphocytes in trout NALT. In response to nasal vaccination, adaptive immune responses at the gene level peak at day 14. At the gene level, IgM but not IgT expression was stimulated in NALT at this time point. However, B-cell and antibody immune responses at the protein level have not been studied to date.

Aves

As mentioned earlier, birds have both diffuse and O-NALT and both compartments take up antigen. Combination of viral antigens with an adjuvant increases antigen uptake in chicken NALT. Innate immune cells are not very abundant in the avian respiratory system compared to rodents, with only a small number of phagocytic cells found throughout the respiratory tract. Most of the myeloid cells present in avian NALTs are polymorphonuclear cells (PMNs), specifically heterophils (Śmiałek et al. 2011). The significance of low phagocytic cell numbers in the bird olfactory system is enigmatic and requires further examination. The nasal adaptive immune responses of birds are better characterized than the innate immune responses. Intranasal vaccination of chickens is an effective method to control infectious diseases such as viral diseases that threaten the poultry industry. As is the case in teleosts and mammals, nasal vaccination is effective at inducing immune responses in other mucosal sites as well as systemically. For instance, upon local nasal immunization with a DNA vaccine against Newcastle diseases, specific IgA titers can be detected in serum, bile, and the Harderian gland. Antibody titers quickly increase within 3 weeks after immunization and peak at 7 weeks post immunization (Zhao et al. 2016). This vaccine results in antigen-specific IgA and IgY antibodies but very low amounts of specific IgM. This immune response shows an isotype switch within plasma cells with no gene conversion or somatic hypermutation in the germinal centers. Ducks are reservoirs of avian influenza virus. Following nasal vaccination of ducks with inactivated influenza vaccine, specific serum hemagglutination inhibition titers are detected as early as 7 days post vaccination (Kang et al. 2012).

Mammals

Mammalian NALT (diffuse and organized) is composed of myeloid and lymphoid cells that are required for the induction and regulation of mucosal immune responses to antigens that invade the nasal cavity. Overall, our knowledge of nasal immune responses in mammals is limited compared to gastrointestinal immune responses. Immune responses in the olfactory organ can also result in changes in the immune molecules secreted into the nasal mucus. Thus, mammalian nasal secretions can be used noninvasively to gain insights into the innate and adaptive immune responses of the upper respiratory tract. Microfold cells (M-cells) are embedded in respiratory and gastrointestinal epithelium joined to neighboring epithelial cells with tight junctions. These cells are responsible for the uptake and transcytosis of antigens and microorganisms from the lumen to the O-MALT (Hathaway and Kraehenbuhl 2000). Targeting nasal M-cells has become an area of great interest for vaccine delivery.

Innate immune cells are abundant in mammalian NALT and are known to play critical roles during immune responses. Mast cells are abundant in the OE of mammals and are known to be active during allergic rhinitis in humans. Additionally, nasal mast cells promote allergic reaction by increasing expression of Fcε and by

inducing synthesis of IgE by B cells. Nasal basophils and eosinophils also respond to allergic disease (Galli and Tsai 2013). Additionally, human nasal epithelial cells appear to be important mediators of innate immune responses against live attenuated influenza virus in humans. These responses are largely driven by type-III interferon and are inflammatory in nature (Forero et al. 2017). Substantial evidence supports the idea that nasal vaccination in mammals also leads to distant mucosal and systemic specific immune responses at the antibody level. Intranasal exposure to viral antigens causes both humoral and cellular specific immune responses in NALT. Antibody responses following nasal immunization can be detected systemically and at other mucosal sites such as the reproductive mucosa (Davis 2001). The ability of nasal vaccines to induce strong systemic antibody responses appears to rely on the highly vascularized nature of the OE. Additionally, cytotoxic T-cell responses can be detected in murine NALT nasally infected with a virus (Asanuma et al. 1997). Finally, chronic foreign antigen exposure results in additional organization of germinal centers in O-NALT structures of mice, with subsequent clonal expansion of antigen-induced IgA^+ B cells.

In summary, as presented in this chapter, the investigation of the phylogeny of NALT in different vertebrate groups is an emerging area of study. NALT is present in both aquatic and terrestrial vertebrates, underscoring the importance of protecting this mucosal site. NALT, like other MALTs, became progressively more organized during the vertebrate transition from water to land. Thus, nasal lymphocytic structures similar to human tonsils evolved prior to the emergence of tetrapods. The presence of NALT in ectothermic and endothermic vertebrates provides an excellent route to deliver mucosal vaccines. The unique aspects of the innate and adaptive immune responses that take place at the local nasal environment as well as how these responses are integrated with other parts of the immune system remain poorly characterized compared to other MALTs.

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Part III

Comparative Immunology: Future Paths, Climate Change, Environmental Influences, Cancer, Therapy

An Introduction to Ecoimmunology

Laura A. Schoenle, Cynthia J. Downs, and Lynn B. Martin

"…variation itself is nature's only irreducible essence. Variation is the hard reality…" from The Median Isn't the Message by Stephen J. Gould

Immunological research has made astounding progress in describing the cellular and molecular mechanisms underlying the defenses to parasites. Researchers have developed life-saving medical procedures based on experiments conducted on genetically identical animals that have been raised in sterile, carefully controlled laboratory housing (National Research Council 2004). Yet, the reality remains that animals, including humans, are not genetically homogeneous, and both develop and live in variable environments. As a result, the ability to defend against parasites (i.e., immunity) varies among individuals, populations, and species (Schmid-Hempel 2003). Why should we care about variation in immunity? The sources and consequences of immune variation have implications for public health, wildlife management, agriculture, and conservation. Understanding the sources of variation in immunity, such as which genes influence the likelihood of developing diseases or how nutrition influences susceptibility to infection, should help identify management strategies to minimize the effects of disease (Jones et al. 2014; Bulik-Sullivan et al. 2015; Farh et al. 2015). Furthermore, integrative approaches to immunology

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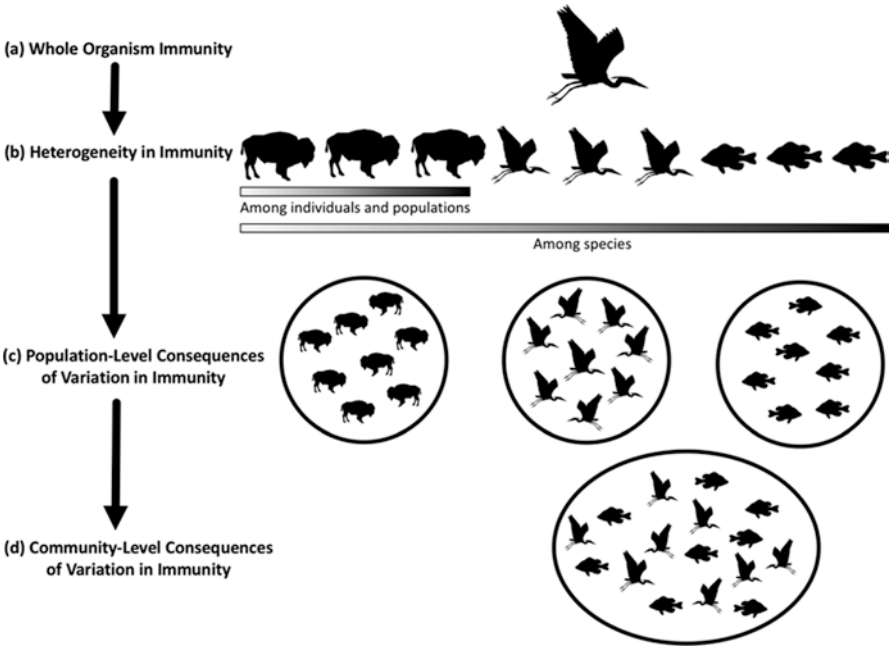

Fig. 1 The central themes in ecoimmunology integrate across levels of biological organization. (**a**) Ecoimmunologists study immunity in the context of the whole organism. Thus, immunity (as defined in the body of the paper) is a product of interacting physiological systems (e.g., nervous, endocrine, and immune systems). These interacting physiological systems underlie complex immune traits, including parasite resistance and tolerance. (**b**) Immunity varies among individual organisms because of individual-specific traits, such as developmental history and genetics, as well as across contexts due to variable environmental conditions and life history stage. Heterogeneity in immunity is also found among species. (**c**) and (**d**) Variation in immunity among individuals and species can influence host–parasite eco-evolutionary dynamics within populations and communities. Animal silhouettes CC by 4.0 to Natasha Sinegina. (Super Coloring 2017)

will enhance our ability to understand the equally intriguing consequences of that variation, such as disease dynamics and the evolution of life history traits.

Ecoimmunology is the study of the causes and consequences of variation in immunity (Brock et al. 2014; Downs et al. 2014). This field places immunology in evolutionary and ecological contexts across levels of biological organization, from the organism to community-level interactions (Fig. 1). Furthermore, ecoimmunology builds upon and complements the field of comparative immunology, the focus of this volume. In this chapter, we review the approaches and tools used by ecoimmunologists and discuss insights obtained from ecoimmunology as they relate to three central themes: (1) immunity in the context of the whole organism, (2) heterogeneity in immunity, and (3) the broad consequences of individual variation in immunity. We also provide a brief overview of the field's history in Box 1. Finally, we will reflect on the future of ecoimmunology.

Immunity in the Context of the Whole Organism

Ecoimmunologists investigate immunity as a characteristic of the whole organism rather than a series of isolated mechanisms. Although this holistic strategy has long been the goal, the approaches used to characterize immunity have evolved over time. Early efforts were relatively simplistic and focused on measuring the strength of responses to immune challenges or the magnitude of available defenses (Martin et al. 2006b). New information about the mechanisms underlying immunity and the costs of parasite defenses have led to fundamental changes in how immunity is studied (Brock et al. 2014). The complexities of immune function and the interdependent nature of physiological systems is facilitating a transition to more integrative approaches today.

Immunocompetence: The Search for a Simple Immune Measure

The quest for a measure of organismal-level immunity began with the concept of immunocompetence, or an individual's ability to prevent or eliminate a parasite infection (Demas et al. 2011). Because immunocompetence is so broad, it is logistically difficult (or even impossible) to assess. Moreover, faith in the validity of the concept led early ecoimmunologists to underestimate the complexities of parasite defenses. Early ecoimmunology studies tended to use one or two immune metrics and interpreted measured variation as an indication of overall antiparasite defense (e.g., Saino and Calza 1999; Johnsen et al. 2000; Møller and Erritzoe 2000). One such immune metric was the phytohemagglutinin (PHA) skin test, which was thought to signal the strength of T-cell-mediated immunity (Smits et al. 1999; Tella et al. 2008). For example, a study of barn swallows (*Hirundo rustica*) demonstrated that adults producing offspring with strong responses to the PHA skin test were less likely to survive until the next breeding season (Saino and Calza 1999). The authors interpreted this result as indicating that producing highly immunocompetent offspring was associated with survival costs. Although this approach pointed to interesting relationships between immunity and other traits, it began to fall out of favor after researchers raised concerns about the general concept of immunocompetence and the interpretation of this and other specific immune assays (Adamo 2004; Viney et al. 2005; Martin et al. 2006b). Whereas immunologists know well that the immune system is complex and different immune pathways are important for responding to different parasites, early ecoimmunologists hoped to be able to overcome this problem and capture something meaningful with simple assays. Now, though, the concept of immunocompetence is understood as too broad to be useful (Demas et al. 2011). This recognition arose when ecoimmunologists began interpreting results of their assays in terms of the specific immune components measured, not immunocompetence in a broad sense (Martin et al. 2006b). This epiphany was reinforced when researchers found that variation in immune measures, such as the PHA skin test or white blood cell counts, only sometimes predicted responses to an actual infection, and usually they did so weakly (Adamo 2004; Adelman et al. 2014).

What the concept of immunocompetence sought to capture, though, that there is an optimal immune response for different species or individuals, still resonates in the field today (Adamo 2004; Demas et al. 2011). But just what is an optimal immune response?

Optimal Immunity: More Isn't Always Better

The foregoing question is probably the main thing that distinguishes traditional immunology from ecoimmunology. Whereas all immunologists appreciate that more immunity is not always better (e.g., overzealousness of the immune system can cause damage to the host), ecoimmunologists have made this and other costs of immunity central to their research and resultant theories. Natural selection favors the optimal immune response, the magnitude and type of immune response that maximizes fitness (the total reproductive output within an individual's lifetime). If there are costs to immunity, the optimal immune response is not necessarily the strongest one (Schmid-Hempel 2003). For example, Soay sheep (*Ovis aries*) with higher antibody concentrations are more likely to survive the winter but also have lower reproductive success (Graham et al. 2010). Thus, mounting an antibody response is an important defense, but it carries a cost. In this and probably other systems, antibodies help control parasite burdens, which in these sheep are most problematic during winters, but they also can harm host tissue if they are autoreactive. The optimal immune response will therefore be balanced between the risk of dying from infection versus producing viable offspring (Fig. 2). Ultimately, any costs required to develop, maintain, or activate defenses will impact the expression and persistence of immune traits (Schmid-Hempel and Ebert 2003). A cornerstone of ecoimmunology is that immunity to parasites has costs at both evolutionary and individual scales (Brock et al. 2014).

The Costs of Immunity Influence Evolutionary Trade-Offs

Evolutionary costs of immunity can arise because of genetic architecture. The genes that influence immune phenotypes can regulate other traits as well or can be inherited with other genes. Similarly, transcription factors that regulate immunity may also regulate other traits (Downs et al. 2014). As a result, there are negative genetic correlations between traits that promote immunity and traits associated with other characteristics that enhance fitness (Rolff and Siva-Jothy 2003; Ardia et al. 2011). Therefore, the evolution of immunity is influenced by genetically correlated traits.

Antagonistic pleiotropy occurs when a single gene causes negative correlations between an immune trait and another trait that can enhance fitness (Rolff and Siva-Jothy 2003; Roff and Fairbairn 2007). Antagonist pleiotropy can force trade-offs that are not easily altered by selection because breaking these trade-offs requires a gene duplication event (Roff and Fairbairn 2007). Fruit fly (*Drosophila melanogaster*) defenses against parasitoid wasps present one of the most extreme examples of antagonistic pleiotropy influencing immunity. A wasp oviposits eggs inside the host fly, and if allowed to develop, the wasp's offspring kill the fly larva by consuming it

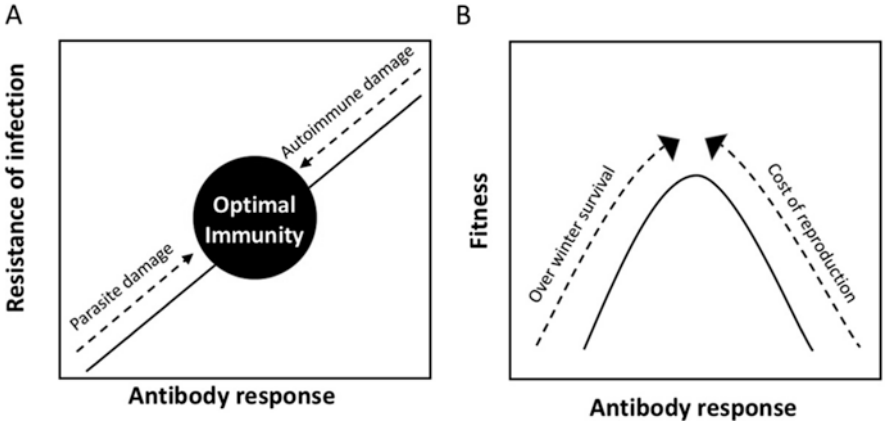

Fig. 2 The antibody response of Soay sheep provides parasite protection but also has fitness costs, and as a result, the strongest immune response does not maximize fitness (Graham et al. 2010). (**a**) Natural selection should lead to intermediate levels of immunity and resistance to parasites when there are both fitness costs and benefits to the immune response. Increasing the costs of parasite damage will favor a stronger antibody response. However, high levels of antibody production that cause autoimmune damage will favor a dampened immune response. (**b**) Individuals producing highest antibody responses had greater overwinter survival but also reduced breeding success. Individuals with the intermediate, optimal antibody response should have the highest fitness. (Adapted from Martin and Coon 2010 with permission from AAAS)

from the inside out. Some *Drosophila* genotypes can resist the wasp's attack by producing hemocytes (blood cells) that encapsulate the eggs, destroying them (Poirie et al. 2000; Kraaijeveld et al. 2001). However, this and other defenses have costs: more resistant *Drosophila* genotypes (i.e., those that can better control infections) also have slower feeding rates than more susceptible *Drosophila*. Subsequently, resistant individuals are less likely to survive in high-competition environments (Kraaijeveld and Godfray 1997; Kraaijeveld et al. 2001). Evidence suggests that a single gene might regulate the trade-off between competition and parasitoid defenses (Hodges et al. 2012). Antagonistic pleiotropy also is suspected to play a role in human aging (Franceschi et al. 2000; Goto 2008). Inflammation, an important part of the innate immune response, also contributes to cellular senescence, tissue damage, and several diseases associated with aging such as arthritis and Alzheimer's (Lambeth 2007; Freund et al. 2010). The protective advantages conferred by inflammation in younger individuals becomes costly as those individuals age, when inflammation can become chronic and more likely to cause disease (Lambeth 2007; Freund et al. 2010).

Often it is unclear whether immune trade-offs with a genetic basis are caused by pleiotropy or linkage disequilibrium, when genes are inherited together because of their proximity on a chromosome (Saltz et al. 2017). In these scenarios, correlations between traits can be broken through genetic recombination. A meta-analysis revealed that artificial selection for rapid growth in chickens led to attenuated immune responses (van der Most et al. 2011). However, selection for various

immune traits was not always associated with reduced growth rates, and the strengths of relationships varied among lines of chickens and forms of immunity, suggesting a mechanism other than pleiotropy (van der Most et al. 2011).

The degree to which immunity is traded off with other traits depends on the relative strength of the evolutionary pressures (e.g., parasite risk, predation, conspecific competition) (Lazzaro and Little 2009). The outcome of evolutionary immune trade-offs thus vary across environments. Defenses of *Drosophila* against the parasitoid wasps vary geographically, indicating that variable selection pressures across environments lead to local optima for immunity (Kraaijeveld and Godfray 1999). For example, humidity influences the biological pathways enabling resistance to parasitoids and, thus, can alter the costs of immunity. As a result, humidity could shape the local evolution of defenses.

The Costs of Immunity Cause Trade-Offs Within Individuals

The aforementioned evolutionary costs of immunity have physiological bases. For instance, the development, activation, and even mitigation of parasite defenses can impose substantial costs on organisms (Lochmiller and Deerenberg 2000). Experimental activation of immune responses can cause increases in metabolic rate and body mass loss, indicating that immunity has energetic costs (Demas et al. 2011; Ots et al. 2001; Freitak et al. 2003; Martin et al. 2003; Eraud et al. 2005; Amat et al. 2007). Nutrient availability is also critical. For example, carotenoid availability limits the expression of immune traits in juvenile (Saino et al. 2003; Klasing et al. 2006; Tyndale et al. 2008) and adult (Blount et al. 2003; Amar et al. 2004) birds and fish. Similarly, the amino acid lysine is integral to leukocyte function and the biosynthesis of proteins associated with innate immunity (Iseri and Klasing 2014). Indeed, in chickens, the lysine required during the acute-phase response to a bacterial infection is equivalent to that in 355 feathers or 17% of an egg (Iseri and Klasing 2014). Because any resources allocated to immunity are unavailable for other activities, the resource costs of immunity can drive allocation trade-offs (Norris and Evans 2000; Lochmiller and Deerenberg 2000; Ardia et al. 2011; Downs et al. 2014). For example, energy limitation underlies a trade-off between wound healing and reproduction in the ornate tree lizard (*Uta ornatus*) (French et al. 2009). Injuries occur frequently in nature, and wound healing involves multiple components of the immune system. Experimentally wounding a lizard's skin reduced egg follicle size if the studied female was food restricted. However, when food was unlimited, there was no trade-off between wound healing and reproductive investment (French et al. 2007a, b). In addition, food restriction had no effect on wound healing in females before reproduction (French et al. 2007a). Similar allocation trade-offs between reproduction and immunity are relatively common and can be found across taxa, including insects, mammals, reptiles, and birds (Schwenke et al. 2016; Norris and Evans 2000; Hayes and Shonkwiler 2001; French et al. 2009).

Allocation trade-offs with immunity are not limited to reproduction. Other energetically costly functions, such as tissue growth or dispersal to new areas (e.g., migration), are also subject to allocation trade-offs (Soler et al. 2003; Moreno-Rueda 2010; Altizer et al. 2011). For example, some forms of immune function in

thrushes (*Catharus* spp. and *Hylocichla mustelina*) are reduced during migration when compared to breeding conspecifics (Owen and Moore 2006), and resource availability is likely to underlie these reductions (Owen and Moore 2008). At a migratory stopover site, thrushes in poor energetic condition (lower fat deposits, muscle mass, body-size-controlled condition index) had lower leukocyte and lymphocyte counts; among birds captured and brought into captivity, mass gain was associated with greater responsiveness to an immune challenge (Owen and Moore 2008). Importantly, the resource costs of immunity will only lead to trade-offs under conditions of resource limitations. If individuals can obtain sufficient resources, they can support immunity in addition to other costly activities, and there will be no allocation trade-off (van Noordwijk and de Jong 1986; Downs et al. 2014).

Immunity not only imposes energetic costs on individuals; activating immune defenses can harm hosts as well. Autoimmune disease is one form of such a cost. For example, Guillain-Barré syndrome is the result of mounting an immune response against neurons (Yuki and Hartung 2012). Lupus occurs when antibodies target multiple host organ systems (Lipsky 2001). Immune responses targeting an infection can also cause damage to hosts. During malaria infections, the loss of red blood cells is often much higher than could be achieved by the malarial parasites alone (Price et al. 2001; Evans et al. 2006). Evidence suggests that the immune response, specifically T-cell activity, damages uninfected red blood cells, increasing the severity of malarial anemia (Evans et al. 2006). In addition, immune responses to malaria and other parasites can increase concentrations of reactive oxygen metabolites (ROMs), often called free radicals (Costantini and Møller 2009). High concentrations of ROMs can damage tissue and even accelerate rates of senescence (Finkel and Holbrook 2000; Monaghan and Haussmann 2006; Haussmann and Marchetto 2010). Because individuals can harm their own tissues by activating parasite defenses, mounting overly strong immune responses could result in a loss of fitness (Råberg et al. 1998; Graham et al. 2010, 2011).

Behaviors associated with immune responses, sickness behaviors, can also be costly. When individuals mount particular types of immune responses, they exhibit specific behaviors, including lethargy, anorexia, and low libido and alertness (Adelman and Martin 2009; Adelman and Hawley 2016). Many of these behavioral changes are regulated by proinflammatory cytokine activity in the nervous system (Adelman and Martin 2009). Sickness behaviors are thought to be adaptive; they can promote energy conservation for defending against parasites (Hart 1988) or possibly support tissue repair (Medzhitov et al. 2012). However, elicitation of sickness behaviors can also reduce survival or reproductive success. For example, immune-challenged field crickets, *Gryllus campestris*, were at increased risk of predation because they slowed responses to predators and spent more time in exposed areas, possibly because of differing thermoregulation needs during infection (Otti et al. 2011). Sickness behaviors can also carry opportunity costs because they directly interfere with activities such as foraging or breeding effort. Immune-challenged house sparrows (*Passer domesticus*) reduced the rate at which they fed their offspring, had higher rates of nest abandonment, and, ultimately, fledged fewer chicks (Bonneaud et al. 2003). However, individuals appear to have evolved to

minimize the opportunity costs associated with sickness behavior. Male song sparrows (*Melospiza melodia morphna*) exhibit greater sickness behaviors in response to an immune challenge during the nonbreeding season than during the breeding season, when sickness behaviors could impair reproduction (Owen-Ashley and Wingfield 2006). This seasonal variation in sickness behaviors might be hormonally mediated; experimental increases in testosterone suppressed sickness behaviors in male Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) (Ashley et al. 2009).

Complex Immune Phenotypes: Using Resistance and Tolerance to Characterize Whole-Organism Responses to Parasites

At both individual and evolutionary scales, the optimal parasite defenses will be those that maximize fitness (Schmid-Hempel 2003; Ardia et al. 2011). Studies of the costs versus benefits of immunity initiated new directions in ecoimmunology. In particular, researchers have started characterizing immunity as a complex, integrated response rather than the activity of a single physiological system operating without constraints of other life processes (Graham et al. 2011; Brock et al. 2014). Central to this new framework are resistance and tolerance (Råberg et al. 2009).

Resistance and tolerance are distinct, but not mutually exclusive, strategies for defending against parasites. Broadly speaking, parasite resistance is fighting infection and involves limiting the number of parasites infecting a host. Before a parasite successfully infects a host, resistance can include avoidance or physically blocking a parasite invasion (Best et al. 2014; Kutzer and Armitage 2016). Infected hosts can resist parasites by removing parasites or limiting their reproduction, often through actions of the host's immune system (Råberg et al. 2009). More resistant individuals will have fewer parasites or a faster rate of parasite clearance, and as a result, increased host resistance has a negative impact on parasite fitness (Råberg et al. 2007). In observational studies of free-living animals, parasite load is often used as an indicator of resistance (e.g., Coltman et al. 2001; Schoenle et al. 2017b). In experiments, resistance is often measured as the change in parasite load over time (e.g., Råberg et al. 2007, 2009). Resistance, perhaps obviously, has been the focus of traditional immunology.

In contrast, tolerance emphasizes the costs of infection rather than control of parasite burden (Råberg 2014; Kutzer and Armitage 2016). The concept of tolerance comes from the plant sciences, where researchers realized that plants could gain fitness by minimizing damage caused by herbivores, fungi, and other threats rather than avoiding, clearing, or otherwise walling off threats entirely (Caldwell et al. 1958; Schafer 1971; Strauss and Agrawal 1999). Tolerance is defined as the minimization of the costs of infection per parasite (Råberg et al. 2009). In other words, tolerance involves the regulation of damage accrued during an infection, independent of parasite load. Tolerance can be achieved by reducing the damage caused by the host's own defense mechanisms (Schneider and Ayres 2008). Consider the example of the Soay sheep mentioned earlier (Fig. 2) in which some of the

antibodies that protected from parasites and promoted overwinter survival were also associated with low fecundity (Graham et al. 2010; Nussey et al. 2014). The immune system of the Soay sheep can cause immunopathology, so preventing antibody levels from becoming too high could increase tolerance. Individuals can also increase tolerance by mitigating damage caused by parasites (Schneider and Ayres 2008). For example, the human intestinal helminth, *Schistosoma mansoni*, causes extensive tissue damage in multiple organ systems as it progresses through its life cycle (Allen and Wynn 2011). The production of Th2 cytokines promotes the repair of parasite-damaged tissues and thereby increases tolerance to the helminths (Allen and Wynn 2011). Although a relatively new area of study, ongoing research indicates that tolerance is an important trait for understanding host–parasite interactions. Tolerance is often heritable (Mazé-Guilmo et al. 2014; Parker et al. 2014), associated with host fitness (Hayward et al. 2014b), and impactful on parasite evolution (Cousineau and Alizon 2014; Cressler et al. 2015).

As forms of immunity, resistance and tolerance have great advantages relative to the concept of immunocompetence and immune variation as measured by specific assays. Because resistance and tolerance assess how individuals manage parasite loads and costs of infection for specific parasites, they are biologically relevant performance metrics. Furthermore, resistance and tolerance are integrative measures of immunity at the level of the whole organism; they incorporate the effects of multiple branches of the immune system, as well as behaviors and other physiological systems (e.g., endocrine and nervous system), on organisms' parasite loads and the costs of infection.

Integrative Ecoimmunology: Immunity as Part of a Complex, Dynamic System

Immunity is the product of interconnected physiological systems. Most people experienced this first hand through unfortunate and uncomfortable encounters with the flu. Although flu viruses do not usually enter the central nervous system, the response to the infection affects the brain, leading to fatigue, reduced alertness, reduced appetite, and poor mood (Kelley and McCusker 2014). Indeed, an infection anywhere in the body, such as the lungs or sinuses, can induce a proinflammatory cytokine response in the brain that results in many unpleasant flu symptoms (Jurgens et al. 2012). This common experience is just one example of how the immune system and parasite defenses are inextricably linked to other physiological systems. Addressing immunity in the context of the whole organism requires understanding interactions between immune defenses and other components of physiology.

An entire field, psychoneuroimmunology, is dedicated to understanding how the immune, endocrine, and nervous systems are related to behavior (Ader et al. 1995). Interest in these connections grew following the discovery of evidence suggesting that stress and depression could influence immune function (Irwin 2008). For example, early work in the field demonstrated that the death of a spouse was associated with weak responses to the PHA skin test (Bartrop et al. 1977; Schleifer et al. 1983).

Evidence also suggests that psychological stress can increase susceptibility to infectious disease (Cohen et al. 1991) and suppress the ability to heal wounds (Kiecolt-Glaser et al. 1996). Physiological links between the nervous and immune systems underlie the relationship between stress and disease. For example, the neuropeptide corticotropin-releasing hormone (CRH) is released from the hypothalamus in response to stress and is elevated in depressed humans (Irwin 2008). When high levels of CRH bind to receptors in the brain, CRH suppresses both innate and cell-mediated immune responses (Irwin et al. 1987; Strausbaugh and Irwin 1992; Irwin 2008).

Ecoimmunologists have also devoted substantial efforts toward understanding the mechanistic links between stress and immune function. Researchers working on nonhuman vertebrates have primarily focused on glucocorticoids. Glucocorticoids increase in circulation when organisms are faced with psychological or physical challenges (Bonier et al. 2009; Boonstra 2012). They promote a suite of physiological and behavioral changes, including alterations in immune function, metabolism, and reproductive capability (Sapolsky et al. 2000). Interestingly, glucocorticoids can enhance or suppress immune function, and in some cases do both simultaneously depending on the threat or context (Sapolsky et al. 2000; Martin 2009). The relationship between glucocorticoids and immune function is highly dependent on past and current environmental conditions (French et al. 2009), host life priorities (Martin et al. 2005), and the amplitude, duration, and frequency of glucocorticoid secretion (Martin 2009; McCormick et al. 2015).

A substantial proportion of ecoimmunology studies investigating connections among physiological systems have focused on endocrine–immune interactions. Ecoimmunologists might emphasize endocrinology because many hormones can act as physiological integrators (Cohen et al. 2012), factors for which many cell types have receptors. Because so many cells carry receptors for the same hormones, the activities of disparate systems can be coordinated for the same tasks (Martin et al. 2011b). Moreover, many hormone concentrations rapidly respond to changes in the physical or social environment, further facilitating a match of the whole-organism phenotypes to current conditions (e.g., Maher et al. 2013). For example, melatonin transduces information about day length into a hormonal signal that mediates seasonal changes in immunity (Guerrero and Reiter 2002; Hardeland et al. 2011). In general, melatonin tends to enhance immune function (Martin et al. 2008; Weil et al. 2015), and by supporting a stronger immune response during winter, when days are short and risk of some infections is relatively high, melatonin might bolster immunity (Nelson 2004). Other hormonal integrators can also create links between immunity and other physiological traits. For example, in male red grouse, *Lagopus lagopus scoticus*, testosterone increased both nematode burden and comb size (Mougeot et al. 2005a, b). Grouse combs are colorful ornaments, and the form of these sexually selected traits influences female mate choice and male–male interactions (Moss et al. 1979; Rintamaki et al. 1993). Thus, the link between testosterone, comb size, and resistance to nematodes might ensure that the comb is an honest signal in this system; only high-quality males, who will have high levels of testosterone and a large comb, can cope with high parasite burdens (Folstad and Karter 1992; Mougeot et al. 2004).

Heterogeneity in Immunity

Parasites are ubiquitous. At least 50% of organisms are parasites in some way, and nearly every organism is infected with at least one parasite at some time in its life (Dobson et al. 2008). Even though all organisms face the threat of infection, extensive variation in parasite defenses endures at the individual, population, and species levels (Brock et al. 2014; Downs et al. 2014). In this section, we will address the causes of such variation, and in the following section, we will discuss the consequences (Fig. 3).

Sources of Individual Heterogeneity in Immunity

Spatiotemporal variation in infection risk as well as the value of particular sickness behaviors or other costly immune responses influence investments in immune traits. Moreover, forms of optimal immunity are apt to vary over the course of an organism's lifespan. Both the rate and extent of immune investments as well as subsequent immune development are contingent on the life history strategy of a population. In all hosts, there can be permanent, semipermanent, and reversible effects of genes and environment, ultimately altering immunity across environments (Fig. 3a).

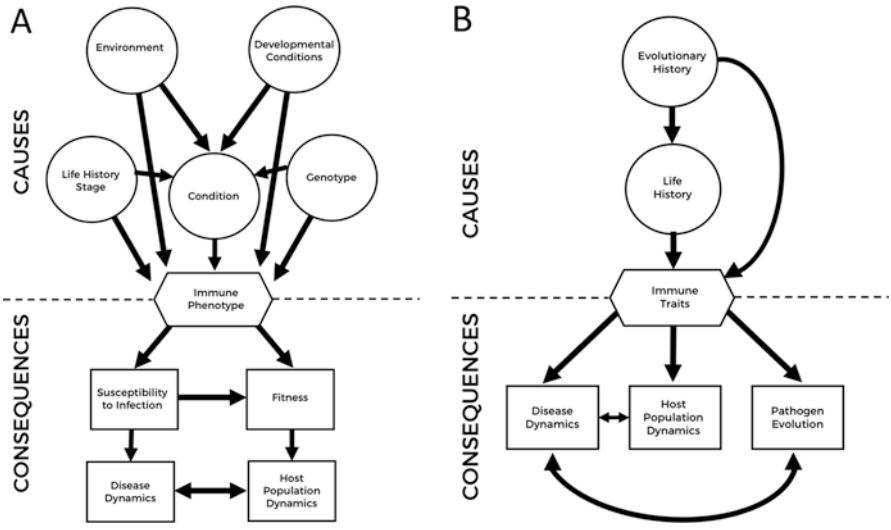

Fig. 3 The purview of ecoimmunology: (**a**) within and (**b**) among species variation in immune defenses. Circles: putative drivers of variation in immunity; squares: potential consequences of that variation; arrows: pathways by which each driver might influence immune phenotype and how variation in immune phenotype could lead to each possible consequence

Genetic Sources of Immune Heterogeneity

Hosts can express a range of immune phenotypes, and the breadth of that range is determined by genotype, developmental history, and maternal and other epigenetic effects. Genotypes vary in many immune traits, including cytokine production (de Craen et al. 2005) as well as organismal traits such as resistance and tolerance (Hayward et al. 2014a; Mazé-Guilmo et al. 2014; Parker et al. 2014). For example, nucleotide polymorphisms were associated with substantial variation in the ability to subdue bacterial infection in wild-sourced *Drosophila melanogaster* (Lazzaro et al. 2004). Furthermore, immune phenotypes are also influenced by the environment and gene-by-environment interactions. For example, *Drosophila* resistance to the ectoparasitic mite, *Macrocheles subbadius*, is also negatively genetically correlated with larval competitive ability (Luong and Polak 2008). However, the relative success of more resistant versus more competitive *Drosophila* varies with density and temperature, resulting in an environment-dependent evolutionary trade-off.

Developmental Environment and Immune Heterogeneity

Early developmental experiences can also affect the expression of immune traits, sometimes throughout life. For example, parents can transmit antibodies to their offspring that shape immunity both immediately and in the long term (Hasselquist and Nilsson 2009). In mammals, some antibody isotypes pass across the placenta and via lactation shortly after birth (Grindstaff et al. 2003; Boulinier and Staszewski 2008). Maternal antibodies can also be transferred to offspring in the egg yolks of birds, fish, and reptiles (Grindstaff et al. 2003; Boulinier and Staszewski 2008). Neonates tend to be particularly vulnerable to parasites in early life. Short-term bolstering of resistance by maternal antibodies can enhance offspring growth rates and maturation (Hasselquist and Nilsson 2009). The effects of maternal antibodies can extend beyond the neonatal phase, shaping immune function into adulthood. In laboratory animals, exposure to antibodies during development altered the immune response to antigens later in life (Wikler et al. 1980; Elliott and Kearney 1992; Lundin et al. 1999). In one study of Wistar Furth rats, the effects of neonatal antibody exposure increased the immune response to a bacterial antigen in the next generation (Lundin et al. 1999). Other components of the developmental environment, such as food availability and parental care, can also influence immune phenotype. In Gambian villages supported by subsistence farming, adult body condition and the mass of newborns were tightly linked to the growing season; body condition was significantly lower during the seasonal postharvest period when little food is available (Moore et al. 1999). People born during the postharvest period were at higher risk of death from infectious disease, suggesting long-term impacts of early-life food limitation. Other types of parental care can also affect immune traits. For example, wood ducks (*Aix sponsa*) incubated at low temperatures were less responsive to immune challenges than those incubated at average temperatures measured in the wild (Durant et al. 2012).

Environmental Conditions Influence Immune Trait Expression

Although genes and the developmental environment constrain the expression of immune traits, immunity remains responsive to fluctuations in the environment throughout organisms' lives. For example, monarch butterflies' (*Danaus plexippus*) ability to resist and tolerate the protozoan parasite *Ophryocystic elektroscirrha* depend on which species of milkweed the butterflies ate (Sternberg et al. 2012). Relationships between resource availability and immunity can be complex, however. Cuban tree frogs (*Osteopilus septentrionalis*) fed a high resource diet (i.e., more crickets) were more resistant to penetration of nematodes through the skin; if an infection was already established, though, they were also more tolerant to infection (Knutie et al. 2017). Environmental effects on immunity are not limited to resource availability. Perceived predation risk induce system-wide changes in physiology and behavior (Clinchy et al. 2013; Newman et al. 2013), including immune functions (Navarro et al. 2003; Stoks et al. 2006; Groner et al. 2013; Adamo et al. 2017). For example, house sparrows (*Passer domesticus*) exposed to predators mounted lower responses to an immune challenge (Navarro et al. 2003).

Life History Stage and Strategy

As discussed previously, parasite defenses are inextricably tied to life history strategy (Sheldon and Verhulst 1996; Ricklefs and Wikelski 2002). Life history can be defined as the patterns of investment an organism makes in growth, reproduction, and survival over its lifetime. Life history stages are periods characterized by specific patterns of investment in some processes over others (e.g., reproduction, immunity, growth, migration) (Ricklefs 1977; Stearns 1992; Roff 2002). The ability to protect against parasites varies across life history stages, including from juvenile to adult as well breeding to nonbreeding. For example, juvenile field voles (*Microtus agrestis*) were more resistant to than tolerant of macroparasites (e.g., intestinal worms, arthropods), whereas the opposite was true of adults, who tended to favor tolerance over resistance (Jackson et al. 2014). This difference could be attributed to the maturation of the immune system with age or to an adaptive strategy mediated by different risk of exposure to parasites or the greater value of growth and maturation versus finding a mate and breeding, depending on individual age. For instance, infections could be more harmful during development, favoring investment in resistance over tolerance in younger animals. Immunity also often changes from the breeding to nonbreeding season, most likely because individuals face different challenges (Zuk and Stoehr 2002; Love et al. 2008). Similarly, we observe sex differences in immunity because males and females engage in different activities during these periods (Zuk and McKean 1996; Zuk and Stoehr 2002). For example, responses to an immune challenge vary across breeding and nonbreeding life history stages in resource-limited female zebra finches (*Taeniopygia guttata*), but not in males (Love et al. 2008). Female zebra finches tend to invest more energy and effort in breeding than males, so differences in immune phenotype might reflect differences in the resources available to the immune system.

Physiology and Body Condition Influence Immune Heterogeneity

All of the sources of individual heterogeneity in immunity discussed here are also likely to influence other components of physiology, such as metabolism or the endocrine system (Ricklefs and Wikelski 2002). As a result, factors that influence immunity might not be acting directly but instead through another mechanism. Body condition is a potential mediator of immunity. For example, short day length (i.e., simulated winter photoperiod) causes a reduction in both body fat and humoral immunity in Siberian hamsters (Drazen et al. 2001). Experimental removal of fat from hamsters also causes a reduction in humoral immunity, suggesting that the presence of fat mediates the effect of day length on immunity (Demas et al. 2003). In this system, the effect of fat on immunity is driven by leptin, a hormone produced by adipose cells (Carlton and Demas 2014, 2015; Carlton et al. 2014). Poor body condition is not always associated with decreasing investment in immunity. North American elk (*Cervus elaphus*) in worse condition invested more in constitutive immune defenses than elk in better condition (Downs et al. 2015). Similarly, mallards in worse condition were less susceptible to infection with an influenza virus and had shorter periods of viral shedding (Arsnoe et al. 2011). Individuals in poor body condition might invest more in less energetically costly components of immunity, or constitutive defenses, to prevent and remove infections quickly because allowing parasites to become established could require more energetically costly specific immune responses (Downs et al. 2015).

Heterogeneity in Immunity Across Species

The fields of ecoimmunology and comparative immunity converge at the study of immune heterogeneity across species. Comparative immunology emphasizes the evolution of immunity and the extent of species-level variation in immune traits (Cooper 2003). Thus, comparative immunology enhances our understanding of how species can defend against parasites. Furthermore, ecoimmunology complements comparative immunology by evaluating why heterogeneity in immunity persists through the study of the selective forces and constraints that influence the evolution of immunity (Martin et al. 2011a; Downs et al. 2014). In this section, we discuss two potential drivers of variation in immunity across species: life history and body size.

Variation in Species' Life History Underlies Heterogeneity in Immunity

Species-level immune variation is the product of evolutionary history, including historic selective pressures and inherited genetic and physiological constraints (Fig. 3b). One way to understand this variation is to consider parasite defenses in the context of life history traits. Life history traits covary along a continuum called the pace-of-life axis (Hille and Cooper 2015). Species with a fast pace of life tend to grow quickly, reproduce early and in high numbers, and have short lifespans. Species with a slow pace of life express the opposite traits: slow maturation, late reproduction, few offspring per reproductive bout, long lifespans, and large body sizes. Thus, evaluating how immunity maps onto the pace-of-life axis will provide

insights into species-level immunity variation (Sheldon and Verhulst 1996; Zuk and Stoehr 2002). The most basic prediction is that individuals with a slower pace of life should exhibit greater immunity because investing in parasite defenses should enhance survival because long-lived organisms should encounter more parasites during their lifetimes (Williams 1966; Ricklefs 1998).

Multiple researchers have hypothesized that fast paces of life are associated with constitutive innate immunity and inflammatory responses, whereas slow paces of life are linked to acquired immunity and anti-inflammatory responses (Lee 2006; Sears et al. 2011). Evidence supporting this hypothesis exists both within and across species (Tieleman et al. 2005; Martin et al. 2006a, 2007; Previtali et al. 2012; Downs et al. 2013; Pap et al. 2015). For example, house sparrows with a slower pace of life exhibit stronger acquired immune responses and lower inflammatory response than fast-living house sparrows (Martin et al. 2006a). Similarly, rodent species with slower paces of life had the lowest innate and strongest acquired immune responses (Martin et al. 2007; Previtali et al. 2012). Multiple cross-species analyses of birds have found that higher mass-adjusted basal metabolic rate, which is associated with a fast pace of life, correlates with lower innate immune responses (Tieleman et al. 2005; Pap et al. 2015; but see Versteegh et al. 2012). There is also evidence that selection for maximal metabolic rate, a trait associated with a fast pace of life, enhanced innate immunity and decreased adaptive immunity in mice (Downs et al. 2013).

Studies have also evaluated the relationship of resistance and tolerance with life history strategy. Because inflammation can cause substantial damage to host tissue as well as to parasites, tolerance is predicted to be associated with anti-inflammatory responses, acquired immunity, and a slower pace of life (Sears et al. 2011). Greater resistance to parasites is predicted to be associated with fast-paced living and stronger innate and proinflammatory immune responses. A comparative study of 13 amphibian species found support for these predictions (Johnson et al. 2012). Fast-living species suffered more severe limb malformations and more pathology during trematode infection than slow-living ones. Counter to predictions for resistance, though, faster-pace-of-life species had greater parasite burdens than slower-pace-of-life species. In another study (Sears et al. 2015), tadpoles with a faster pace of life were more resistant, but slower-pace-of-life species were more tolerant of the same type of trematode infection.

Species-Level Variation in Immunity Can Scale with Body Mass

Body size, as a trait in itself, might affect inter- and intraspecific variation in immunity (Langman and Cohn 1987; Wiegel and Perelson 2004; Perelson et al. 2006; Han et al. 2015). Numerous traits of animals change in a predictable manner, or scale, with body size (Schmidt-Nielson 1984; Calder 1996). We can predict with remarkable accuracy the lifespan, heart rate, sleeping patterns, metabolism, and many other characteristics of organisms from body size alone. Several theoretical models of the immune system suggest that the relationship between body size and immunity is the result of physiological structure (e.g., vasculature, tissue organization) or metabolic rate, which could drive immune cell generation and transport throughout the body (Langman and Cohn 1987; Wiegel and Perelson 2004; Perelson

et al. 2006). Scaling of immune function might also be related to the role that immune function plays in regulating life history traits. Immune function supports survival, so longer-lived individuals are predicted to invest more in immunity. In addition, lifespan increases with body mass and immunity might scale in the same way because the immune system is required to function for a longer time (Wiegel and Perelson 2004).

Some empirical evidence suggests that some aspects of immunity, such as white blood cell numbers (Nunn 2002; Nunn et al. 2003; Tian et al. 2015) and the costs of immune responses (Brace et al. 2017), are related to body size. Presently, all conclusions about body mass and immunity are premature. We do not yet know how size affects defenses against parasites. However, it is important to generate such information because it has important implications for public health. For instance, the effectiveness and toxicity of most pharmaceuticals are assessed initially in small mammals such as rodents. Drug effects do not scale directly with size, though, but we rarely take this into account when developing human treatment plans (Blanchard and Smoliga 2008; Mahmood et al. 2016; Smoliga and Blanchard 2017). Similarly, the most dangerous novel infectious diseases (e.g., Ebola, Nipah, SARS, Hendra viruses, among others) have spread recently from animals to people. Information on immune scaling could help us predict which species are most likely to dilute and amplify infection risk for other species, including humans (Han et al. 2015).

Broad Consequences of Individual Variation in Immunity

Variation in immunity is important ecologically. Heterogeneity in immunity can influence disease transmission through populations and communities, which can alter rates of survival and reproduction of resident organisms (Fig. 4a, b) (Ezenwa and Jolles 2011; Hawley and Altizer 2011; Adelman 2014). Many mechanisms can mediate these outcomes, but one of the most conspicuous is host competence. Host competence represents the ability to transmit parasites to another host or a vector (Hawley and Altizer 2011; Gervasi et al. 2015; Martin et al. 2016; VanderWaal and Ezenwa 2016). Immunity is a, but not the only, key part of host competence. Individuals' immune responses obviously influence their susceptibility to infection (e.g., Savage and Zamudio 2011) and subsequent parasite loads (e.g., Bichet et al. 2012), both of which are important to the ability to transmit infection (Gervasi et al. 2015). However, individuals that are more tolerant or maintain rather than eliminate an infection (e.g., Graham et al. 2010; Jackson et al. 2014) also have more opportunities to transmit parasites (Hawley and Altizer 2011; Martin et al. 2016). Further, immune responses are also linked to sickness behaviors that could influence contact rates between infected individuals and susceptible hosts or vectors (Barron et al. 2015).

Competence is therefore an organismal trait comprised of processes that operate at multiple levels of organization (VanderWaal and Ezenwa 2016). Moreover, it seems so plastic that in some cases individuals within species could be as distinct as groups of species (Gervasi et al. 2015). Depending on where an organism (genotype) finds itself, its role in a disease epidemic can be quite different. For instance, differences in diet quality among laboratory mice (*Mus musculus*) influence eosinophil levels, and

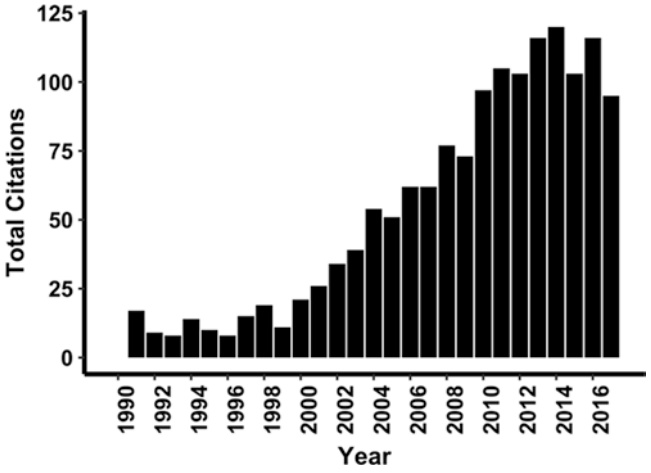

Fig. 4 Annual citations in ecoimmunology from 1990 through October 2017. Citations were identified in the ISI Web of Science database using the search terms ecol (ecology and ecological) and immunol (immunology and immunological)

variation in eosinophils predicts the amount of helminth eggs shed by mice (Budischak et al. 2015). In Grant's gazelles (*Nanger granti*), nematode shedding rates are higher in territorial males than in nonterritorial males and females (Ezenwa 2004). This particular difference in competence among classes of gazelle could be attributed to differences in testosterone, which is associated with immune phenotype as well as the likelihood of defending a territory and parasitism (Ezenwa et al. 2012). Species differences in immunity and competence also underlie spatial variation in Lyme disease risk (LoGiudice et al. 2003; Keesing et al. 2010). Highly disturbed forest sites in the Northeastern United States tend to have less species diversity overall and higher densities of the white-footed mouse (*Peromyscus leucopus*). The white-footed mouse is a highly competent host for Lyme, at least in part due to its immune profile (Donahue et al. 1987; Martin et al. 2007; Previtali et al. 2012; Ostfeld et al. 2014). When there are both a low diversity of hosts available for the vector of Lyme disease, the tick *Ixodes scapularis*, and high densities of the white-footed mouse, then a higher proportion of ticks carry the Lyme-causing bacterium, increasing the risk of disease for all hosts in the community (LoGiudice et al. 2003).

Variation in immunity has the potential to influence population dynamics independent of disease (Lochmiller 1996). Individuals require resources to both develop their immune system and maintain its ability to respond to threats (Demas et al. 2011; Klasing 1988; Ots et al. 2001; Klasing et al. 2006). These costs of immunity can influence individuals' ability to invest in reproduction or other traits important to survival, thereby influencing birth and death rates of populations (Downs and Stewart 2014). For example, environmental conditions, such as precipitation or human disturbance, can shift trade-offs between investment in immunity and reproduction in multiple species of reptiles (Smith and French 2017). Immunity also contributes to survival and population dynamics through influences on recruitment and longevity, which in turn affect lifetime reproductive success. For example,

cell-mediated immunity was a better predictor of recruitment nesting into a breeding population of pied flycatchers (*Ficedula hypoleuca*) after 2 years than mass or hatch date, more traditionally used predictors of recruitment (Moreno et al. 2005).

Few empirical data sets link variation in host immunity to host population dynamics directly, an application of comparative immunology that remains relatively unexplored. However, immunity is linked to both disease transmission and infection outcomes, and parasite effects on host fitness can influence population dynamics. Parasites influence host fitness both by directly killing hosts and through sublethal effects that indirectly reduce survival rates or alter reproductive output (Hatcher et al. 2006). For example, avian malaria (*Plasmodium* spp.) can cause rapid mortality, particularly in areas where the parasites were recently introduced (Atkinson and Van Riper III 1991; Atkinson et al. 1995; LaPointe et al. 2012). Avian malaria is particularly deadly for many Hawaiian birds, and mathematical models indicate that the parasites are a major factor causing population declines and blocking the recovery of at-risk species (Samuel et al. 2011). In addition, climate warming is extending the reach of the parasites and their vectors in Hawaii, further restricting the host-population distributions. Over most of its range, avian malaria infections tend to be chronic, but infection has sublethal effects that can reduce fitness (Bennett et al. 1993; Merino et al. 2000; Valkiūnas 2005; Marzal et al. 2005; Asghar et al. 2015; Schoenle et al. 2017a). Even sublethal effects of parasites can alter population dynamics, though. For example, intestinal nematodes reduced reproductive success by 2–13% in the Svalbard reindeer (*Rangifer tarandus*) (Albon et. al 2002). This reduction in fecundity was sufficient to limit reindeer population growth, even without any measurable effects of nematodes on reindeer survival. In some cases, it is the interaction between the effects of parasites and other environmental challenges that underlie population-level patterns. A study of white-footed and deer mice (*Peromyscus maniculatus*) demonstrated that population crashes were caused by the combination of nematode infections and decreases in resource availability (Pedersen and Grieves 2008). Experiments manipulating both food availability and parasite infection indicated that while each stressor was involved in population regulation, both were required to replicate population crashes.

Conclusions and Future Directions

The field of ecoimmunology has begun to explain why we observe substantial variation in immune defenses and provide some understanding of the implications of that variation. Perhaps the greatest contribution of the field has been to establish that immunity has costs, and those costs can lead to trade-offs within individuals and at an evolutionary scale (Lochmiller and Deerenberg 2000; Schmid-Hempel 2003; Brock et al. 2014). Identifying the costs of parasite defenses led the field in new directions. One of the most influential developments has been the concept of tolerance, which incorporates the costs of infection into measures of immunity. The study of tolerance has expanded beyond ecoimmunology, reaching into human biomedicine, wildlife ecology, and public health. For example, public concern has been growing around an “antibiotic crisis” caused by the rise of drug-resistant microbes

and a decline in the discovery of effective antibiotics (Martens and Demain 2017). Shifting the focus of treatments from resistance to tolerance presents a potential solution: the development of drugs that reduce the virulence of infections rather than parasite elimination (Vale et al. 2014, 2017; Totsika 2017). Furthermore, ecoimmunology has begun to evaluate the constraints that shape immunity and, by combining forces with disease ecology, to use information about immunity to predict the spread of infectious disease (Hawley and Altizer 2011; VanderWaal and Ezenwa 2016).

An important goal of future research is to understand the fitness consequences of different immune phenotypes and the resulting consequences for infectious disease dynamics. Individuals' ability to resist or tolerate infection can change with environmental conditions and life history stage or strategy (Blanchet et al. 2010; Jackson et al. 2014; Sears et al. 2015; Knutie et al. 2017). However, very few studies compare the fitness benefits of a more tolerant to a more resistant phenotype. Studies of Soay sheep defenses against intestinal nematodes find that resistance to nematodes is associated with lower reproductive success (Hayward et al. 2014a), whereas tolerance is associated with greater reproductive success (Hayward et al. 2014b). Furthermore, resistance is independent of tolerance in this system, indicating that individuals are not forced to trade off investment in these strategies (Hayward et al. 2014a). Thus, to understand how defense strategies affect disease dynamics, it might be important to address how resistance and tolerance influence fitness independently. To our knowledge, no studies have assessed the fitness benefits of resistance and tolerance across contexts. Like other components of immunity, resistance and tolerance could vary adaptively across environments or life history stages. Because resistance and tolerance influence hosts' parasite load and the duration of infection, these strategies can have important implications for infectious disease transmission, parasite prevalence, and even the evolution of virulence (Restif and Koella 2004; Miller et al. 2006; Adelman 2014; Gopinath et al. 2014). Determining how investment in resistance and tolerance within populations scales to disease dynamics within populations is an important future direction.

Another important frontier in ecoimmunology is to understand how environmental change influences disease dynamics. Chemical pollutants from industry and agriculture can alter immune function and have been associated with multiple disease outbreaks in wildlife (Grasman 2002). For example, the industrial contaminants polychlorinated biphenyls have been linked to infectious disease deaths in numerous marine mammal species, including harbor porpoises (*Phocoena phocoena*) and striped dolphins (*Stenella coeruleolba*) (Ross 2002). Urbanization is linked to changes in disease dynamics (Bradley and Altizer 2006), and traits of the urban environment, such as light pollution, alter immune function (Bedrosian et al. 2011). Climate change can also alter immunity (Hernroth et al. 2012), which could amplify the risks of infectious disease outbreaks as the ranges of parasites and vectors change in response to a warming climate (Greer et al. 2008). The frameworks are already in place to understand the relationships between environmental factors and immunity as well as immunity and disease dynamics. By improving our understanding of the causes and consequences of immune variation in the context of anthropogenic change, ecoimmunology could provide insights that have implications for public health and conservation.

Finally, building on the connections between the fields of ecoimmunology and comparative immunology will continue to advance our understanding of diversity in immune function. Both fields have made substantial progress through research on both model and nonmodel organisms and by addressing how multiple physiological systems interact to produce immune defenses (Cooper 1984, 2002, 2006; Martin et al. 2006b). In this chapter we discussed two areas of research that link ecoimmunology and comparative physiology: (1) the selective forces and constraints that influence heterogeneity in immunity and (2) how variation in immunity can influence disease dynamics. Characteristics inherent in species, such as body size or life history, can constrain or influence selection on immune traits and, thus, help explain patterns of immunity and disease spread across species that are not explained by phylogenetic relationships (Calder 1996; Han et al. 2015; Brace et al. 2017). Investigating comparative immunology in the context of community ecology is changing how we evaluate infectious disease risk. Because multiple species within a community can be at risk for the same parasitic infections, evaluating heterogeneity in host immunity can help parameterize models predicting infectious disease spread (Adelman 2014; VanderWaal and Ezenwa 2016). Incorporation of ecological factors and organismal traits into comparative studies of immunity can provide insights into both the evolution of immune defenses and community-level consequences of cross-species variation in immunity.

Box 1 The Origins and Central Tenets of Ecoimmunology: A Brief History

Ecoimmunology emerged when researchers began formally considering immunology in an ecological and evolutionary context. One of the first papers to link immunology to ecology was published in 1973 and addressed age-related variation in the prevalence and intensity of schistosome infections in humans (Martin et al. 2011a). It suggested that the lower infection prevalence and intensity observed in older humans could be driven by ecological factors (e.g., parasite exposure, death of hosts with high-intensity infection) in addition to or instead of acquired immunity (Warren 1973). More recent work has confirmed these claims: patterns of human contact with water, management of waterways, and antibody levels are all important in predicting the risk of schistosome infection (Brooker 2007; Moira et al. 2010). Although it was not established as a field until the 1990s, other early studies addressed ideas that remain at the core of ecoimmunology, including trade-offs between physiological functions (Williams 1966), mechanistic links between the immune system and other aspects of physiology (Grossman 1985), the costs and benefits of controlling parasite loads (Behnke et al. 1992), and the effects of resource availability on immunity (Klasing 1988).

Advances in behavioral ecology and endocrinology associated with sexual selection theory were key to the formalization of the field of ecoimmunology. The Hamilton and Zuk hypothesis proposed a role for parasite defenses in influencing the evolution of sexually selected traits, which enable the opposite sex to assess the quality of potential mates (e.g., colorful feathers in birds,

"push-up" display in some lizards) (Hamilton and Zuk 1982). Hamilton and Zuk postulated that sexually selected traits could serve as honest signals of resistance to parasites. The later publication of the immunocompetence handicap hypothesis (ICHH) in 1992 promoted interest in immune mechanisms that could mediate honesty in sexual traits (Folstad and Karter 1992). The ICHH posited that androgens (e.g., testosterone) support both the expression of male secondary sexual traits and suppress immune function, and as a result, sexual trait expression depends on parasite exposure. A meta-analysis revealed weak support for the hypothesis, probably because the ICHH is too simple to capture the complexities of how endocrine–immune interactions influence phenotype (Roberts et al. 2004). Nevertheless, the ICHH was significant to the development of ecoimmunology because it was one of the first widely tested theories linking immunological mechanisms to larger-scale biological processes.

In 1996, Sheldon and Verhulst published an influential paper coining the phrase "ecological immunology," which is arguably the beginning of ecoimmunology as a field. In this paper, Sheldon and Verhulst discussed the costs of immune responses and suggested that immunity is subject to trade-offs. Furthermore, they highlighted that trade-offs can take both physiological and evolutionary forms (Sheldon and Verhulst 1996). Early in the development of ecoimmunology, research on physiological trade-offs primarily involved allocation trade-offs: how organisms distribute limited resources across various functions (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Schmid-Hempel 2003). As the field grew, research expanded to include other costs individuals pay to develop, maintain, and activate parasite defenses (Adelman and Martin 2009; Graham et al. 2010). Around the same time, evolutionary trade-offs attracted attention and were found to occur when selection favored the investment in one trait to the detriment of another (Schmid-Hempel 2003). This form of trade-off is represented by negative genetic correlations often seen among traits (Schmid-Hempel 2003; Ardia et al. 2011). Today, research addresses the consequences of costs and trade-offs for host–parasite coevolution as well as host population cycling and disease dynamics (e.g., Cousineau and Alizon 2014; VanderWaal and Ezenwa 2016).

Ecoimmunology has continued to grow since its inception in 1996, and the integrative nature of the field endures. The number of ecoimmunology publications produced annually (identified using the search terms ecol* and immunol* in the ISI Web of Science database) has increased more than tenfold since the establishment of the field in 1996 (Fig. 4). Ecoimmunology research incorporates techniques and theories from diverse fields, including ecology, evolutionary biology, epidemiology, comparative physiology, animal behavior, neurobiology, genomics, and proteomics. In 2009, the National Science Foundation funded a Research Coordination Network in Ecological Immunology, and in 2014, the Society for Integrative and Comparative Biology introduced a Division of Ecoimmunology and Disease Ecology (Martin et al. 2014). Going forward, we anticipate that ecoimmunology will continue to make valuable contributions to our understanding of host–parasite interactions.

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Annelida: Environmental Interactions and Ecotoxicity in Relation to the Earthworm Immune System

Radka Roubalová, Barbara Płytycz, Petra Procházková, Natividad Isabel Navarro Pacheco, and Martin Bilej

Introduction

Earthworms are the most abundant invertebrates in the soils of temperate regions. They are extremely important for soil formation, and they can be found in all types of soil habitats (Edwards 2004). Earthworms have a major impact on soil structure, water movement, nutrient cycling, and plant growth. In addition, earthworms affect microbial activity and change microbial populations and the community structure of microorganisms during the decomposition of organic substances (Castillo et al. 2013). Earthworms are divided into three ecological groups according to their different behavioral patterns and feeding strategies. Epigeic earthworms (e.g., *Dendrobaena octaedra*, *Eisenia andrei*, *Eisenia fetida*, *Lumbricus rubellus*) live above the mineral soil, rarely form burrows, and preferentially feed on plant litter. This top layer of soil is rich in decaying organic matter and is characterized by a high variability of microbiota. Endogeic species live below the surface, where they build predominantly horizontal burrows and ingest large amounts of mineral soils and humified material. This environment is characterized by a lower amount of organic residues and by decreasing variability of microbiota. To endogeic earthworms belong species like *Aporrectodea caliginosa*, *Aporrectodea rosea*, and *Octolasion lacteum*. Anecic earthworms (e.g., *Aporrectodea longa*, *Fitzingeria platyura*, *Lumbricus terrestris*) build permanent vertical burrows deep into the mineral soil layer characterized by the lowest microbial load but come to the surface to feed on decomposed plant litter and other organic residues, which they drag into their borrows (Lee 1985).

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Interactions of Earthworms with Soil Microbiome

It was found that the gut microbiota of earthworms differ greatly from the microorganisms present in surrounding soil and that earthworms modify the composition of microbial communities in soil during their transit through the earthworm gut, where some microorganisms are digested while others survive (Fig. 1) (Drake and Horn 2007). Changes in microbial composition may accelerate the turnover of organic matter in soil systems as the modified microbial communities are released to the environment as part of earthworm casts (Aira et al. 2015). A relatively small number of studies have quantified the microbiota of earthworms. However, current data show the massive reduction of bacterial diversity in the cast microbiome in *E. andrei* and *L. rubellus* in comparison with the surrounding environment (Aira et al. 2015; Pass et al. 2015). Most of the earthworm-associated microbiome belongs to phyla Actinobacteria and Proteobacteria, as opposed to already described animal core gut microbiomes composed mainly of Firmicutes and Bacteroidetes (Hanning and Diaz-Sanchez 2015). The core microbiome appears to be a result of selection from the pool of ingested bacteria. Moreover, the gut environment of earthworms differs from the surrounding soil as a result of many factors such as neutral pH, anoxia, and higher levels of organic carbon (Drake and Horn 2007; Brown et al. 2000). This can result in the presence of increased levels of methanogenic, fermentative, and nitrate-reducing bacteria in earthworm gut (Depkat-Jakob et al. 2012, 2013). Some gut bacteria of earthworms possess antimycobacterial (Fiolka et al. 2010) and anti-*Candida albicans* (Fiolka et al. 2012) activity, so vermicomposting earthworms may be

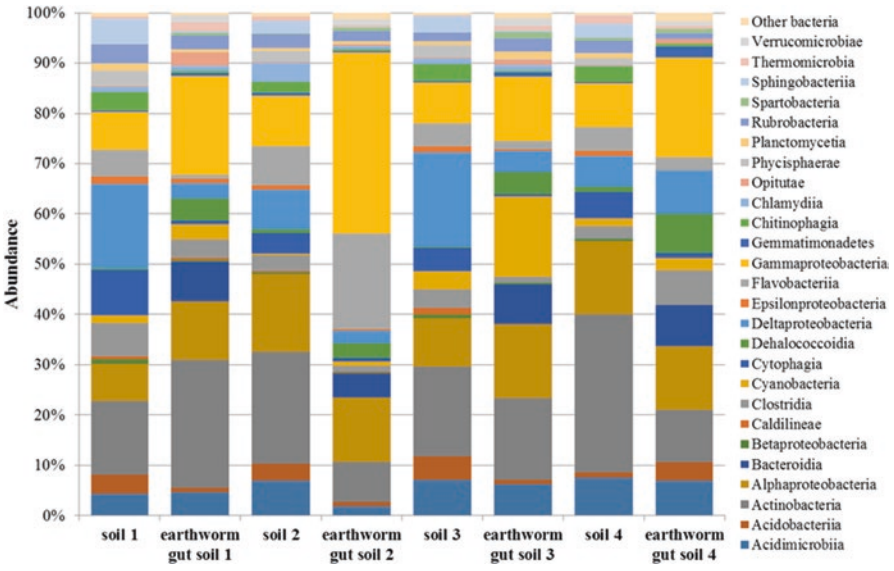

Fig. 1 Profile of microbial communities in four soil samples and composition of microorganisms isolated from intestines of earthworms living in corresponding soils. Values represent relative abundance of microorganisms

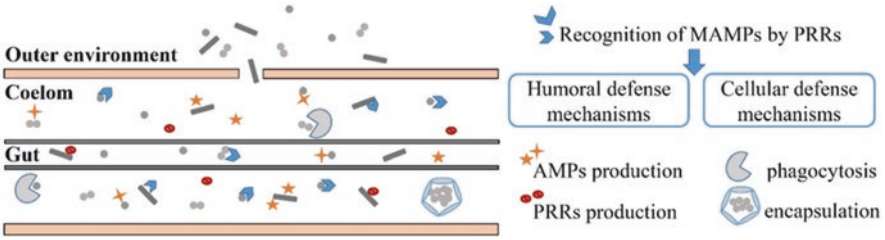

Fig. 2 General scheme of innate defense mechanisms in earthworms. Invading microorganisms can be recognized by pattern recognition receptors, phagocytozed by coelomocytes, or agglutinated and subsequently encapsulated. Different humoral factors are also involved in the elimination of invaders such as AMPs and various pattern recognition molecules, including TLRs, LBP/BPI, and CCF triggering the activation of the prophenoloxidase cascade

used for the reduction of pathogens in septic tank sludge (Rodriguez-Canche et al. 2010). All animals exist in close relationship with microorganisms that play an essential role in the normal development and tissue physiology of hosts. In all likelihood, the indigenous microbiota of both earthworms and mammals serves similar functions in the digestive tract: to ferment polysaccharides to short-chain fatty acids (Hooper et al. 2002) and to confer protection against infection by pathogens (McCracken and Lorenz 2001).

From an immunological perspective, soils are perhaps the most complex microbial habitats comprising the broad spectrum of soil bacteria, algae, fungi, and protists that exhibit almost endless variations in potential interactions with earthworms (Drake and Horn 2007; Zirbes et al. 2012).

Earthworm Immune System

Both skin and gut of earthworms are in permanent contact with soil. Coelomic fluid, which fills the coelomic cavity, is not aseptic (Dvorak et al. 2016), as each segment of the cavity interfaces with the outer environment via excretion organs and dorsal pores that enable microorganisms to enter the coelom. As a consequence, the epithelial surfaces of both the coelom and the gut interact with naturally occurring soil microorganisms. The microorganisms that pass through the epithelial barrier into the coelom can be eliminated by efficient innate defense mechanisms that are ensured by coelomocytes and by different humoral antimicrobial factors since the coelomic fluid of earthworms contains a great variety of molecules involved in the direct elimination of invading microorganisms (Fig. 2) (Dvorak et al. 2016).

Earthworm Immune Cells

Various stressing factors, like predator attack and physical/chemical stimuli like alcohol, ultrasounds, or mild electric current, induce expulsion of coelomic fluid with cells and soluble factors followed by a gradual restoration of the lost elements (Eyambe et al. 1991). A convenient method of stressing earthworms for quantitative

purposes is a mild electrostimulation of earthworms immersed in physiological saline with anticoagulant agent ethylenediaminetetraacetic acid (EDTA) (Cholewa et al. 2006). Such treatment induces sudden expulsion of coelomic fluid containing freely floating morphotic elements, coelomocytes, countable in a hemocytometer and suitable for analysis by flow cytometry and spectrofluorimetry (Fig. 3) (reviewed in Plytycz and Morgan 2011). The coelomocytes of all earthworm species contain macrophage-like amoebocytes. In addition, some species possess a second and morphologically distinct highly granulated cells freely floating in coelomic cavity, that is, the eleocytes, which are considered to be mature chloragocytes detached from chloragogenous tissue (Fig. 3a–c). Eleocytes, but not amoebocytes, exhibit autofluorescence under fluorescence and laser confocal microscopy, which is confined to their granules called chloragosomes, which are lysosome-derived vesicles (Fig. 3b2, c2) that predispose them to cytofluorimetric studies (Fig. 3d). Studies by spectrofluorimetry have revealed that riboflavin, that is, vitamin B2, stored in the chloragosomes of chloragocytes and eleocytes is one of the fluorophores responsible for their autofluorescence (Fig. 3d). Vitamin B2, as a precursor of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), has an indirect effect on many metabolic processes and determines the proper functioning of several systems, including the immune system (Plytycz and Morgan 2011; Mazur et al. 2011). Among other functions, riboflavin acts as chemoattractant for immunocompetent cells, including coelomocytes (Mazur et al. 2011). In response to massive bacterial load or larger parasites, coelomocytes encapsulate them in multicellular capsules called brown bodies (Fig. 3b1, b2). The formation of brown bodies might be facilitated not only by the chemotactic action of riboflavin (Mazur et al. 2011) but also by extracellular traps formed by stimulated coelomocytes, composed of extracellular DNA, histones, and antibacterial proteins, recently discovered in *E. andrei* (Homa et al. 2016a).

The percentage of autofluorescent eleocytes among coelomocytes and the absolute amount of riboflavin stored within eleocytes are both species-specific parameters (Plytycz and Morgan 2011; Rorat et al. 2014). Both the frequency of eleocytes and their riboflavin content are high in the endogeic species *Allolobophora chlorotica*, while these granular cells are apparently absent or very uncommon in another endogeic species, *Aporrectodea caliginosa*. A high frequency of riboflavin-rich eleocytes was also discovered in *Dendrobaena veneta*, *E. andrei*, *E. fetida*, and *Octolasion* spp., while freely floating eleocytes are very scarce among coelomocytes (usually <1%) in *Aporrectodea longa* and several *Lumbricus* spp. Lack of riboflavin-storing freely floating eleocytes is compensated in these species by riboflavin stored in chloragocytes of chloragogenous tissue (Plytycz and Morgan 2011).

Electrostimulation-induced drastic depletion of coelomic fluid with its morphotic elements is followed by a recovery process lasting several weeks that is much faster for amoebocytes due to their proliferation in coelomic fluid than for chloragocyte-derived eleocytes and eleocyte-stored riboflavin. Amoebocytes are crucial for immune functioning, while functions of eleocytes may be temporarily replaced by chloragocytes of chloragogenous tissue. The numbers and composition of coelomocytes in coelomic fluid are age-dependent, fluctuate in an annual cycle, and may be modified by several external factors (reviewed in Plytycz et al. 2016).

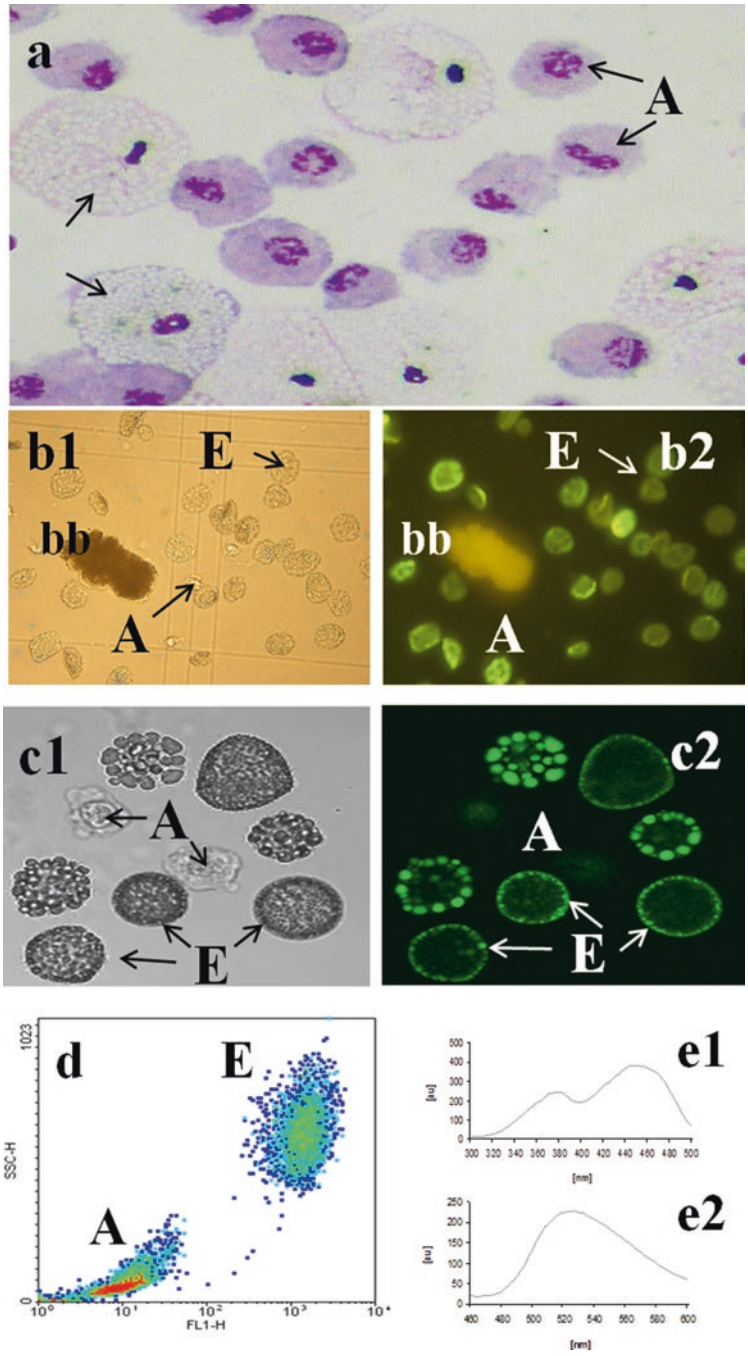

Fig. 3 Earthworm coelomocytes; A – amoebocytes, E – eleocytes. (**a**) MGG stained cytospin preparation; (**b**) coelomocytes in hemocytometer bright light (b1) and blue light (b2); bb – brown body; (**c**) confocal microscope bright light (c1) and blue light (c2); (**d**) flow cytometric density plot; X – fluorescence intensity, Y – cell complexity; (**e**) spectrofluorimetric spectra of riboflavin (RF); emission (e1), excitation (e2)

Antimicrobial Peptides and Proteins

Antimicrobial peptides (AMPs) represent a first-line innate immune response. Their amino acid composition, amphipathicity, cationic charge, and size allow them to attach to and insert into membrane bilayers to form pores (Lockey and Ourth 1996). AMPs are generally considered to kill their microbial targets through insertion and damage/permeabilization of the cytoplasmic membranes of target cells (Jelinek and Kolusheva 2005). However, a number of defense peptides may also interact with intracellular targets such as DNA and RNA, presumably interfering with their metabolic functions and thus leading to cell death (Brogden 2005; Hale and Hancock 2007). They can alter cytoplasmic membrane septum formation, inhibit cell wall, nucleic acid, or protein synthesis, or inhibit enzymatic activity (Brogden 2005). Only a limited number of bioactive peptides have been described in annelids to date.

In *Lumbricus rubellus*, the AMP named lumbricin I was identified (Cho et al. 1998). Lumbricin I is a proline-rich AMP that exerts in vitro antimicrobial activity against a broad spectrum of microorganisms. Furthermore, lumbricin I is constitutively expressed in adult animals and is not inducible upon bacterial infection.

A lumbricin I analog named PP-1 was found in the Asian earthworm *Pheretima tschiliensis* (Wang et al. 2003) and showed 77.6% homology with lumbricin I. PP-1 is synthesized only in the body wall, and its localization in the mucus of the epidermis suggests its role in the mucosal defense. Another homolog of lumbricin I, lumbricin-PG, was identified in earthworm *Pheretima guillelmi* (Li et al. 2011). Furthermore, an antimicrobial short peptide, OEP3121, of only five amino acids was found in *E. fetida* earthworm (Liu et al. 2004). In the marine annelid *Nereis diversicolor*, an AMP named hedistin was identified (Tasiemski et al. 2007). Hedistin is constitutively expressed in circulating natural killer (NK)-like cells, and an antimicrobial effect against a large spectrum of bacteria was shown.

One of the widely distributed AMPs is lysozyme. This protein cleaves the β-1,4-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine of peptidoglycan of bacterial cell walls and thus efficiently contributes to protection against infections caused mainly by Gram-positive bacteria. In earthworms, lysozyme was described to exert also isopeptidase activity hydrolyzing isopeptide bonds present in bacterial cell walls (Fiolka et al. 2012; Joskova et al. 2009).

The coelomic fluid of earthworms also contains molecules exerting bacteriostatic and bacteriolytic activities connected with the hemolytic activity of glycoproteins originally described as fetidins (Roch et al. 1981) and lysenins (Sekizawa et al. 1996). These proteins exert antimicrobial activity against both Gram-positive and Gram-negative bacteria, particularly against strains pathogenic to earthworms (Roch et al. 1991; Valembois et al. 1986). Moreover, they are able to bind sphingomyelin, a major lipid constituent of plasma membranes of most mammalian cells. Upon binding sphingomyelin, they polymerize and form channels through the lipid bilayer (Roch et al. 1989; Yamaji-Hasegawa et al. 2003). The presence of lysenin and lysenin-related proteins (LRP-1, PRP2/fetidin, and LRP3) was recently evidenced on the level of gene expression, proteins, and hemolytic functions in representatives of *E. andrei* and *E. fetida*, but not in *Dendrobaena veneta* (Swiderska et al. 2017).

Pattern Recognition Receptors

Invading microorganisms can be also recognized by both soluble and membrane-bound pattern recognition receptors (PRRs) that sense microorganism-associated molecular patterns (MAMPs) (Beschin et al. 1998; Bilej et al. 2001; Skanta et al. 2013, 2016). MAMPs are represented by specific bacterial components such as peptidoglycan, lipopolysaccharide, outer-membrane proteins, flagellins, and other molecules that are expressed by a wide range of bacteria. Upon this recognition, downstream signaling pathways are triggered, often activating cellular and humoral innate immunity effectors such as phagocytes, AMPs, and reactive oxygen species (ROS) (for review see Leulier and Lemaitre 2008 and Royet et al. 2011).

A key role in the innate immunity of both invertebrates and vertebrates is played by Toll-like receptors (TLRs). TLRs were originally characterized as mammalian orthologs of the *Drosophila melanogaster* transmembrane protein Toll that was discovered as a molecule essential in the development of embryonic polarity (Anderson et al. 1985), and later on its role in antifungal and Gram-positive bacteria protection was described (Lemaitre et al. 1996; Michel et al. 2001). TLRs can be found in both plants (Gassmann et al. 1999) and animals (Coscia et al. 2011; Satake and Sekiguchi 2012). In annelids, the high number of genes encoding TLRs have been described (Davidson et al. 2008; Skanta et al. 2013).

One of the structural components localized in the outer membrane of Gram-negative bacteria is lipopolysaccharide. This molecule can be recognized by both lipopolysaccharide-binding protein (LBP) and bacterial permeability-increasing protein (BPI). In mammals, these proteins serve an antagonistic biological function (Elsbach and Weiss 1998; Fenton and Golenbock 1998), whereas in invertebrates and nonmammalian vertebrates, the distinction between LBP and BPI has not been established to date. In the earthworm *E. andrei*, *Ea*LBP/BPI was described. It was shown to be predominantly expressed in the coelomocytes, in the seminal vesicles, and in the anterior part of the digestive system. The levels of *Ea*LBP/BPI mRNA increased in the coelomocytes upon challenge with both Gram-negative *E. coli* and Gram-positive *B. subtilis* (Skanta et al. 2016).

In earthworms, cell wall components of Gram-positive bacteria, Gram-negative bacteria, and yeast are recognized by coelomic cytolytic factor (CCF) (Beschin et al. 1998; Bilej et al. 2001). CCF also acts as an opsonin in coelomic fluid (Bilej et al. 1995). It has been shown that CCF triggers the activation of a prophenoloxidase cascade (Beschin et al. 1998), which is a basic defense mechanism in many invertebrate species. Further, the mRNA level of CCF in coelomocytes was detected to be upregulated upon the injection of microorganisms into the coelomic cavity (Kohlerova et al. 2004).

Ecotoxicologic Effects on Earthworms

Earthworms are in permanent close contact with soil via both their highly permeable skin and alimentary tract. They are therefore excellent model organisms in ecotoxicological studies of soil toxicity (ISO 1993, 1998; OECD 1984, 2004).

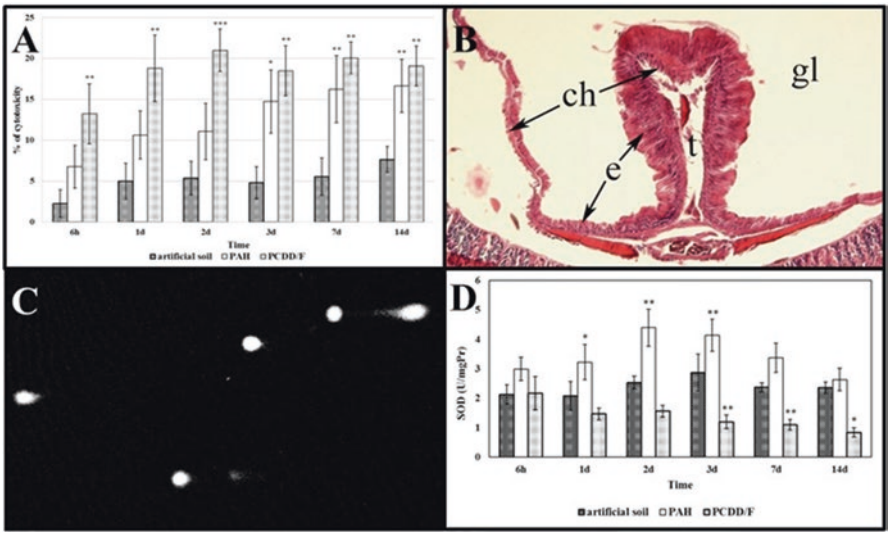

Fig. 4 Examples of methods used in detection of ecotoxicity of contaminants in earthworms. (**a**) Cytotoxicity of coelomocytes. (**b**) Histological alterations of earthworm gut (gl – gut lumen; ch – chloragogenous tissue; e – endothelial cells, t – typhlosolis). (**c**) Comet assay. (**d**) Detection of superoxide dismutase activity

Earthworms have been shown to bioaccumulate contaminants, such as various organic pollutants (Jager et al. 2005; Henriksson et al. 2017; Chen et al. 2017), heavy metals (Nahmani et al. 2007; Rahman et al. 2017), and nanoparticles (Makama et al. 2016; Zhang et al. 2017). Contaminants entering the earthworm body disturb their major physiological functions that can be reversible (Olchawa et al. 2006). The effect of contaminants can be monitored on various levels (Fig. 4). First is the whole-body level, in terms of, for example, viability, weight loss, inhibited or delayed maturation, reduction of reproduction, and avoidance/escape reaction. Second, the organ and tissue damage can be seen in histopathologic alterations (Rodriguez-Seijo et al. 2017; Roubalova et al. 2014; Zhang et al. 2015). Third are the changes in cellular physiological conditions (Duan et al. 2017; Mincarelli et al. 2016), while at the fourth level of contaminant effects, the up- and downregulation of the expression levels of genes sensitive to environmental changes and transcriptome profiling (Zhang et al. 2015; Hayashi et al. 2016) can be used to monitor the effect of pollution on earthworms.

Cellular Response to Pollutants

The coelomic fluid of earthworms contains different types of cells that are generally termed coelomocytes. The nomenclature of coelomocytes is largely based on differential staining, ultrastructure, and granular composition. There are two basic categories of coelomocytes: eleocytes with a mainly nutritive function and amoebocytes with a primarily immune function (Sima 1994).

Coelomocytes have been described to respond to a wide range of pollutants and therefore are often used in soil ecotoxicological assessment. As mentioned earlier, coelomocytes can be easily retrieved, and their numbers, the amoebocyte/eleocyte ratio, riboflavin content, and gene expression may be subjected to ex vivo analyses. It turns out that these factors can be affected in species-specific ways by soil quality, including metal contamination or by experimental exposures to metal-spiked soil or metal-spiked filter paper (Homa et al. 2010, 2015).

Contaminants present in soil enter the earthworm body according to their charge. Hydrophilic compounds enter the body mostly through the skin, whereas the major route for hydrophobic compounds is soil ingestion (Belfroid et al. 1995; Thomann 1995). Coelomocytes present in coelomic fluid are therefore highly affected by pollutants reaching the coelomic fluid.

Comet assay, or single-cell gel electrophoresis (SCGE) assay, is a very sensitive technique for the evaluation of DNA damage on the level of individual eukaryotic cells, and it has also proven effective for measuring the DNA integrity of coelomocytes. Comet assay is an essential tool used in ecotoxicological research. In earthworms, it is used to assess DNA damage in worms exposed to soil samples contaminated with, for example, organic pollutants (Wang et al. 2016; Ma et al. 2016), heavy metals (Li et al. 2015), and nanoparticles (Correia et al. 2017).

Besides the comet assay, the effect of pollutants on the immune function of amoebocytes can be detected in earthworms. The inhibition of phagocytosis in earthworms exposed to various metals and organic substances, such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins/dibenzofurans (PCDD/Fs), has been described (Belmeskine et al. 2012; Fournier et al. 2000; Sforzini et al. 2017). Some coelomocytes were described to possess cytotoxic activity similar to that of NK cells. This NK-like cell activity has been demonstrated to be affected by polycyclic aromatic hydrocarbons (PAHs) (Patel et al. 2007), PCBs (Suzuki et al. 1995), and PCDD/Fs (Belmeskine et al. 2012).

At the subcellular level, the lysosomal membrane stability system has been identified as a specific target of toxic contaminants (Moore 1990). Lysosomal membrane integrity can be measured by a neutral red retention assay (Weeks and Svendsen 1996; Plytycz et al. 2011). The stability of membranes has been shown to decrease with increasing stress owing to the presence of pollutants in the environment (Booth et al. 2003; Booth and O'Halloran 2001; Moore 1985).

It turns out that eleocyte-rich species are especially valuable for the investigation of soil quality. The most striking results were obtained in the case of epigeic *Dendrodrilus rubidus* that contains a dense population of autofluorescent eleocytes in worms from both unpolluted and metalliferous sites from Wales, while spectra characteristic of riboflavin were evident only in samples from unpolluted sites. However, riboflavin was lost after transferring earthworms from unpolluted soil to soil samples from polluted habitats, while eleocytes were still autofluorescent, perhaps due to aging pigment lipofuscin (Plytycz et al. 2009, 2010). It was concluded that riboflavin depletion in coelomocytes of *D. rubidus* is a biomarker of metal soil contamination. Any generalizations and extrapolations may, however, be misleading, as this relationship does not seem to hold in other species under different experimental conditions.

Coelomocytes and riboflavin content are affected by soil quality in species-related ways. Moreover, it is apparent that fluctuations in riboflavin content in coelomocytes occur not only in metal-polluted soil samples but also in metal-free sandy-clay or loamy-sand natural soil samples. Significant riboflavin depletion was visible in *A. chlorotica* coelomocytes. In contrast, the riboflavin content increased in the eleocytes of *E. andrei* or *D. veneta* transferred from lab soil to metal-polluted soil samples and to unpolluted sandy clay or loamy-sand soils (Fig. 5a). In conclusion, various edaphic factors other than elevated metal concentrations can effectively act as stressors, and this may lead to species-specific alterations in riboflavin metabolism. Hypothetically, riboflavin status (storage/mobilization) may depend on parasite–immune system balance, which is disrupted by soil-derived stressors, including metals (Plytycz and Morgan 2011).

Environmental stressors induce protective mechanisms in coelomocytes, among them increased levels of stress factors (Nahmani et al. 2007; Zhang et al. 2017), detectable by the level of stress proteins (Fig. 5b, c) (Olchawa et al. 2006; Homa et al. 2005, 2016b) or gene expression (Homa et al. 2010, 2015). Stress proteins act in species-specific and metal-dependent manners. The omnipresent endogeic *A. chlorotica* could serve as a novel distinctively susceptible species for environmental contamination studies. In this species decreased cell counts and riboflavin content are molecular biomarkers of Cu exposure, while induction of MT-mRNA is a molecular biomarker of worm cadmium exposure (Homa et al. 2010). The presence of MT-2 was detected not only in coelomocytes but also in the intestine, blood vessels, and epidermis. In conclusion, *A. chlorotica* coelomocytes are adapted to respond differentially to various heavy metals, generating powerful responses to the potentially most dangerous exogenous, nonessential elements (Homa et al. 2010, 2016b). In another ecotoxicologically important species, *E. andrei*, coelomocyte parameters were unaffected by dermal exposure to zinc, copper, lead, and cadmium, but variations of defense gene expression, phytochelatin synthase, and especially metallothionein were evidenced (Homa et al. 2015).

Lumbricid earthworms are often exposed to the simultaneous action of various environmental stressors like soil contamination, temperature fluctuation, or predator attacks, which may induce extrusion of coelomocyte-containing coelomic fluid or loss of tail segments. Exposure of *E. andrei* to cadmium-polluted soil inhibited the maturation of juvenile earthworms and reproduction of adults but had no effect on the kinetics of restoration of depleted coelomocytes or the regeneration of amputated tail tips, which was connected with significantly upregulated expression of Cd-metallothionein (but not of catalase, lysenin, and phytochelatin) in coelomocytes (Rorat et al. 2017). Hypothetically just efficient detoxification with the participation of defense proteins is responsible for the successful application of *Eisenia* spp. in vermicomposting (Rorat et al. 2016; Suleiman et al. 2017).

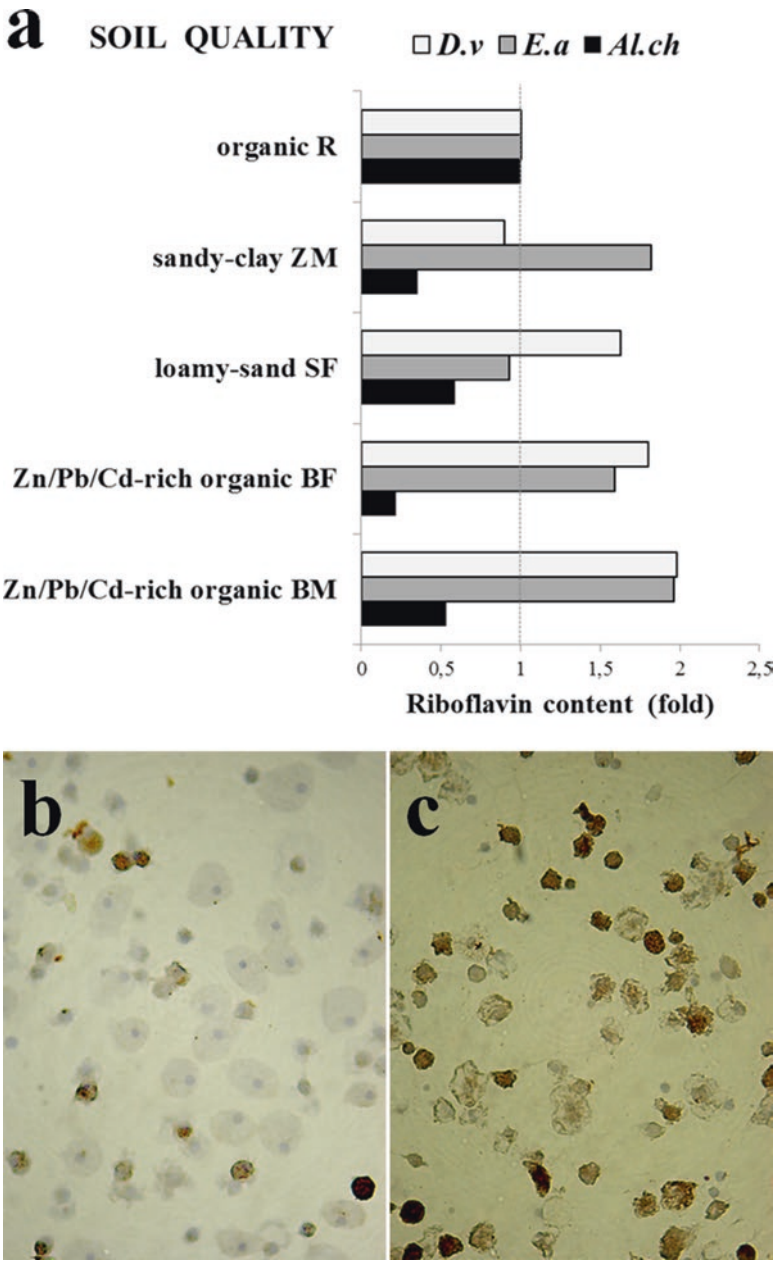

Fig. 5 Effects of environmental pollution on riboflavin content (**a**) and expression of metallothionein (**b**, **c**) in earthworm coelomocytes. (**a**) Riboflavin content in coelomic fluid retrieved 4 weeks after transferal of adult specimens of *Dendrobaena veneta* (*D.v*), *Eisenia andrei* (*E.a*), and *Allolobophora chlorotica* (*Al.ch*) from commercial organic soil to fresh soil samples of the same quality (organic R) or natural soil from unpolluted sites (ZM and SF) or sites heavily polluted with metals (BF and BM); (**b**, **c**). Immunohistochemical detection of metallothionein 2 (wMT2) expression (brown deposits) in coelomocytes extruded after earthworm exposure to water (**b**) or cadmium chloride (**c**)

Humoral Response to Pollutants

The coelomic fluid of annelids exhibits numerous biological activities that participate in the defense mechanisms against invaders. Many of the substances involved in these defense responses are affected by the presence of contaminants in soil. Pollutants entering the earthworm body induce the formation of ROS and subsequent oxidative stress results in the production of antioxidant enzymes. Also, other molecules that participate in the maintenance of cell and tissue integrity, such as the two chaperone molecules calreticulin and heat shock protein 70 (Hsp70) (Roubalova et al. 2014; Wang et al. 2016), riboflavin, and various molecules involved in immune reactions, have been reported to be affected in earthworms living in contaminated soils.

Oxidative Stress

Oxidative stress is defined as a disturbance in the balance between the production of ROS and the oxidant defense system. This system is developed in all aerobic organisms to protect themselves against free radicals. ROS is a common term for both oxygen radicals, such as superoxide, hydroxyl, peroxyl, and hydroperoxyl radicals, and certain nonradical peroxyl agents, such as hydrogen peroxide, that can be easily converted to radicals. The major source of intracellular ROS is the mitochondrial respiratory chain (Han et al. 2001; Ott et al. 2007). These compounds are, however, produced in other cell compartments as well, such as the endoplasmic reticulum and peroxisomes (del Rio et al. 2006; Zeeshan et al. 2016). ROS production is essential to normal cell physiology (Sena and Chandel 2012). ROS are important for cell signaling and can function as second messengers. Moreover, they also participate in the process of inflammation as they eliminate invading microorganisms (Kodiha and Stochaj 2012). However, elevated amounts of ROS can damage a wide variety of molecules. They cause damage to nucleic acids and proteins (Bohr 2002), promote lipid peroxidation (Barrera 2012), interfere with active sites of some enzymes (Guttmann 2010), and induce apoptotic events by means of caspase-dependent pathways (Kagan et al. 2002).

Under stressful conditions, such as biotic and abiotic stress, the levels of ROS become elevated, which results in the promotion of oxidative stress and subsequent damage to cells and tissues. In earthworms, histopathological changes have been reported as a valuable marker of soil contamination. It has been shown that hydrophobic dioxins cause damage to intestinal and adjacent chloragogenous tissue that serves as a center of synthesis and storage of glycogen and lipids and the excretion of the waste products of cell catabolism (Morgan and Morgan 1989; Sturzenbaum et al. 2004). Histopathological alterations of the intestinal tract were observed also in worms exposed to the presence of microplastics (Rodriguez-Seijo et al. 2017). The histological changes of the body wall, specifically epidermis and muscle fibers, were described in earthworms exposed to soils contaminated with heavy metals (Markad et al. 2015; Sharma and Satyanarayan 2011).

The main defense system against ROS is provided by antioxidant enzymes. They are able to deactivate free radicals before they attack cellular components. Three groups of antioxidant enzymes play a significant role in the protection of

cells against oxidative stress: superoxide dismutases (SOD), catalases (CAT), and peroxidases (POD). The activity of these enzymes was described as being affected in earthworms living in soil contaminated with both organic and inorganic substances. Both the enzyme activity and gene expression levels of these antioxidant enzymes are frequently used to determine the effects of pollution on earthworms (Wang et al. 2015, 2016; Shi et al. 2013; Homa et al. 2016c).

Environment and Pathogen Recognition Receptors

Earthworms possess various substances of innate immunity that are reportedly affected by the contaminants present in soil. As mentioned earlier, one of the most important molecules of the earthworm immune system are so-called PRRs that recognize molecular patterns common to the microorganisms. One of these PRRs, CCF, was shown to be significantly downregulated in *L. rubellus* following lifelong exposure to C_{60} nanoparticles, which suggests the induction of immunosuppression (Van Der Ploeg et al. 2013). The mRNA levels of CCF were also detected to be increased in earthworm *E. andrei* living in dioxin-polluted soil. However, the dioxin-contaminated soil samples contained increased amounts of microbial mass compared with the artificial soil, which could explain the higher expression of CCF molecules rather than the direct action of dioxins (Roubalova et al. 2014). Further, the transcriptional responses of genes involved in TLR signaling were described in *E. fetida* coelomocytes to the representative nanosilver NM-300 K. The effector role of TLRs in nanosilver pathophysiology in earthworms was suggested by Hayashi et al. (2016).

Also, one antimicrobial protein, lysenin, present in the coelomic fluid of earthworm *E. andrei* is frequently used in the monitoring of soil ecotoxicity (Brulle et al. 2011; Bourdineaud et al. 2017). The alteration of the expression of this molecule may affect its ability to eliminate invading microorganisms.

Conclusions and Further Challenges

Taken together, the disruption of defense mechanisms of earthworms due to exposure to pollutants can result in the immunosuppression and subsequent reduction of reproduction, decreased growth, and increased mortality of earthworms living in polluted soils.

Mechanisms of earthworm response to environmental factors are stressor-dependent and species-specific, so proper species identification is crucial. Growing evidence reveals that cryptic/sibling speciation (genetically distinct, but morphologically very similar or indistinguishable organisms) is widespread among some earthworm "species" (King et al. 2008; Perez-Losada et al. 2009; Sturzenbaum et al. 2009). One of the important implications of these findings is the possibility that the genetically distinct "lineages" are differentially responsive to environmental contaminants (Morgan et al. 2007). Therefore, comparative studies on the stress response of closely related species *E. andrei* and *E. fetida* and distinct clades within the *E. fetida* phylogenetic tree are of great importance.

Series of transplantation experiments (Cooper 1969; Cooper and Rubilotta 1969) led to the discovery of some specificity and short-term "memory" in earthworm immunity as the rejection of second-set allografts performed soon after the first-set grafts was accelerated (Cooper and Roch 1986). These early pioneer achievements are in line with later studies on other invertebrates, mainly insects (Faulhaber and Karp 1992; Little and Kraaijeveld 2004; Moret and Siva-Jothy 2003), leading to the conclusion that past experience with an antigen can provide individual invertebrates with enhanced immunity, referred to as immune priming, that is functionally similar to the acquired immune response in vertebrates (Little and Kraaijeveld 2004; Cooper and Eleftherianos 2017). It would be worth studying further the phenomenon of immune priming in earthworms and its hypothetical modifications by environmental factors.

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Mollusca: Disseminated Neoplasia in Bivalves and the p53 Protein Family

Annette F. Muttray and Katerina Vassilenko

Introduction

The immune system plays an important role in the detection and control of neoplasia in vertebrates and invertebrates. The occurrence of neoplasia in invertebrates appears to be far less common and not as diverse in nature as it is in vertebrates. Neoplasia and, in some cases, neoplasia exhibiting malignant characteristics have been found in several invertebrate species, from corals to nematodes, mollusks, and arthropods (for a summary of known neoplasia in invertebrates see Robert 2010). Invertebrate neoplasms exhibit characteristics or "hallmarks" of cancer, including undifferentiated cells, abundant mitotic figures, and rapid, invasive, and proliferative growth resulting in the death of the individual (Aguilera 2017).

Invertebrates possess an innate immune system primarily consisting of cellular (hemocytosis, phagocytosis, encapsulation, nodule formation) and humoral (antimicrobial peptides, lectins, and complement-like factors, prophenoloxydase cascade) defenses (Rowley and Powell 2007). Hemocytes provide the first line of immune defense against foreign substances or organisms in bivalves via phagocytosis and hemocytic infiltration (Gosling 2003). The mechanisms of cellular defense in bivalves are predominantly hemocytosis (increase in the number of circulating hemocytes), phagocytosis (uptake and destruction of foreign particles), and encapsulation (Gosling 2003). In some cases, pathogens remain alive within the phagolysosome, continue to develop, and eventually kill the host. When the invading organism or particle is too large to be phagocytosed, encapsulation by hemocytes can be activated. Under this scenario, hemocytes rearrange and create concentric

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layers of cells around the invader, disintegrate it, and remove the cellular debris (Gosling 2003).

It is commonly accepted that invertebrates are predominantly affected by benign neoplasias, while vertebrates can be affected by metastatic malignancies or cancer (Robert 2010). Noteworthy exceptions appear to be tumors with metastatic potential in the arthropod (insect) *Drosophila melanogaster* and neoplasias in mollusks. In addition to several known benign neoplasms, such as adenomas, polyploid tumors, papillomas, and mesenchymal tumors, several tumors with apparent malignancy have been described in mollusks, including epithelial carcinomas, gill carcinomas, gliomas, gonadal neoplasia, and disseminated neoplasia (Aguilera 2017). In particular, the characteristics of disseminated neoplasia and gonadal neoplasia can be thought of as metastatic as neoplastic cells or undifferentiated germ cells, respectively, infiltrate tissues and continue to divide in an uncontrolled manner throughout the animal.

Little is known about the role of the innate immune system in detecting and controlling neoplasia in invertebrates. It has been hypothesized that the pressure exerted by the immune system on neoplasia selects for increased tumorigenicity and malignancy and may generate new variants that can escape the immune system, as was observed in a comparative study between immunocompetent and immunodeficient mice challenged by chemical carcinogens (Robert 2010). A similarly selective role can likely be attributed to the innate immune system elements in invertebrates as well. Because of the tremendous variety of body patterns, life histories, and ecological niches within the 1.3 million-plus species of living invertebrates, there is a similarly high potential for diversity in their immune strategies (Rowley and Powell 2007).

Of the malignant tumors in invertebrates, disseminated neoplasia in marine bivalves is one of the most prevalent cancers, with often serious implications to natural and aquaculture populations (Bower 2010). It is also the most extensively studied naturally occurring invertebrate cancer, in particular its distribution, etiology, and cellular and molecular processes contributing to the disease. As a result, although some initial studies elucidating the molecular mechanisms of malignant gonadal neoplasia in bivalves were undertaken (Rhodes and Van Beneden 1997), this chapter will focus on disseminated neoplasia of hemocytes. Disseminated neoplasia will serve as a case study of an invertebrate cancer for which recent findings point to an infectious etiology and which is capable of overcoming innate immune responses by debilitating their key cellular player, the hemocytes. We will further examine what is known about the role that the p53 protein and gene family may play in the innate immune system response to the development of this cancer.

Bivalve Disseminated Neoplasia

Characteristics of Disseminated Neoplasia

Disseminated neoplasia, often referred to as hemic neoplasia or bivalve leukemia, is a malignant cancer of circulating cells in the hemolymph of bivalve species, the hemocytes. Bivalve hemocytes do not contain a respiratory pigment and are

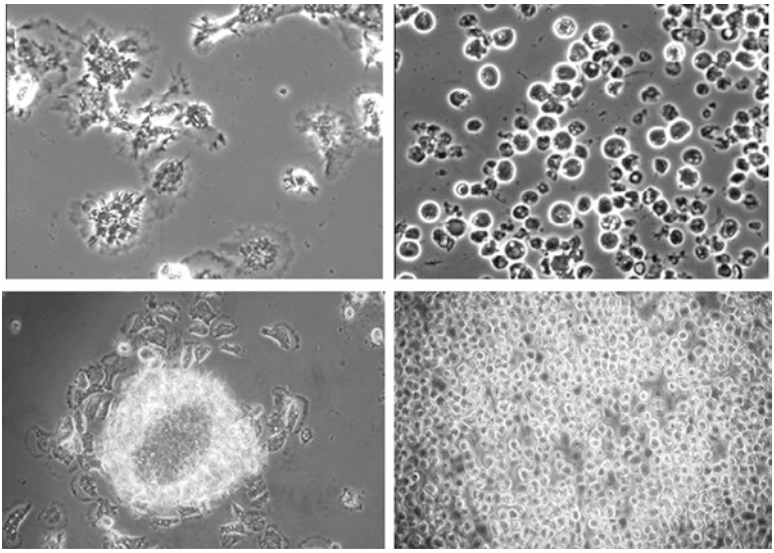

Fig. 1 Nondiseased (top left), late stage (top and bottom right), and transitional stage (bottom left) of disseminated neoplasia in the bay mussel *M. trossulus.* (Phase contrast microscopy, photos by K. Vassilenko)

predominantly characterized as phagocytic granulocytes and agranulocytes floating in a colorless hemolymph (Gosling 2003). Hemocytes play an important role in the innate immune system response, primarily in phagocytosis and encapsulation of pathogens. The hemolymph and hemocytes perform many physiological functions, including the initiation of wound repair, the transport of nutrients and metabolic waste products, oxygen transport, gas exchange, and osmoregulation. Disseminated neoplasia is generally characterized by an uncontrolled proliferation of abnormally large and anaplastic cells of unknown, but likely hemic, origin (section "Origin of Neoplastic Hemocytes") (Barber 2004; Carella et al. 2017; Miosky et al. 1989; Reinisch et al. 1983) in the hemolymph, sinuses, and tissues of the affected animals (Fig. 1). These anaplastic cells display a high nucleus-to-cytoplasm ratio and have lost the functionality of normal hemocytes circulating in the hemolmph (Mix 1983). In particular, neoplastic cells showed limited immune activity (Díaz et al. 2011). Also, in many cases neoplastic cells had abnormal ploidy, meaning up to ten times increased DNA content (Carella et al. 2017; Vassilenko and Baldwin 2014). As the disease advances, normal hemocytes are progressively replaced by neoplastic cells, eventually at significantly higher cell densities compared to normal cell density, and the normal function and integrity of hemocytes, hemolymph, tissues, and organs are lost. This progression can lead to a mortality rate of up to 80% in affected populations (Bower 2010). The disease has been identified in a number of marine mollusks: in Eastern and Pacific oysters *Crassostrea virginica* and *C. gigas* (Farley 1969), in blue and bay mussels *Mytilus edulis* and *M. trossulus* (Elston et al. 1988a), in soft-shell clam *Mya arenaria* (Miosky et al. 1989), in European oyster *Ostrea*

edulis (Alderman et al. 1977), the common cockle *Cerastoderma edule* and lagoon cockle *C. glaucum* (Carballal et al. 2016; Poder and Auffret 1986), the Baltic clam *Macoma balthica* (Christensen et al. 1974), the golden carpet-shell clam *Polititapes aureus* (formerly *Venerupis aurea*) (Carballal et al. 2013), the razor shell clam *Solen marginatus* (Ruiz et al. 2015), and in cultured mussels *Mytilus platensis* in southern Argentina (Vázquez and Cremonte 2017), occurring in all oceans except the Arctic (Carballal et al. 2015). Evidence of remission of neoplasia has been detected in *M. edulis* (Elston et al. 1988a) and *M. arenaria* (Brousseau and Baglivo 1991), although a high incidence of disseminated neoplasia is commonly associated with massive mortalities in the affected bivalve shellfish population.

Like any other cancer, a genetic alteration or mutation causing dysregulation of gene expression is required for neoplasia to occur. The genetic alteration may be spontaneous, hereditary, or acquired as a result of exposure to external agents (Aguilera 2017). Various external agents, also called stressors, of a chemical (pollution, biotoxins), physical (increased temperature and overcrowding) or biological nature (genetics, infections) have all been implicated in disease development, may not be exclusive to each other, and thus point to a potentially complex etiology. The following sections will briefly review the current state of knowledge about the potential contributions of each stressor to disseminated neoplasia in bivalves, but for a more in-depth examination of the issue in several bivalve species, the reader is advised to consult the comprehensive review by Maria Carballal and coworkers (Carballal et al. 2015).

Species-Specific Genetic Background

Species-specific genetic background is one of the determining factors affecting the prevalence of the disease in different bivalve species. Disseminated neoplasia was reported at high prevalence in oysters *C. virginica* and mussels *M. trossulus*, while it is nearly, but not entirely, absent in closely related species, *C. gigas* and *M. edulis*, respectively (Barber 2004). Also, disseminated neoplasia is much more prevalent in the common cockle *C. edule* than in the lagoon cockle *C. glaucum* located within the same geographic area (Galicia) (Carballal et al. 2016).This difference in susceptibility to the disease between closely related species may provide evidence of the genetic basis of resistance against initiation of the disease (Carballal et al. 2015). In addition, considering that the disease appears to be transmissible between species (section “Horizontal Transmission and Infection”) (Metzger et al. 2016), the differential prevalence of disseminated neoplasia between closely related species may point to a functional aspect of innate immune response during disease exposure. The immune system of the related species encountering “foreign” neoplastic hemocytes released by nearby affected animals of the other species likely has a higher resistance to the transformed hemocytes than the donating host species itself.

Origin of Neoplastic Hemocytes

The tissue origin of neoplastic hemocytes in shellfish has been a matter of debate for many years, at least since 1969, when CA Farley suggested the gonad as the tissue of origin (Farley 1969). There are several hypotheses about possible neoplastic cell origins (Carballal et al. 2015). Generally, these neoplastic cells are considered to be sarcomas (neoplasias of mesoderm-derived tissues), and within this classification, undifferentiated mesenchymal cells, hematopoietic stem cells, and differentiated vesicular connective tissue cells. Because neoplastic cells are first observed in the hemolymph, with increasing prevalence over normal hemocytes as the disease progresses, and because normal and neoplastic hemocytes share receptors for the same monoclonal antibodies (Noël et al. 1994; Reinisch et al. 1983; Smolowitz et al. 1989), it is believed that normal and neoplastic hemocytes are ontogenetically related and that neoplastic cells are of hemocytic origin. However, similar neoplastic diseases in other bivalves, *M. balthica* (Christensen et al. 1974) and *V. aurea* (Carballal et al. 2013), appeared to have the gill epithelium as the origin of neoplastic cells, which subsequently spread to other organs.

Effect of Environmental Stressors and Contamination

A wide range of environmental stressors and contaminants are thought to be implicated in disease development. Stressors such as decreased salinity, hypoxia, and lower pH were associated with an increased incidence of disseminated neoplasia (Carballal et al. 2015; Smolarz et al. 2005; Sunila 2003; Wolowicz et al. 2005). The increase in disease incidence can be explained, at least in part, by higher survival and growth rates of neoplastic cells under physiologically unfavorable conditions, while healthy cells may be suppressed (Seton-Rogers 2016). Also, under conditions of limited food supply, allocation of energy favors neoplastic cells over healthy gonadal cells in several species of bivalves (Cremonte et al. 2011; Elston et al. 1992; Ford et al. 1997; Potts 1996).

Similar to environmental stressors, exposure to anthropogenic contaminants and algal biotoxins may suppress the immune system in bivalves and create conditions favoring neoplastic cells over healthy ones and, thus, enhance susceptibility to the disease. Dinoflagellate biotoxins deposited in bivalve tissues correlated with the presence of disseminated neoplasia (Landsberg 1996). Dinoflagellate biotoxins from a group called Azaspiracids (AZAs) were associated with the expression and activity of proteins critical for organism defense, cellular regulation, apoptosis, and cancer development, such as cathepsin D and p53 family proteins (Nzoughet et al. 2009). Association of AZA toxins with the self-defense system and carcinogenesis indicates that the toxins may act as tumorigenic compounds. As will be discussed in the following sections, the p53 family is highly conserved among all species, and therefore exposure to AZA toxins may have analogous consequences for other species, including humans (Nzoughet et al. 2009).

Chemical stress appeared to be a significant contributing factor to the development of neoplasia according to several studies, reviewed in detail by Bruce Barber (2004), and additional studies conducted since (Böttger et al. 2013; Muttray et al. 2012; St-Jean et al. 2005). These studies showed that higher prevalence of the disease was associated with higher concentrations of contaminants such as fuels, oil spills, petroleum-derived hydrocarbons, polycyclic aromatic hydrocarbons, chlordane, pesticides, cadmium, or polychlorinated biphenyls (Carballal et al. 2015). For example, intensity of disseminated neoplasia was higher in Prince Edward Island estuaries located downstream of high-intensity potato-farming watersheds than in estuaries of lower-farming-activity areas (Muttray et al. 2012). As will be discussed subsequently in sections "Viral Induction of Disseminated Neoplasia" and "Horizontal Transmission and Infection", one of the key events of disease initiation and progression is the induction of a retrotransposon called *Steamer* (Arriagada et al. 2014). It is hypothesized that the initiation of *Steamer* and its genome-destabilizing effects could be triggered by environmental contaminants (Arriagada et al. 2014).

Viral Induction of Disseminated Neoplasia

Viral induction of disseminated neoplasia in bivalves, and specifically in clams, was hypothesized and investigated as one of the primarily suspected etiologies relatively early on in the field. For in-depth reviews on the subject the reader is referred to Carballal et al. (2015) and Walker et al. (2011). The initial finding by Oprandy and coworkers (Oprandy and Chang 1983; Oprandy et al. 1981) indicated that retroviruses could be causative agents in disseminated neoplasia in *M. arenaria*, but those studies could not be repeated, which cast doubt on the hypothesis. Subsequently, researchers were able to transmit the disease from affected to healthy animals by injection of cell-free hemolymph or lysed hemocytes in *M. trossulus* (Kent et al. 1991), *M. edulis* (Elston et al. 1988b), *C. edule* (Collins and Mulcahy 2013), and *M. arenaria* (Taraska and Böttger 2013; Walker et al. 2009). Induction of disease by the retroviral inducer 5-bromodeoxyuridine (BrdU) (Oprandy and Chang 1983) or by injection of filtered hemolymph isolated from BrdU-treated animals (Taraska and Böttger 2013) also suggested the possibility of a retroviral element or retrotransposon as the causative agent. In addition, a number of studies have reliably detected reverse transcriptase (RT) activity, which is indicative of retroviral genome replication, and physiological as well as pathological processes (Spadafora 2004), in neoplastic clam tissues and hemolymph (AboElkhair et al. 2009a, b; House et al. 1998; Manso et al. 2012; Oprandy et al. 1981; Romalde et al. 2007), further bolstering this hypothesis. Although there appears to be background RT activity in *M. arenaria*, the activity increases with an increase in tetraploid hemocytes (polyploidy being an indicator of the presence of disseminated neoplasia) (AboElkhair et al. 2009a, b). In addition, expression of other genes indicative of retrotransposons (transposase and polyprotein genes) was upregulated in hemocytes from *M. arenaria* with moderate and heavy disseminated neoplasia (Siah et al. 2011). Despite these findings,

numerous tests revealed no evidence for the presence of retroviral particles in *M. arenaria* (AboElkhair et al. 2012), and it was concluded that the RT activity may have been endogenous and not considered conclusive evidence of retroviral infection leading to disseminated neoplasia in the clams. In contrast, retrovirus-like particles had been observed in neoplastic hemocytes in *C. edule* (Romalde et al. 2007). Recently, Gloria Arriagada and coworkers (Arriagada et al. 2014) found sequences of a novel retrotransposon, called *Steamer*, in RNA from hemolymph of *M. arenaria*, which are marked by long terminal repeats and encode a single large protein with similarity to mammalian retroviral Gag-Pol proteins. Activation of a high expression level and an increased copy number of *Steamer* mRNA were correlated with disease status. Retrotransposons act by copying and inserting themselves throughout the genome of the host with potentially catastrophic induction of genetic instability that may initiate or advance the course of disseminated neoplasia, similarly to events during tumorigenesis in many human cancers. Injection of BrdU would likely induce expression of *Steamer* retrotransposons in host cells carrying *Steamer* in their genome, leading to an observed increase in DNA copies (Metzger et al. 2015). Host cells are then transformed *de novo* into neoplastic hemocytes, resulting in disseminated neoplasia.

Horizontal Transmission and Infection

As can be concluded from the studies reviewed in section "Viral Induction of Disseminated Neoplasia", it may not actually be a virus particle that acts as the transmissible infectious agent causing outbreaks of disseminated neoplasia. It was shown recently that it is more likely that disseminated neoplasia in clams as well as in mussels is transmitted horizontally between animals as contagious cancer cells (Metzger et al. 2015, 2016). These cancer cells appear to have arisen by clonal expansion from a single individual and are nearly identical to each other, but they are curiously distinct from their hosts (Metzger et al. 2015, 2016), indicating that they belong to a natural neoplastic cell line that is transferred between animals. This is highly unusual, and not many transmissible tumors are known to exist: The exceptions are canine transmissible venereal tumor (Murgia et al. 2006), transmitted by sexual contact, Tasmanian devil facial tumor disease (DFTD) (Pearse and Swift 2006), transmitted between individuals by bites, and a contagious reticulum cell sarcoma in Syrian hamsters (Copper et al. 1964). Not only were clam and mussel disseminated neoplasias infectious within the same species, but it was further shown that these contagious cancer cells could cross species boundaries in marine bivalves (Metzger et al. 2016), that is, cancer cells in the clam *P. aureus* were derived from *Venerupis corrugata*, a different clam species living in the same geographical area (Fig. 2). (Interestingly, no cases of disseminated neoplasia have so far been found in *V. corrugata* from the same region.) This contagious cancer cell transmission may be more widespread in the marine environment, where ocean currents can potentially transport neoplastic cells over large distances.

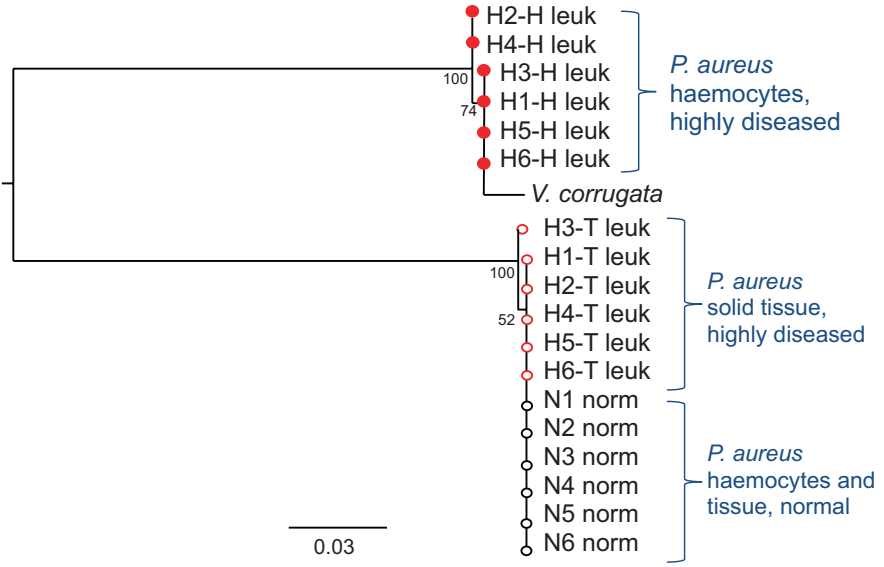

Fig. 2 Phylogenetic analysis of neoplastic cells in *P. aureus* and *V. corrugata*. Maximum-likelihood tree of sequences from solid tissue (T) and hemocyte (H) DNA from six samples of normal (N1–N6 norm) and highly diseased (H1–H6 leuk) *P. aureus* and normal *V. corrugata*, based on mtCOI (658 bp). Open black circles, tissue and hemocytes of normal *P. aureus*; open red circles, tissue of diseased *P. aureus*; filled red circles, hemocytes of diseased *P. aureus*; scale bars show genetic distance. (Adapted from Metzger et al. 2016)

The p53 Gene Family as Part of an Innate Tumor Defense Mechanism

The desire to understand the etiology of disseminated neoplasia in bivalves has been fueled by the notion that understanding the common mechanisms that likely exist in invertebrate and in vertebrate cancers will ultimately lead to a much more cohesive understanding of the fundamental processes that are altered during tumorigenesis and of tumor immunity. In addition, the refinement of a naturally occurring outbreeding bivalve cancer model that can more easily be studied and manipulated than highly regulated vertebrate cancer models where tumors are induced (e.g., mouse) (Walker et al. 2009, 2011) would contribute greatly to the fields of comparative immunology and comparative oncology.

One gene family that has been studied widely in human cancers for reasons outlined in section "Overview of the p53 Protein and it's Relatives p63 and p73" is the tumor suppressor protein p53 and its relatives. Given p53's central role in the regulation of cell growth and cell death, it is not surprising that p53 homologs have been discovered in a large variety of other species, including many invertebrates. The following sections provide an overview of the p53 family of proteins, their structure, evolution, and how their crucial roles may be abrogated in disseminated neoplasia in bivalves.

Overview of p53 Protein and Its Relatives p63 and p73

Protein p53 is a transcriptional activator and tumor suppressor, first discovered as a suspected oncogene in 1979 (DeLeo et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979) and since investigated in over 50,000 studies. Many studies illustrate the importance of the p53 gene, as it is either mutated or inactivated in over 50% of human cancers (Hollstein et al. 1991; O'Brate and Giannakakou 2003). In addition to mutation, differential expression and degradation (Shieh et al. 1997), phosphorylation and acetylation (Burns and El-Deiry 1999), and translocation and exclusion from the nucleus (Lu et al. 2000; Moll et al. 1996) can lead to inhibition of p53 activity and aberrant cellular proliferation. Because of its central role in the molecular networks that determine the fate of cellular life and death, p53 has been termed the "gatekeeper of the genome" as well as a "network hub" (Vogelstein et al. 2000). As a transcriptional activator, p53 is normally "turned off" (deactivated or rapidly degraded) but becomes activated upon sensing damage to DNA by radiation or chemical treatments, hypoxia, or activation of oncogenes. As a transcriptional regulator, the p53 protein binds to promoters of downstream genes involved in cell growth arrest and programmed cell death in response to sensing DNA damage. Thus, it will prevent cells from passing on incorrect DNA messages that can potentially turn these cells into malignant tumors. p53 is at the intersection of many cellular stress response pathways that lead to cell cycle arrest, DNA damage repair, apoptosis, or senescence (Hofseth et al. 2004). Additionally, genes responding to p53 transcriptional regulation are involved in anti-angiogenesis, autophagy, and cellular antioxidant metabolism (Joerger and Fersht 2016).

Tumor suppressor p53 is part of an expanding protein family that also contains proteins p63 and p73 and many C- and N-terminal isoforms produced by differential splicing (Fig. 3). The family members share high sequence similarity in their core sequences, but multiple splice variants produce protein isoforms with differing functions dependent upon the particular tissue type in which they are found (Marin and Kaelin 2000). While p53 is ubiquitously expressed, p73 and p63 are restricted to certain tissues and developmental stages. While p63 is essential for various aspects of ectodermal differentiation, p73's role is predominantly in neuronal differentiation. However, p73 and p63, respectively, can also respond to DNA damage by controlling apoptosis and cell cycle arrest (Moll and Slade 2004).

Structure and Function of p53 Family Genes

The functional domains of p53 family proteins include an N-terminal transactivation domain (TAD), proline-rich domain, central DNA-binding domains (DBDs), and an oligomerization (or tetramerization) domain (OD) (Fig. 3). The physical distinction between p53- and p63/73-like sequences is based on the presence of a

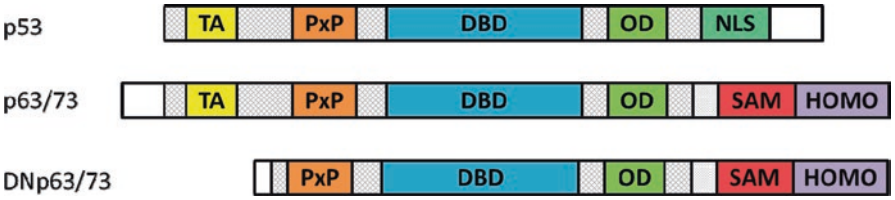

Fig. 3 Structure of known members of p53 protein family in *Mytilus* spp. TA, transactivation domain; PxP, proline-rich domain; DBD, DNA-binding domain; OD, oligomerization domain; NLS, nuclear export signal; SAM, sterile alpha motif; HOMO, homodimerization domain. Hatched areas and colored functional domains are conserved between family members, while open areas contain a unique sequence. Domains are not to scale. (Adapted from Vassilenko et al. 2010)

sterile alpha motif (SAM) domain in the carboxy terminus of the latter (Nedelcu and Tan 2007; Thanos and Bowie 1999). As a transcriptional regulatory protein, p53 is able to transactivate downstream genes by binding to specific DNA response elements (REs) (El-Deiry et al. 1992) or to repress transcription by binding to promoters that lack REs. p53 is active as a tetramer, facilitated by the OD at the carboxy terminus of the protein, and only the tetrameric form can bind efficiently to DNA. The OD contains a nuclear export signal (NES), which is masked during oligomerization, preventing nuclear export of p53. The TA domain located at the N-terminus is critical for binding coactivators and corepressors. Binding partners of the TA domain include the repressor MDM2 (section "p53's Negative Regulator MDM2") and coactivator CBP/p300. The TA has several phosphorylation sites, which become phosphorylated in response to DNA damage, thus altering p53 activity. The proline-rich domain located between the TA and the DBD has several PxP motifs that are required for the ability of p53 to cooperate with antineoplastic agents to promote cell death (Baptiste et al. 2002). The DBD is p53's core domain that provides a scaffold for a DNA-binding surface (Pavletich et al. 1993). The four DBDs of the active tetrameric form of p53 bind to DNA in a highly cooperative manner, increasing p53's affinity to DNA 100-fold over that of monomeric p53. Most p53 cancer mutations are located in the DBD. The C-terminal domains provide a platform for post-translational modifications and protein–protein interactions, which further regulate p53 activity.

The p63 and p73 family members possess a domain structure similar to that of p53 but contain variable C-terminal extensions with a SAM, a homodimerization domain (HOMO), and a sumoylation site (SUMO) domain and N-terminal deletions/additions (deltaN or TA isoforms) (Jessen-Eller et al. 2002; Muttray et al. 2007; Thanos and Bowie 1999). SAM domains are known to associate with other SAM domains, forming both homo-oligomers and hetero-oligomers. For a detailed discussion of the structure and function of individual domains the reader is referred to Chillemi et al. (2017) and for a comparative structure and function analysis of p73 to Yoon et al. (2015).

Evolution of p53 Family Genes

p53 family genes have been identified in modern-day descendants of early metazoan sponges (*Amphimedon queenslandica*), placozoans (*Trichoplax adhaerens*), cnidarians (*Nematostella vectensis*), and even protists (*Monosiga brevicollis*), clearly indicating that this gene family is ancient and had important roles long before the need for protection against the development of cancer arose (Joerger and Fersht 2016). The emerging picture of the evolution of the p53 gene family from early protist and metazoan life to invertebrates and finally vertebrates, spanning some 1–2 billion years, can be viewed as an example of how one rather simple defense mechanism can be expanded and modified to serve in the control of a myriad of complex cellular functions. This is not unlike the evolution of the immune systems, where the most primitive innate responses to recognize self versus nonself gradually evolved and diversified into the complex acquired immunity responses observed in mammals today. As it is a central part of the innate immune response, let us examine the evolution of the p53 family genes more closely.

Although p53 has attracted most of the attention in the literature owing to its relevance in human cancers, it is its evolutionarily older homolog, p63, that has been shown to be the likely origin of this family (Fig. 3). The discovery that p63 is not only expressed in the basal compartment of stratified epithelial tissues but also in oocytes (Suh et al. 2006) indicates that the ancestor of the p53 family genes may have controlled the integrity of germ cells. This is supported by the recent discovery that the choanoflagellate *Salpingoeca rosetta,* a close relative of *M. brevicollis,* has a sexual life cycle and transitions between haploid and diploid states (Levin and King 2013) (sexual reproduction of *M. brevicollis*, in which the p63-like gene has been characterized, remains unknown). Further evidence is supplied by a study on the starlet sea anemone *N. vectensis*, in which a p63-like protein selectively induces apoptosis in germ cells upon UV radiation (Pankow and Bamberger 2007). In the nematode *Caenorhabditis elegans*, the p63/p73-like protein Cep-1, which contains a SAM domain (Ou et al. 2007), is expressed in its germ cells where it is required for DNA-damage-induced apoptosis (Greiss et al. 2008). It appears that the gene family had an important role long before the need of protection against cancer in early short-lived organisms and that this role is likely to protect the female germ line and genomic integrity in response to DNA-damaging stress (Joerger and Fersht 2016).

Within the diverse invertebrate phylum, strategies appear to have evolved to employ p63-like proteins not only in germline protection but also in protection against neoplasia. *M. arenaria* has a single p63/p73-like hybrid gene but can splice the transcripts from this gene into a p53-like sequence and a p63/p73-like sequence (Kelley et al. 2001). Although the gene sequence is not resolved for *Mytilus* ssp., it appears that *Mytilus* can similarly produce p53- and p63/p73-like isoforms and in addition an N-terminally truncated deltaN p63/p73-like isoform through alternative splicing (Muttray et al. 2007). The surf clam *S. solidissima* has additional splice variants, p97 and p120 (Cox et al. 2003), and interestingly the p120 is only expressed in the embryonic stages of clam development in response to stress and appears to be involved in neuronal development (similar to vertebrate p73) (Jessen-Eller et al. 2002).

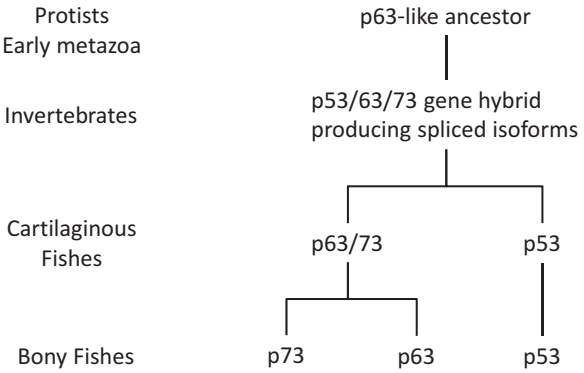

Fig. 4 Simplified p53 family gene evolution illustrating gene duplication events

At the junction with the early chordates and vertebrates, the tunicate sea squirt *Ciona intestinalis* still has only one p63 gene expressing two isoforms in eggs and early embryos (Noda 2011), but what follows is a first gene duplication in cartilaginous fish, resulting in two paralog genes (Belyi et al. 2010), giving rise for the first time to a candidate p53 gene (Fig. 4). The evolutionary advent of bony fishes sees a second gene duplication of the original p63 and clearly establishes the presence of a third paralog gene, p73. All three genes are present throughout the vertebrate phylum with very few known exceptions, and this genetic expansion of the family tree likely led to the differentiation of functions that we see in mammalian species today. Although germ cell quality control was likely the original function of the ancestral gene, as the lifetime of organisms increased and started to exceed the average lifetime of individual cells, evolution developed renewable tissues requiring the establishment of stem cells (Belyi et al. 2010). Thus, p63 and p73 developed into essential factors for epithelial cells and neuronal stem cell maintenance, respectively. As Belyi et al. (2010) summarize eloquently, with the appearance of renewable tissue, tumorigenesis became an increasing problem, with about 80% of human cancers originating from epithelial tissues. In the most recent stage of the evolution of the p53 protein family, the tumor-suppressor function was added to its repertoire, with p73 and, in particular, p53 being assigned to the surveillance of the genetic and cellular quality of somatic cells.

p53 Family in Disseminated Neoplasia in Bivalves

At the molecular level, bivalve disseminated neoplasia has been associated with changes in the family of p53 and associated proteins. p53's association with disseminated neoplasia in bivalves has been investigated along four different lines of evidence: (1) expression of p53 family members mRNA and protein, (2) mutations identified in the p53 transcript, (3) p53's translocation from the nucleus to the cytoplasm, and (4) p53's interaction with other protein members of the p53 pathway. A

summary of our current understanding of the functions of the molluscan p53 family members will be presented here in an effort to illustrate that the molluscan p53 shares not only structural but also functional similarities with mammalian p53 family genes, placing the mollusks at a critical juncture in the evolution of this gene family.

Expression of p53 Family Members and Regulators in Disseminated Neoplasia

All molluscan p53 gene family member isoforms are likely derived from one gene by differential splicing and potentially other transformations (sections "Structure and Function of p53-Family Genes" and "Evolution of the p53-Family Genes"). Deregulation of expression of different p53 isoforms has been shown in many human cancers (Bourdon 2007), and it was thus hypothesized that expression of the structurally similar molluscan p53 and its known isoforms may similarly be deregulated in bivalve disseminated neoplasia. Expression levels of p53 isoforms as well as genes that are thought to be transcriptionally regulated by p53 have been investigated in the mussel *M. trossulus,* the clam *M. arenaria,* and the cockle *C. edule.* Results of these investigations will be reviewed in this section. Both proteins and mRNAs of p53 family members were quantified using anibodies for proteins and primers specific for family members mRNAs, and results were not always congruent between the methods, likely due to additional post-transcriptional protein modifications regulating the isoforms' activity (Muttray et al. 2008).

Concentrations of p53 protein do not appear to be different between hemocytes of healthy *M. arenaria* clams and clams with disseminated neoplasia (Kelley et al. 2001; Stephens et al. 2001). In contrast, hemocytes of cockles *C. edule* with moderate and late stages of disseminated neoplasia expressed a mutant 53 kDa protein, reacting with antibody Pab240 raised against human mutant p53, that was not observed in healthy hemocytes (Díaz et al. 2010). A smaller-size protein with high affinity to the antibody was also detected in hemocytes of healthy and early-stage disseminated neoplasia. At the mRNA level, hemocytes of *Mya* and *Mytilus* with disseminated neoplasia showed an increase in p53 mRNA (Muttray et al. 2008, 2012). p53 mRNA levels were also significantly higher in samples of digestive gland tissue from neoplastic cockles *C. edule* compared to samples from healthy cockles, in particular at moderate stages of the disease (Ruiz et al. 2013). The increase in mRNA levels of the p53 isoform in neoplastic hemocytes may be counteracted by genetic changes, such as mutations, that inhibit the activity of the tumor suppressor protein, such as in Burkitt's lymphoma in humans (Balint and Reisman 1996). Interestingly, concentrations of a p63/73-like protein were also increased in neoplastic hemocytes in *Mya* and *Mytilus* (Kelley et al. 2001; Stephens et al. 2001), which was consistent with an increase observed in deltaNp63-p73 (but not TAp63/p73) mRNA levels in those species (Muttray et al. 2008, 2012). Similarly, an antibody to the p73 HOMO-domain of the surfclam *S. solidissima* (Cox et al. 2003) detected an increased amount of p63/73 in neoplastic *Mytilus* hemocytes (St-Jean et al. 2005). Thus, the increase in p63/73-like proteins observed in *Mya* and *Mytilus* neoplastic hemocytes may be correlated with the increase in the truncated

deltaNp63/73 mRNA. Another study used flow cytometry to estimate the proportion of neoplastic tetraploid clam hemocytes in the hemolymph and correlated the proportion with mRNA expression levels of p53 and p63/73 (Siah et al. 2008). Both p53 and p63/73 levels increased in clams with neoplastic tetraploid hemocyte levels of 15–50% compared to clams with neoplastic tetraploid hemocyte levels of either greater than 50% or less than 15% (Siah et al. 2008).

To summarize these studies, it appears that an overexpression of some of the p53 isoforms is correlated with disseminated neoplasia in bivalves, notwithstanding that there may be differences between bivalve species in mRNA or protein concentrations that have not been resolved. The transcriptional upregulation of p53 in mollusks affected by disseminated neoplasia might constitute a defense or adaptive reaction to tumor initiation by arresting cell cycle progression or inducing apoptosis. However, mutations in the protein may limit its effectiveness.

Monitoring of mRNA expression levels in bivalve hemocytes provides only limited understanding of the potential activity of proteins that are regulated by p53 family members, and hypotheses are generally based on extrapolation from results in mammalian functional studies.

Expression levels of proteins that are known to be regulated by or interact with p53 in mammals, specifically the proto-oncogene RAS and the heat shock protein mortalin, were investigated in mussels, cockles, and clams, (Böttger et al. 2008; Muttray et al. 2012; Siah et al. 2008). RAS belongs to a class of proteins called small GTPases involved in signal transduction pathways between cells that lead to cell growth, differentiation, and survival. Mutations in RAS can result in aberrant cell growth and are often identified in human cancers. In mussels *M. trossulus*, RAS was found to have a higher number of silent polymorphic variations and elevated expression levels (estimated by band intensity after polymerase chain reaction amplification) in neoplastic hemolymph samples than in normal samples (Ciocan et al. 2006). One of the polymorphic variations coincided with a known mutational hotspot (codon 13) that in mammals leads to diminished GTPase activity and uncontrolled cell division (Bos 1989). In contrast, RAS mRNA (estimated by quantitative polymerase chain reaction) was expressed at a higher level in normal compared to neoplastic hemocytes of mussels and digestive gland tissue in cockles (Muttray et al. 2010; Ruiz et al. 2013). Thus, the role of RAS expression and silent mutations remain an interesting but unsolved component of the processes that might contribute to disseminated neoplasia in bivalves.

Heat shock proteins 70 and 90 kDa in size were detected by western blot analysis in hemocytes of cockles *C. edule* with different stages of disseminated neoplasia, but not in healthy hemocytes, when using the anti-CgHsc72 and human Hsp90 antibodies (Díaz et al. 2010).

Mortalin is a member of the heat shock Hsp70 family of proteins. It has been shown to serve as a cytoplasmic tether for p53 by binding to p53's cytoplasmic sequestration domain (Wadhwa et al. 2002), thus keeping it sequestered in the cytoplasm where it is inactive (Walker et al. 2006). Investigations in neoplastic clam hemocytes showed that mortalin expression was considerably higher in neoplastic clam hemocytes compared to normal hemocytes (Böttger et al. 2008), potentially

inactivating clam p53. In another study, Ahmed Siah and coworkers observed that mortalin levels increased in clams with neoplastic tetraploid hemocyte levels of 15–50% compared to clams with neoplastic tetraploid hemocyte levels of either greater than 50% or less than 15% (Siah et al. 2008). In addition, it was shown that clams with heavily proliferated disseminated neoplasia had a significantly higher expression level of mortalin than clams with moderate neoplasia or healthy clams (Muttray et al. 2012). While not providing functional data, these studies indicate that transcriptional functions of p53 in tumor suppression are silenced when clam mortalin proteins are overexpressed.

Mutations in p53 Gene Transcript

Point mutations on the p53 gene are widely implicated in cancer development in mammals. For instance, mutations in the coding region of p53 are associated with the majority of nonviral tumors in humans, particularly variations in the highly conserved DNA binding domain (Cetin-Atalay and Ozturk 2000; Greenblatt et al. 1994). As discussed earlier in section "Evolution of the p53-Family Genes", this ancient gene family is present in all taxa from protists to humans and has highly conserved functional domains and gene sequences. Moreover, some p53 gene family members are expressed at increased levels in neoplastic hemocytes (section "Expression of p53 Family Members and Regulators in Disseminated Neoplasia"). Therefore, by analogy with human cancers, it was suggested that at least one cause of the dysregulation of p53 gene family members in bivalve neoplasia can be associated with mutations.

Indeed, analysis of the coding region of the p53 gene family in the bay mussel *M. trossulus* revealed that one particular genotype was associated with 94% of late-stage disease cases (Vassilenko et al. 2010). Three single nucleotide polymorphisms (SNPs) were detected, one located in a highly variable region and the other two in highly conserved domains, in the proline-rich domain and in the DBD, close to mutation hotspots in the human p53 gene. All three SNPs were synonymous substitutions, meaning they did not change the coding amino acid. However, silent mutations can induce changes in splicing mechanisms or cause changes in the secondary structure of the mRNA molecule, affecting its interaction with cellular components for RNA processing, transport, stability, and expression (Shabalina et al. 2006). In *Mytilus*, the two SNPs located in the highly conserved domains altered methylation sites on the DNA strands, an important aspect observed in human cancers, where variations in the p53 gene are frequent at methylation sites (Greenblatt et al. 1994). Methylation of DNA strands may occur at CpG and CCTGG sites and is known to be one of the key mechanisms of gene expression regulation. In the human p53 gene, all cytosines on both DNA strands are methylated regardless of the tissue type (Kouidou et al. 2005, 2006). Therefore, SNPs detected in the mussel p53 gene that create additional methylation sites may significantly alter regulation of p53 gene expression if all CpG and CCTGG sites become completely methylated.

A closer look at the three SNPs in the p53 coding region of the mussel *M. trossulus* revealed another interesting fact. Analysis of the haplotypes of the p53 coding region showed that all mRNAs of the p53 family were expressed from a single p53

gene in healthy hemocytes (Vassilenko et al. 2010). This was exactly as expected according to the earlier finding of a single gene copy coding for all p53 family isoforms in invertebrates (Belyi et al. 2010; Kelley et al. 2008; Lu et al. 2009; Muttray et al. 2005; Nedelcu and Tan 2007; Stifanic et al. 2009). In contrast, up to four additional haplotypes were observed in neoplastic hemocytes, which could not be coded by only one gene copy (Vassilenko et al. 2010). This means that there are additional copies of the p53 gene present in the hemocytes' gene pool. The additional copies of the p53 gene may be present in neoplastic hemocytes acquired during the transmission of the disease and undergoing clonal expansion during the disease progression (sections "Viral Induction of Disseminated Neoplasia" and "Horizontal Transmission and Infection").

Yet another surprising fact was observed when two closely related species of mussels, the blue mussel *M. edulis*, and the bay mussel *M. trossulus* were kept side by side in submerged nets, and the p53 sequences in healthy and neoplastic hemocytes from both species were compared with each other (Vassilenko and Baldwin 2013). It was found that the mRNA sequence of p53 in neoplastic hemocytes of *M. edulis* were similar to that of *M. trossulus* instead of healthy *M. edulis* (Fig. 5) (Vassilenko and Baldwin 2013). This likely represents an additional example of horizontal transmission of disseminated neoplasia between mussel species discovered for several other bivalve species (Metzger et al. 2015).

p53 Is Overwhelmed by Heat Shock Protein Mortalin

As a transcriptional regulatory protein, p53 is primarily active in the cell nucleus, where it binds to DNA at p53 binding domains of genes involved in apoptosis and cell cycle arrest. However, p53 is also key in the nontranscriptional mitochondrial activation of apoptosis by disrupting the Bcl2/Bax protein complex and activating caspases. To survive, cancer cells must be able to block both transcriptional nuclear and nontranscriptional mitochondrial apoptosis pathways.

Indeed, in neoplastic hemocytes of the clam *M. arenaria*, p53 is located in the cytoplasm and bound to clam mortalin (Böttger et al. 2008; Walker et al. 2006). This sequestration of wild-type p53 in the cytoplasm away from the nucleus is one of the mechanisms of p53 inactivation that contributes to disseminated neoplasia in clams (Walker et al. 2012). Interestingly, there are similar vertebrate cancers where p53 protein is tethered to mortalin in the cytoplasm and, thus, inactivated. It is especially common in human colorectal cancers and primary and secondary glioblastomas (Kaul et al. 2007; Wadhwa et al. 2002). Cytoplasmic tethering of p53 mediated by mortalin or Parc protein anchors also occurs in undifferentiated neuroblastoma cells in mice and humans and in human colorectal adenocarcinoma cell lines. For additional details on p53 tethering in mice and humans, see Böttger et al. (2008), Gestl and Boettger (2012), and Walker et al. (2006).

Mortalin has an exceptionally high sequence homology between widely divergent vertebrate and invertebrate taxa (Walker et al. 2012), indicating that this protein evolved early on in the evolution of heat shock proteins and possibly the function of p53 binding. Full-length clam mortalin is 91% conserved with human mortalin and has highly conserved functional domains. Functional data for mortalin

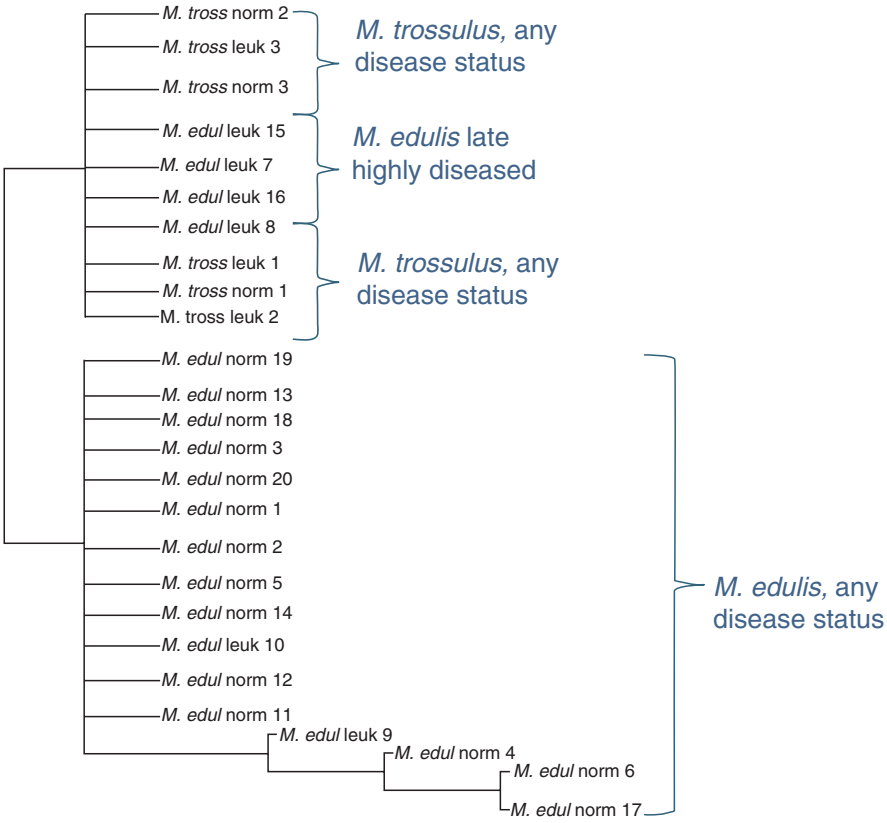

Fig. 5 Phylogenetic analysis of p53 sequences in hemocytes of *M. trossulus* and *M. edulis*. The tree was made using Neighbor-Joining method with Jukes-Cantor model of nucleotide substitution and 1000 bootstraps, no scale. Hemocytes from highly diseased (or leukemic; leuk) and nondiseased (or normal; norm) animals were used for the analysis. (Adapted from Vassilenko and Baldwin 2013)

exist for very few invertebrates, where its activities relate to p53 and apoptosis (identified in nematode worms, echinoderms, clams, and a free-living flatworm). The high sequence homology and conservation of function between invertebrate and vertebrate mortalin makes disseminated neoplasia in bivalves an attractive and simple preclinical model to further elucidate molecular mechanisms of p53–mortalin interaction and to study the effects of chemotherapeutic drugs on this naturally occurring cancer.

Interestingly, in disseminated neoplasia, the distribution of p53 is perinuclear (Walker et al. 2006), a pattern that is also seen in some human cancers. It is thought that nuclear import signals of p53 guide it to the nuclear membrane, but since the tethering of p53 is to a truncated variant of mortalin, which is lacking the ATPase binding site, mortalin cannot release p53, effectively plugging nuclear pores and arresting the mortalin–p53 complex at the outside of the nucleus (Walker et al.

2012). It was further shown that blocking the p53 binding site of mortalin with MKT-077, a cationic inhibitor of mortalin that competes with p53 for binding to mortalin, results in the translocation of p53 to the nucleus and to an increase in the apoptosis of the neoplastic clam hemocytes. Interestingly, treatment of neoplastic hemocytes with DNA damage-inducing etoposide increased the expression of p53 protein to a much greater level than the expression of clam mortalin, and *de novo* p53 was shown to overcome the cytoplasmic tethering by mortalin and translocated to the nucleus inducing apoptosis (Böttger et al. 2008). However, nontranscriptional apoptosis at the mitochondria by p53 (Moll and Zaika 2001) seemed not to be recovered this way. The treatment of neoplastic clam hemocytes with MKT-077 resulted in p53's translocation to the nucleus, with very little accumulation at the mitochondria. However, if the nuclear access was blocked by wheat germ agglutinin (a nuclear pore blocker) prior to treatment with MKT-077, clam p53 was found only in the cytoplasm with increased mitochondrial localization, where it could induce apoptosis via interaction with antiapoptotic Bcl-2 and proapoptotic Bax proteins (Walker et al. 2011).

In the context of comparative immunology, it is important to review mortalin's guardian innate immune system functions that have been discovered in mammals (Kaul et al. 2007) and may similarly be present and awaiting discovery in invertebrate innate immune responses. It has been shown that mortalin is associated with the interleukine-1 (IL-1) receptor type I and was hypothesized to be involved in endocytosis of the receptor because it appeared not to affect IL-1-dependent phosphorylation cascades (Sacht et al. 1999). IL-1 is a major proinflammatory cytokine mediating local and systemic responses of the immune system in vertebrates and invertebrates. Mortalin was also found to be upregulated in isolated rodent pancreatic islets exposed to cytokines. In two rat strains that showed different sensitivities to the toxic effects of cytokines, a significant differences in IL-1 beta-mediated mortalin expression was observed, suggesting its role in cytokine-induced beta-cell destruction (Johannesen et al. 2004).

Another major function of surface-expressed mortalin is its captivating role in antigen presentation and in innate immunity. Complement-mediated cell death is caused by a membrane attack complex (MAC) that inflicts damage on target cells. For protection, cells eliminate the MAC from their surface either by ectocytosis (direct emission of membrane vesicles) or by endocytosis. Recently, the involvement of mortalin in MAC elimination has been suggested. Mortalin was shown to bind to complement and is shed in vesicles containing complement MACs. These results suggest that mortalin promotes the shedding of membrane vesicles loaded with complement MAC and protects cells from complement-mediated lysis (Kaul et al. 2007, and references therein).

There are some noteworthy parallels between the mortalin–p53 tether and another p53 sequestration process. Multiple studies (reviewed in Miciak and Bunz 2016) in vertebrates have shown that adenoviruses are able to suppress the antiviral interferon (IFN) immune response by expressing the viral proteins that assemble an active E3 ubiquitin ligase complex and mark nuclear p53 for degradation. However, these adenoviral proteins are also able to interfere with the location of p53 and

export it to the cytoplasm. As we have seen with the mortalin tether in clams, the viral E1B-55 K protein suppresses mitochondrial destabilization by p53 and, thus, inhibits nontranscriptional mitochondrial apoptosis. p53, together with E1B-55 K, instead localizes in perinuclear regions of the cytoplasm and appears to be retained in a stable form. There are surprising similarities between the perinuclear colocalization of clam p53 and mortalin (potentially mediated by retroviral activation) and the perinuclear colocalization of vertebrate p53 and adenoviral E1B-55 K protein.

Clearly, mortalin is a central player in innate immune responses protecting cells from apoptosis in diverse vertebrate and invertebrate taxa. One of the questions remaining with regard to bivalve disseminated neoplasia is why or how mortalin is induced to sequester p53 in the cytoplasm, where it is inactive, to allow neoplastic hemocytes to survive and ultimately kill the affected animals and in some cases (Barber 2004; Böttger et al. 2013) large proportions of a local population.

p53's Negative Regulator MDM2

Mammalian p53, p63, and p73 induce expression of MDM2, a negative regulator of the p53 family proteins (reviewed in Harms et al. 2004 and Levrero et al. 2000). MDM2 represses p53 and p73 activity by binding to their N-termini, obstructing the transactivation domain and, hence, the ability of p53/p73 to transactivate downstream target genes. In addition, MDM2 functions as a p53-specific ubiquitin ligase destining p53 for degradation and thereby maintains p53 at low levels in unstressed cells to prevent undesired apoptosis (Calabro et al. 2002; Marine and Jochemsen 2005).

Many invertebrate genomes, including sea squirts, mollusks, placozoans, annelid worms, and deer ticks, encode a single MDM2-like protein (Lane et al. 2010a, b; Momand et al. 2011; Muttray et al. 2010), but it appears suspiciously absent from the genomes of the invertebrate model organisms *Drosophila melanogaster* and *Caenorhabditis elegans,* which also have widely divergent p53s (Joerger and Fersht 2016). All four functional domains of MDM2—the N-terminal, acidic region, zinc finger, and RING domains—are conserved in the invertebrate MDM2 homologs. Based on studies in the most primitive metazoan animal *Trichoplax adhaerens* (von der Chevallerie et al. 2014), the p53–MDM2 interaction and its regulation had already been established in some of the earliest animals. With the emergence of vertebrates, a duplication of the ancestral MDM2 gene gave rise to two paralogs, MDM2 and MDMX (MDM4), and a more complex regulatory mechanism (Coffill et al. 2016; Momand et al. 2011). This is an example of a tightly linked coevolution of two regulatory proteins, where the duplication of the ancestral MDM2 gene appears to have coincided with the duplication of the ancestral p53 gene.

Of the bivalve MDM proteins, only bay mussel *M. trossulus* MDM was demonstrated to exist experimentally and shown to form a complex with p53 in vitro (Muttray et al. 2010). To date, it has not been possible to show that this complex leads to ubiquitination and degradation of the invertebrate p53 by MDM2's E3 ligase activity (Muttray, unpublished data). Reasons for the failure to induce invertebrate p53 ubiquitination may include that the invertebrate MDM may act more like a mammalian MDMX, which is structurally very similar to MDM2, but by itself cannot degrade mammalian p53 (Shadfan et al. 2012). However, transfection

of a p53-null, human non-small-cell-lung-carcinoma cell line with molluscan p53 isolated from the clam *M. arenaria* induced expression of endogenous human HDM2, suggesting that the molluscan p53 might be able to participate in the MDM2 feedback loop (Holbrook et al. 2009). At a minimum, the E3 ligase activity of MDM2 was established in the common ancestor of vertebrates more than 500 million years ago (Joerger and Fersht 2016). It was further shown that MDM expression levels were directly correlated with p53 expression levels in healthy and in neoplastic hemocytes, but not with other p53 isoforms or with the proto-oncogene RAS in *M. trossulus* (Muttray et al. 2010).

Implications for Innate Immune Defense

Although the origin of the transformed cells has still not been resolved (section "Origin of Neoplastic Hemocytes"), it is becoming more evident that disseminated neoplasia spreads as a clonal transmissible cell derived from a single original clam or mussel that experienced *de novo* retroviral infection. This cell can be transmitted between animals of the same species or even between species (Metzger et al. 2015, 2016). Normally, virus infections trigger an innate controlled cell death response by activating the p53 tumor suppressor protein to limit the "growth" of viruses, but viruses, in turn, have evolved strategies to express proteins that can counter p53 and block the apoptosis response (Anand and Tikoo 2013; Everett and McFadden 1999). In bivalve disseminated neoplasia, the innate p53-mediated apoptotic response appears to have been severely curtailed as the dysregulated cancerous hemocytes multiply uncontrollably within the animal's hemolymph and solid tissues. This inability to enter the apoptotic response pathway is passed on to a new individual by transmission and clonal expansion of the neoplastic hemocytes, and this step does not require activation by a *de novo* viral infection.

In vertebrate models, p53 was revealed as a central mediator and amplifier of the global innate immune response against viral infections via the IFN pathways (Miciak and Bunz 2016). Since genes for the IFN pathway and IFN-induced genes exist and are functional in a range of mollusks (but not in nematodes and arthropods) (Owens and Malham 2015), p53's mediating function in the antiviral immune response can hypothetically be extended to mollusks. The well-studied p53 promoter in vertebrates contains a functional IFN-stimulated response element, and therefore p53 can be considered an IFN-stimulated gene (ISG). As an ISG product itself that upregulates other ISGs via a regulatory cofactor, p53 serves to amplify the intracellular IFN response to suppress replication of RNA viruses even if cells fail to undergo apoptosis following infection. However, during the development of disseminated neoplasia, the retroviral *Steamer* is somehow able to subvert the IFN response.

The rarity of transmissible tumors in vertebrates can be explained by the fact that the adaptive immune system can generally recognize self from nonself via the major histocompatibility complex (MHC) presented on the surface of each cell. In the case of DFTD, tumor cells do not express MHC molecules on their surface due to a

downregulation of genes essential to the antigen-processing pathway (Siddlea et al. 2013), making it difficult for the recipient's immune system to recognize them as nonself. Further, these cells appear to be able to evade the innate immune system's natural killer cell recognition and defense. Bivalves are not known to have a self/nonself recognition system similar to the MHC system, which may make them more susceptible to infections by transmissible cancer cells from other individuals of the same species. Whether natural killer cells or other innate immune functions play a role in the defense against such infections by transmissible cancer cells remains to be resolved. Within highly affected clam or mussel populations, disseminated neoplasia represents a significant selective pressure, which supports the hypothesis that histocompatibility could have evolved in part due to selective pressure to prevent malignancy (Murgia et al. 2006), rather than simply being a secondary consequence of pressure by infectious diseases. As hypothesized by Michael Metzger and coworkers (Metzger et al. 2015), despite the lack of MHC, mollusks and other invertebrates may employ other self/nonself recognition mechanisms, perhaps similar to the fusion/histocompatibility (Fu/HC) system of colonial ascidians, which protects ascidians from stem cell parasitism, which can occur when unrelated individuals fuse (De Tomaso et al. 2005; Voskoboynik et al. 2013).

In conclusion, recent progress in unraveling the molecular mechanisms of disseminated neoplasia in bivalves has further developed this natural system into an excellent model for the study of the development of retrovirally induced and transmissible cancers, cumulative effects from confounding environmental factors, and functions of the p53 family members related to innate immunity, thus advancing our understanding of human cancers. As echoed by many researchers, by corroboratively applying comparative phylogeny, immunology, and oncology approaches, scientific findings from molecular human and marine biology will be combined to better understand cancer and immune system responses and to translate our growing understanding into novel therapies to benefit humans and mollusks.

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Amphibia: Global Amphibian Declines Caused by an Emerging Infectious Disease and Inadequate Immune Responses

Jonathan Edward Kolby

Introduction: Global Amphibian Declines

Amphibians have been declining globally since the mid-twentieth century, and this phenomenon has been described as a component of the Earth's sixth mass extinction (Stuart et al. 2004; Wake and Vredenburg 2008). Most amphibians experience a biphasic life cycle, wherein larvae are exclusively aquatic and adults emerge into semi- or entirely terrestrial or arboreal habitats, returning to water to reproduce. Amphibian skin is highly permeable and sensitive to the surrounding environment, closely intertwining the health of these animals to the health of the landscape in which they live. In recent decades, a multitude of independent and additive stressors have been contributing to amphibian declines, including habitat loss and degradation, pollution, and overexploitation (Stuart et al. 2004). Traditionally, habitat protection has long been regarded as the most important aspect of safeguarding species in the wild, but a new global threat—the spread of emerging infectious wildlife diseases—cannot be mitigated by physical or legal boundaries erected to delineate a protected area. Pathogens can invade national parks and wilderness areas and negatively impact wildlife populations quickly, silently, and in the absence of prolonged symptoms that might alert the need for a rapid conservation response.

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Discovery and Description of *Batrachochytrium dendrobatidis*

Globally significant amphibian population declines began to occur in the 1970s, but nearly three decades passed before an emerging infectious disease was found to be the likely culprit in many of these instances (Berger et al. 1998). In the early 1990s, a group of Australian researchers studying amphibian declines in Queensland, Australia, determined that the emerging pattern of decline was consistent with the spread of an infectious disease invading new regions at a rate of nearly 100 km/year (Laurance et al. 1996). After documenting an amphibian mass mortality that resulted in the extinction of the sharp snouted dayfrog (*Taudactylus acutirostris*), material collected during this event led to the description of chytridiomycosis, a novel epidermal disease of amphibians (Berger et al. 1998). Then, in 1998, frog mass mortality events in the wild in Panama and in a captive population at the National Zoo in Washington, D.C., also showed signs consistent with chytridiomycosis. Examination of samples from the National Zoo mortality event allowed for the identification of the etiological agent of chytridiomycosis as a novel species chytrid fungus, described as *Batrachochytrium dendrobatidis* (Bd) in 1999 (Longcore et al. 1999). Although hundreds of species of saprophytic chytrid fungi exist around the world, Bd is the first known species to parasitize and cause disease in living vertebrates.

Bd Infection and Development of Chytridiomycosis

Bd is predominantly aquatic in nature and spreads through the release of uniflagellated motile zoospores expelled from mature zoosporangia that grow in the skin of infected amphibians (Longcore et al. 1999). These zoospores are shed at the skin surface and, if released into the water, can actively swim short distances toward a new amphibian host or be passively spread and transmitted via water currents (Rachowicz and Vredenburg 2004). Upon physical contact, Bd encysts on the skin surface and sends a germ tube down into the layer of skin where keratin is produced (Greenspan et al. 2012). Inside this tissue, Bd's chitin-walled zoosporangia mature for approximately 5 days and asexually reproduce by growing new uniflagellated aquatic zoospores, which are then released back at the skin surface, where they either reinfect the same amphibian host or become shed into the environment (Berger et al. 2005).

In amphibians, the skin is a highly permeable vital organ that aids in respiration, osmoregulation, and electrolyte balance, and damage to this tissue's form and function can be catastrophic to amphibian health. Infected larval amphibians sometimes experience oral deformities but generally seem to tolerate Bd infection without experiencing increased mortality, and most animals succumb to chytridiomycosis during or following metamorphosis (Fig. 1) (Lamirande and Nichols 2002; Marantelli et al. 2004; Rachowicz and Vredenburg 2004). This is most likely due to concurrent changes in both the skin and immune system development. The presence of keratin in larval amphibians is confined to the oral structures, but during metamorphosis from larvae to adult, keratin-bearing tissue begins to form throughout the

Fig. 1 Dead exquisite spike-thumb frog (*Plectrohyla exquisita*) from Cusuco National Park, Honduras, found with chytridiomycosis

amphibian's skin, allowing Bd to invade a significantly larger surface area. Around the same period, the immune system undergoes a dramatic reorganization, and the amphibian's defenses are temporarily suppressed during development of the adult immune system (Rollins-Smith et al. 2011). When Bd infection advances and begins to damage the skin, this earmarks the onset of the disease chytridiomycosis.

Chytridiomycosis often causes hyperkeratosis, a thickening of the skin that prevents an amphibian from shedding properly and contributes to death by cardiac arrest due to electrolyte imbalance (Voyles et al. 2009). This disease also sometimes manifests behavioral symptoms, including lethargy, loss of righting reflex, and anorexia, as well as physical symptoms, most notably skin lesions and irregular shedding in adults and oral deformities in larvae, but these are all nonspecific to chytridiomycosis and cannot alone confirm infection or disease (Altig 2007). A Bd-specific conventional polymerase chain reaction (PCR) test can be used to confirm the presence of infection, and a quantitative PCR test can be used to also measure infection load, whereas histological examination of affected tissue is needed to confirm the presence of disease.

Response to Bd Exposure

Bd infection is generally lethal to amphibians, but the response to Bd exposure can vary considerably between species. Certain species, such as the Panamanian golden frog (*Atelopus zeteki*), are extremely susceptible to Bd infection, rapidly develop chytridiomycosis, and experience mortality in nearly all instances of laboratory experimental exposures. Even a minimal inoculation dose, as little as a single Bd zoospore, can catalyze disease and mortality in laboratory exposure of susceptible

species, such as the boreal toad (*Bufo boreas*) (Carey et al. 2006). On the other end of the spectrum, some amphibian species are susceptible to infection but resistant to disease, including the American bullfrog (*Lithobates catesbeianus*) (Daszak et al. 2004) and the African clawed frog (*Xenopus laevis*) (Rollins-Smith et al. 2009). These two species commonly persist with low-intensity infections and can also sometimes resist or clear infections. As a result, these species are generally asymptomatic Bd reservoir hosts and act as vectors of Bd dispersal.

Immune Response and Resistance to Bd

Most amphibian species experimentally exposed to Bd have demonstrated an immune response insufficient to suppress Bd and entirely clear infections. In laboratory inoculation experiments, rates of mortality from exposure to Bd varied from 0 to 100%, with most species exhibiting an intermediate response (Berger et al. 2016). Bd is able to successfully evade host immune recognition and inhibit antifungal defenses due to the excretion of a soluble inhibitory factor from its cell wall into the supernatant that inhibits lymphocyte proliferation and induces apoptosis (Fites et al. 2013, 2014; Rollins-Smith et al. 2015). The discovery of this cytotoxic material has helped to illustrate that Bd is indeed manipulating the immune response of susceptible amphibians, which could otherwise be misinterpreted as the absence of an innate immune response. This phenomenon is clearly demonstrated by the rapid morbidity and mortality exhibited by the Panamanian golden frog (*Atelopus zeteki*) following Bd exposure in laboratory trials (Ellison et al. 2014).

Whether vaccination-type therapy can help boost an amphibian's adaptive immune response enough to increase survival following subsequent Bd exposure remains under investigation. In a laboratory experiment where Booroolong frogs (*Litoria booroolongensis*) were infected with Bd, cleared of infection using itraconazole, and then reinfected, a stronger adaptive immune response was not stimulated and animals did not experience increased survival (Cashins et al. 2013). In contrast, another study that exposed Cuban tree frogs (*Osteopilus septentrionalis*) to Bd, cleared them by exposure to elevated temperatures lethal to Bd (30 °C), and then reexposed them found that this activity did significantly increase lymphocyte proliferation in response to infection, reduced Bd prevalence among preexposed animals, and increased rates of survival (McMahon et al. 2014). These results suggest that some amphibians can acquire a level of increased immunity to Bd that may overcome pathogen-induced immunosuppression if treated with heat but not with itraconazole, which can sometimes express confounding immunosuppressive properties. In another immunization investigation, Poorten et al. (2016) performed experiments to determine whether mountain yellow-legged frogs (*Rana muscosa*) were able to mount a robust adaptive immune response to Bd reexposure. Animals were immunized by exposures to killed Bd before inoculation with live Bd, and in no trials did increased antibody production occur. Immunization of amphibians to prompt an adaptive immune response to Bd exposure might be an effective tool to help increase survival of some species, but not others. Additional studies are needed

to evaluate its potential effectiveness in a field conservation framework to determine whether preexposure to Bd can elicit enough of an adaptive immune response for amphibians to overcome Bd-induced immunosuppression once returned to a Bd-contaminated natural habitat.

Resistance to Bd infection and chytridiomycosis can also be affected by secretion of antimicrobial peptides (AMPs) produced by the skin of some amphibians (Woodhams et al. 2016; Rollins-Smith et al. 2011) and by secondary metabolites secreted by symbiotic bacteria that naturally coexist on amphibian skin (Brucker et al. 2008). In addition to the anti-Bd properties of some AMPs, the type and amount secreted on the skin play an important role in the composition of microbiota that inhabit the amphibian's skin. The most notable example of a naturally occurring secretion that provides significant anti-Bd properties is a pigment called violacein, produced by the Gram-negative bacterium *Janthinobacterium lividum* (Brucker et al. 2008). This bacterium can be found in high densities on the skin of red-backed salamanders (*Plethodon cinereus*) in the Northeastern USA. Violacein is toxic against fungi, and when *J. lividum* colonies are abundant on the skin of an amphibian, violacein is effective at suppressing Bd development. Experimental treatments are now being tested that involve probiotic inoculation of frog skin with *J. lividum* isolated from salamanders in an attempt to transfer cutaneous resistance to Bd infection from species that naturally coexist with these bacteria to those that do not, such as the mountain yellow-legged frog (*Rana muscosa*) (Vredenburg et al. 2011). Laboratory and field trials have shown that probiotic inoculation can be successful in some circumstances, but the long-term persistence of bacterial colonization on amphibians under natural field conditions requires further investigation.

Veterinary Treatment of Chytridiomycosis

Methods of treatment to reduce morbidity and mortality in captive amphibians suffering from chytridiomycosis are limited. Since Bd does not form a protective encapsulated spore, infectious particles are vulnerable to desiccation and exposure to temperatures above 28 °C. Extended exposure to elevated air or water temperatures (>28 °C) is the single proven nonchemical method to suppress Bd reproduction or kill zoospores (Johnson et al. 2003), but many species of amphibians affected by Bd inhabit cool montane forests and may be unable to tolerate these conditions. The most widely applied treatment involves brief daily submersion in a diluted itraconazole bath for approximately 10 days (Baitchman and Pessier 2013). This method has proven effective for most adult amphibians, but concerns have been raised that larval amphibians and even some adults may be negatively affected if doses are too high (Woodhams et al. 2011; Brannelly et al. 2012). Additional medications have shown some efficacy in reducing the load of Bd on infected animals including chloramphenicol (Holden et al. 2014) and voriconazole (Martel et al. 2011), but at present, itraconazole remains the most effective medication to fully clear Bd infections.

Virulence of Bd and Regional Strains

Although primarily clonal, Bd displays a complex evolutionary history involving multiple instances of regional isolation and genetic drift (Rosenblum et al. 2013). This has resulted in the emergence of multiple Bd lineages that now express variable degrees of virulence when introduced to amphibians from different regions. The most abundant and commonly detected lineage in field surveys is also the one that expresses the greatest virulence, referred to as the globally virulent panzootic lineage, or Bd-GPL. Several clades exist therein, generally characterized by broad regional distributions. Other lineages found in more limited distributions include those characteristic of Switzerland (Bd-CH), South Africa (Bd-CAPE), and multiple countries in Asia (e.g., Japan, China, India, Korea), all of which appear to be generally less virulent than Bd-GPL (Rosenblum et al. 2013; Berger et al. 2016).

The most likely reason for Bd-GPL's comparatively exceptional virulence and global ubiquity is that it formed from a rare sexual reproduction event between two different Bd lineages (Farrer et al. 2011; Schloegel et al. 2012), and this hybrid offspring, Bd-GPL, was able to outcompete longstanding endemic hypovirulent Bd strains. The international trade in live amphibians has provided ample opportunities for Bd-GPL to hitchhike among the frequently traded high-volume, high-density commercial amphibian shipments and rapidly emerge globally. Researchers continue to investigate mechanisms of Bd virulence and have yet to identify the biological catalyst for increased Bd virulence expressed by some isolates relative to others.

Global Dispersal of Bd

The spread of Bd involves multiple natural and anthropogenic pathways, but the most common mode of contemporary global dispersal likely to result in new outbreaks of disease is the international trade in live Bd-positive amphibians (Kolby 2014; Kolby et al. 2014; Kolby and Daszak 2016). Millions of amphibians are traded globally each year, primarily for use as exotic pets, food for human consumption of frog legs, and biomedical research test subjects. In the USA, possibly the greatest destination country of live amphibians by volume, nearly 50% of the approximately five million amphibians imported annually are American bullfrogs (*Lithobates catesbeianus*), farmed overseas and then shipped back to be consumed as frog legs (Schloegel et al. 2009), and, to a lesser extent, African clawed frogs (*Xenopus laevis*), imported for the biomedical trade. Both of these species are aclinical reservoir hosts of Bd, are traded globally in high densities likely to increase disease transmission during transport, and commonly become invasive upon release or escape, allowing for the establishment of feral populations. In 2007, chytridiomycosis was listed as a notifiable disease by the World Organization of Animal Health (OIE), and recommendations were made to help control its spread (Schleogel et al. 2010), but negligible action has been formally adopted by the international community to mitigate the continued global spread of Bd through trade.

The international wildlife trade allows Bd to quickly cross environmental boundaries inhospitable to survival and traverse expansive geographical distances otherwise likely to have slowed the pathogens' global emergence. Following introduction to a new environment, Bd can exploit a diversity of dispersal avenues in nature. Aquatic Bd zoospores can be passively spread long distances by water currents and adhere to the feet of waterfowl and be transported through flight across inhospitable terrestrial zones to noncontiguous water bodies (Garmyn et al. 2012). Metamorphic amphibians emerging from a body of water carry exceptionally high loads of Bd on their skin and frequently shed infectious material onto vegetation shared with terrestrial species that would not normally be expected to encounter an aquatic pathogen (Kolby et al. 2015a). Bd has also been detected in rainwater, demonstrating the potential for long-range transport during tropical storm events (Kolby et al. 2015b).

Conclusion

Bd is now present in 60+ countries and continues to spread, largely unabated. Over 500 amphibian species have been confirmed to be susceptible to infection (Olson et al. 2013), and the true number of species at risk of disease and decline is likely much higher. Most amphibians tested have not demonstrated sufficient innate resistance to chytridiomycosis, and this disease often leads to gradual or rapid decline of naïve amphibian populations following Bd introduction. At present, no targeted efforts have been made to prevent the introduction and establishment of Bd-GPL at a naïve location, and thus it remains uncertain whether an endangered species highly susceptible to Bd could feasibly be protected in situ from an approaching wave of Bd. Never before has a single pathogen demonstrated such high virulence and low host species specificity and acutely threatened an entire class of organisms. In the absence of efficient treatment options to boost the immune responses of amphibians to Bd exposure in the wild, developing solutions to mitigate the continued emergence of this global disease event now represents one of the greatest conservation challenges and should be made a priority to prevent irreparable biodiversity decline.

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Biotherapy: Medicinal Maggots and Invertebrate Immunology from the Clinician's Perspective

Ronald A. Sherman and Edwin L. Cooper

Biotherapy is the term used to denote the practice of using live animals to treat or diagnose illness. The term, and the formal discipline, are only about 30 years old, but the practice itself dates back thousands of years. The use of leeches and honey bees, for example, can be traced back to ancient Greek, Egyptian, Persian, and Asian societies (Gileva and Mumcuoglu 2013; Kim 2013). Biotherapy encompasses a variety of living creatures, from microbes to mammals (Fig. 1). Examination of one of these modalities, maggot therapy, offers an opportunity to examine how invertebrate immunology and physiology have been harnessed to benefit human health.

Maggot therapy, also known as maggot debridement therapy (MDT), larval therapy, biodebridement, and biosurgery, is the application of live fly larvae to wounds in order to aid in wound debridement (cleaning), disinfection, or healing. A maggot infestation on a living vertebrate host is called myiasis. When that infestation is limited to a wound, it is called wound myiasis. Maggot therapy is basically a therapeutic wound myiasis, controlled to optimize efficacy and safety (Sherman et al. 2013). We control myiasis by carefully selecting the species and strain of fly (Table 1), disinfecting the larvae, using special dressings to maintain the larvae on the wound, and integrating quality control measures throughout the process.

Maggot therapy treats chronic wounds through three main actions: debridement (removal of dead tissue and debris), disinfection, and stimulation of healthy tissue growth (Sherman 2014). These actions are brought about through the maggots' digestive secretions/excretions and through the physical action of the maggots

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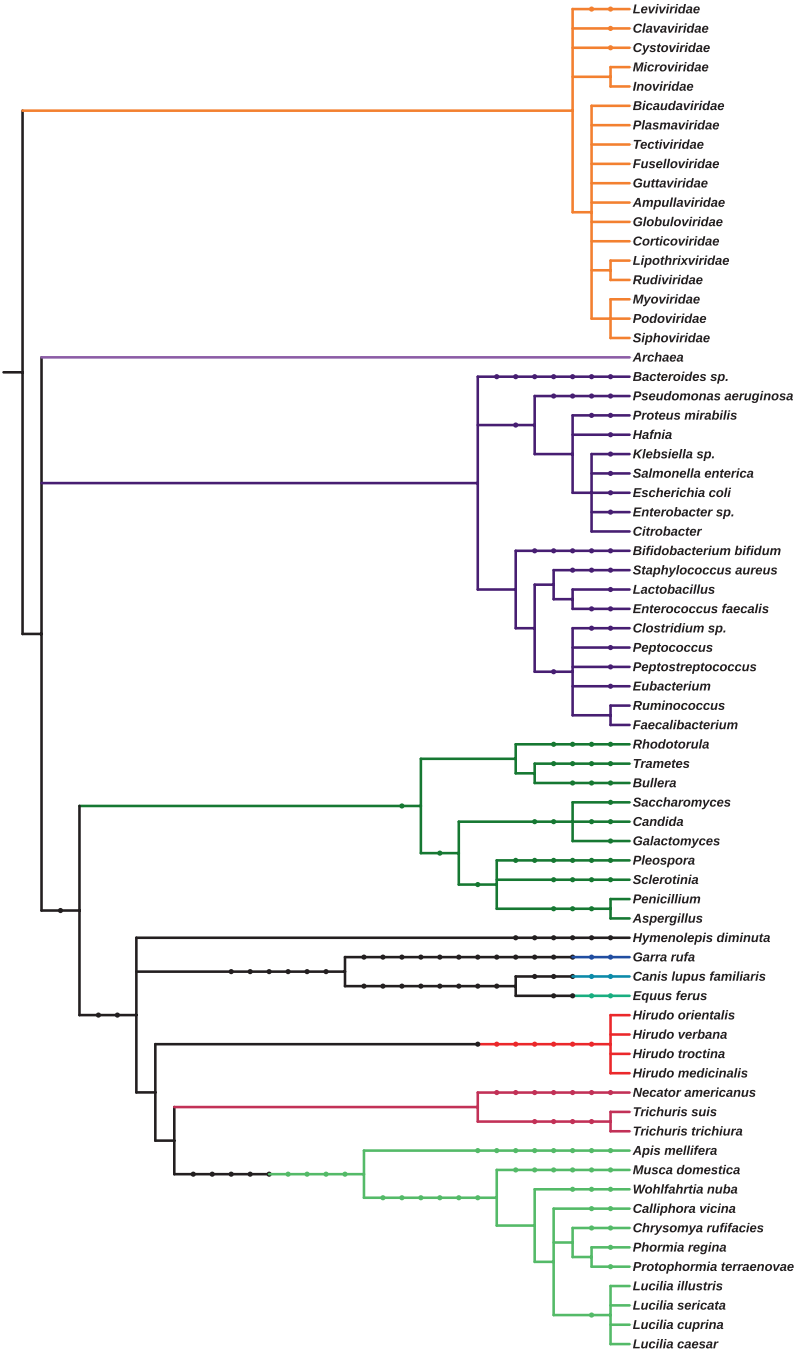

Fig. 1 Phylogenetic diagram of most commonly used biotherapeutic animals. Major clades include viruses, archaea, bacteria, fungi, platyhelminths, annelids, nematodes, insects, cyprinids (carp), canines, and equines

Table 1 Fly species (Order: Diptera) that have been used in maggot therapy

Family	Species
Calliphoridae	
	Calliphora vicina
	Chrysomya rufufacies
	Lucilia caesar
	Lucilia cuprina
	Lucilia illustris
	Lucilia sericata
	Phormia regina
	Protophormia terraenovae
Sarcophagidae	
	Sarcophaga haemorrhoidalis
	Sarcophaga bullata
	Wohlfahrtia nuba
Muscidae	
	Musca domestica

After Sherman et al. (2013)

crawling about the wound bed. Maggot debridement is thorough and precise. It is accomplished, in part, through the proteolytic action of the maggots' digestive enzymes, liquefying the necrotic wound tissue but not the viable tissue. The maggot's spined body also aids in debridement by dislodging dead cells and debris.

Microbial killing was extensively reviewed by Nigam and associates (Nigam et al. 2006a, b). In the decade since, we have come to learn that *Lucilia sericata* secretions/excretions not only kill bacteria in cultures but also dissolve and inhibit the formation of biofilms (Cazander et al. 2009, 2010; Bohova et al. 2014). It was long assumed that medicinal maggots produced antimicrobial peptides like many other insects, especially given the fact that species members, used medicinally, would need to protect themselves from microbes that abound in their natural environments: corpses, feces, and other decaying organic matter. A variety of antimicrobial peptides have now been identified, and some have been completely characterized, including lucifensin (Cerovsky et al. 2011), lucimycin (Pöppel et al. 2014), alloferons (Chernysh et al. 2002), and others (Ratcliffe et al. 2014).

Medicinal maggots alter the immune system of the host as well. Clinically, maggot therapy has been associated with decreased inflammation, though this may in part be merely a result of the antimicrobial and debriding actions that also reduce inflammatory stimuli. Recently, laboratory studies demonstrated that medicinal maggots might suppress the immune response directly by affecting the complement cascade. By combining secretions/excretions from *L. sericata* larvae with serum from preoperative (healthy) and postoperative (immune-activated) patients, Cazander et al. (2012) demonstrated that the maggot excretions reduced complement activation up to 99.9%, via all pathways, breaking down C3 and C4 proteins in a cation-independent, temperature-tolerant manner. Dauros Singorenko and coworkers (2017) demonstrated that maggot excretions/secretions upregulated gene expression in several human wound cell types in vitro, especially monocytes, and partially reduced the interleukin 8 transcription otherwise seen in response to bacterial lipopolysaccharide exposure.

In 2004, medicinal maggots became the first live animal to be granted marketing clearance by the US Food and Drug Administration (FDA) (Sherman 2014). Leeches (*Hirudo medicinalis*) are the only other live animal cleared by the FDA for marketing in the USA (Mumcuoglu 2014).

The use of these animals as food or nutritional supplements and the use of their tissues and extracts are not considered to be biotherapies (since it is not the living animal that is used). But their medicinal benefits may very well be based, at least in part, on some of the same biochemical and immunological mechanisms of action. For example, leech saliva has been shown to contain antimicrobial, anti-inflammatory, and anticoagulant proteins, useful in treating osteoarthritis (Michalsen et al. 2003, 2008; Andereya et al. 2008; Stange et al. 2012; Cooper and Mologne 2016), and a few hirudin-based drugs have been approved as anticoagulants (Eldor et al. 1996; Fields 1991). With continued research, the list of medicinal animals and animal products making their way into mainstream medical practice should continue to grow.

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Pathogens and Cancer: Clonal Processes and Evolution

Edwin L. Cooper

Introduction

Clonal evolutionary processes exist when we consider three prime players, cancer, pathogens, and immunity, and how they interact. The immune system is considered to be an evolutionary necessity that allows all living organisms to guard and protect against mostly externally (but not excluding internally) derived pathogens (e.g., viruses, bacteria, fungi, yeasts, and protozoan parasites). Protection from an internal threat like cancer is an equally powerful force in the armamentarium of all multicellular animals. Invoking Darwinian theory, pathogens and immune reactivity against threats may influence the clonal process. Clonal expansion of cancer cells may occur when the genetic plasticity of these cells allows mutations that provide selective advantages for survival and proliferation. Clonal selection (characteristic of the immune system) is relevant since, when applied to potentially cancerous cells, it allows them to escape from control, thereby favoring peregrinations or metastases more appropriately committed to cancer.

Animal models, especially *Drosophila*, have played a crucial role in shedding light on the evolution of cancer, especially when we benefit from less anthropocentricity. Cancer development is clearly (not) an exclusive characteristic of long-lived vertebrates that are bolstered by a highly *specific* immune system. Certain short-lived invertebrates like marine mollusks (clams) with *nonspecific* immune systems also develop cancer. Another invertebrate, an annelid, the earthworm, will develop specific immune clones; responses can be transferred by clonal leukocytes primed by nonpathogenic antigens.

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Interactions Between Hosts, Pathogens, and Clonality

Interactions between hosts and pathogens are often characterized by substantial phenotypic plasticity. Pathogens may alter their exploitation strategies as a response to essential immune-defensive strategies that have evolved by their hosts and vice versa (Taylor et al. 2006). In a simple host–pathogen system, pathogens exhibit levels of virulence, and in turn the host exhibits complementary levels of immune clearance. Embracing immune clearance to the fullest extent is credible as soon as more experimental inclusion is implemented. This can only come about as evolutionary biologists openly and willingly foster an amalgam of or relaxation of views, thereby embracing at the outset a more "fuzzy" explanation characterized by "blurring"; this approach will promote inquiry and benefits from less anthropocentricity (Kvell et al. 2007, Cooper 2016).

Clearly pathogens can serve to initiate this state, that is, induction of immunity, and in certain instances there occurs an aberrant response, the induction of cancer. Held in check, the induction of an immune response is beneficial to the host; the opposite, meaning unchecked, that is, cancer, is detrimental. That fine line of distinction between induction of clones as immune vs. neoplastic conversion remains a mystery. To compound this problem, a consideration of innate immunity primarily in invertebrates perpetuates the confusion since invertebrates with their innate response rapidly reproduce reactions to pathogens as germline not clonal, yet a substantial number of invertebrates, especially marine mollusks, develop cancer. So what holds a putative repressor in check or releases it, unleashing a neoplasm?

History of Cancer as Clonal Processes

Cancer as an Evolutionary Process

Within the context of evolutionary theory, explanations concerning the origins of cancer that are reasonable are relatively recent. After all, the scourge of cancer has attracted more recent attention. In 1976, Peter Nowell first proposed that cancer could be explained by invoking an evolutionary mechanism driven by clonal selection (recall the use of the term *clonal selection*) associated with specificity, and memory in the adaptive immune system is considered a Darwinian corollary by Burnet (1976). Moving from this mechanism, more aggressive cell generations may be produced by selecting for genetic changes associated with tumor progression (Nowell 1976). Genetic instability in populations of tumor cells (compared to regular and normal somatic cells) could undergo clonal selection, thereby producing increasingly more mutated subpopulations (Nowell 1978). We must take care not to compare or confuse, in the rigid sense, clonal selection of cancer development with clonal selection in the normal somatic immune system. Steps in both processes may share similar, superficial attributes; however, outcomes are undoubtedly different: demise vs. protection. Clearly this process should recognize the source and influence of the inducer. After all, pathogens that could induce clonal selection within the context of the immune system could be different from those that normally initiate cancer.

Darwinian Evolution

As we search for explanations that are not fanciful (e.g., worms as sources of cancer, sufficiently serious to merit the 1926 Nobel Prize!) (Williams 2001), there are views whose platform is rooted in biological theory. A Darwinian model involving selection pressures, rather than the quantity of initiator cells, might more reasonably predict any varying successes or lack of clonal cancer cells. The principles of Darwinian evolution can explain the emergence of increased proliferation and invasive abilities. One strong and reasonable observation recognizes that especially malignant cancers apparently possess unique and effective resistance to therapy (Greaves and Maley 2012). Thus, when considering evolutionary theory, which may govern clonal expansion of cancer, the focus should be the cancer cell as the unit of selection. Cancer cells are under selective pressure that favors the capacity for maximum clonal replication but may result in a loss of the ability to differentiate. Thus raw material for clonal evolution is not composed entirely of genetic changes; heritable epigenetic changes, which can also be selected and usually occur more frequently than genetic changes, could lead to several selective advantages observed in cancer cells, for example, resistance to cancer therapies. Therapies destroy competitors of therapy-resistant cells (an example of strong selective pressure); tumor evolution and proliferation could occur more rapidly in the direction of resistance to therapy (Greaves and Maley 2012).

Oncogenic Selection Influences Evolution of Normal Cells

More insights have been gained. Greaves and Maley (2012) proposed that cancers evolve by a reiterative process of clonal expansion, genetic diversification, and clonal selection within the adaptive landscapes of tissue ecosystems. A crucial environmental variable now enters this scenario: with therapeutic intervention, cancer clones may be destroyed but simultaneously erode their habitats. This ecological niche *perforce* thus provides a potent and unintended selective pressure for any resistant variants to expand in this new and different but temporary and perhaps more favorable environment. Assuming that cancer is inherently Darwinian, the primary reason for this seemingly therapeutic failure, suggests only clues to more effective control, still without plausible insights (Fig. 1) (Greaves and Maley 2012).

p53: A Well-Known Tumor Suppressor Gene

Although p53 is a well-known tumor suppressor gene, one question remains concerning p53 and the more general topic related to the evolution of life forms. What role if any did p53 play in the evolution of multicellular organisms? This process occurred until two problems imposed limitations on analytical progress: (1) organization to support a harmonious whole and (2) the presence of renegade cells that

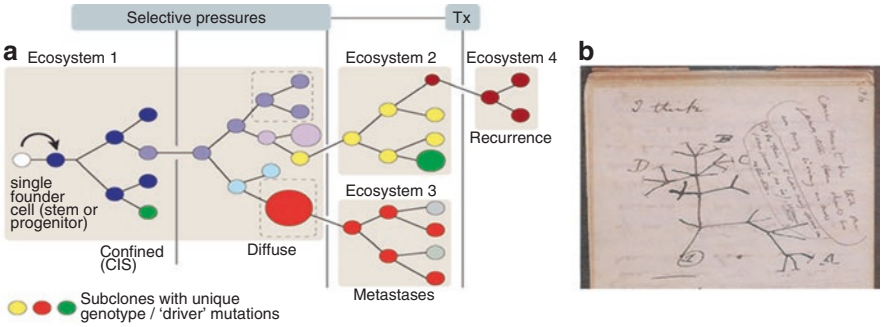

Fig. 1 (**a**) Cancer clones. Selective pressures allow some mutant subclones to expand while others become extinct or remain dormant. Vertical lines represent restraints or selective pressures. This is a representative pattern for common, solid cancers; as recognized by Nowell, leukemic clones may expand over a shorter timeframe (years versus decades) and be subject to fewer restraints and mutational events. Ecosystems 1–4 (boxes) represent the different tissue ecosystems or habitats. Smaller boxes within Ecosystem 1 represent localized habitats or niches. Each differently colored circle represents a genetically distinct subclone. Metastatic subclones can branch off into different time points in the sequence from either minor or major clones in the primary tumor. Tx, therapy; CIS, carcinoma in situ. (**b**) Darwin's branching evolutionary tree of speciation from his 1837 notebook. (Greaves and Maley 2012)

arose owing to the base rate of genomic instability. p53 is a DNA-binding protein that can repress or upregulate numerous genes, so it can respond to stresses that can cause DNA damage, arrest cellular growth, or bring about death (Assaily 2005). Thus, p53 is pervasive and crucial in its influence; its function is sufficiently clear, leaving us the freedom to perhaps assign significant other functions when analyses and plausible results are less controversial.

The primary role of p53 is to prevent the formation of tumors by employing its gene-regulating properties. Levine et al. (2006) suggest that the p53 pathway is composed of hundreds of genes and their products that respond to numerous stress signals, including apoptosis, cellular senescence, or cell cycle arrest. p53-regulated genes serve several functions: (1) produce proteins that communicate stress signals to adjacent cells, (2) prevent and repair damaged DNA, (3) create feedback loops capable of modifying p53 activity, and (4) communicate with other signal transduction pathways. Substantial work remains to be done to understand this network of genes and the role they play in protection from cancers and therapy. There is a need to integrate the homeostatic mechanisms of stress management and fidelity in a cell and in all organisms (Levine et al. 2006).

p53 Has Impressive Origins

Let us move now to the more analytic view of Nedelcu and Tan (2007), who focus on the evolution of the p53 tumor suppressor gene family. Clearly, similarities in

sequence, gene structure, and expression potential are crucial when analyzing the early diversification and complex evolutionary history of the p53 tumor suppressor gene family. The three p53 members differ in domain organization and functional roles (p63 and p73 are also crucial in development). In addition to vertebrates, p53-like sequences occur as single genes, of either the p53 or the p63/p73 type. The p53 tumor suppressor plays a unique role during malignant transformation and in maintaining the genome's integrity and stability. As mentioned earlier, diversification may not be considered to be restricted to vertebrates since both p53 and p63/p73 sequences occur even in a unicellular group, the choanoflagellates: *Monosiga brevicollis* is a choanoflagellate that has long fascinated evolutionary biologists (King et al. 2008).

Choanoflagellates share a marked similarity to the feeding cells (choanocytes) of sponges, so they could conceivably represent the closest living relatives of metazoans (James-Clark 1869; Saville-Kent 1881). Because of their position on the tree of life, analyses of choanoflagellates provide an unparalleled window into the nature of the unicellular and colonial progenitors of metazoans (King 2004). Multiple independent duplication events involving p53-type sequences occur in other simpler animal lineages (cnidarians, flatworms, and advanced insects). Selective factors in other invertebrates play a role in the diversification of this family. Understanding this family's evolution begins by learning about the selective pressures associated with multiple independent duplication events. Surely these must have occurred in the p53 family, and the roles of p53-like proteins in vertebrates are essential to and will help understand the family's evolution. Since both a p53 and a p63/73 copy occur in the unicellular *M. brevicollis*, it is a model system that will clarify the functions of p53 members and their mechanisms, especially those associated with functional diversification with wide evolutionary implications.

p53 Mutation: Homologs p63 and p73

p53 is frequently mutated in cancer, so it is one of the most intensely analyzed tumor suppressors. Slee et al. (2004) investigated the primitive forms of p53 found in *Caenorhabditis elegans* and *Drosophila*; other research using transgenic mouse models indicates that induction of apoptosis is both the most conserved function of p53 and vital for tumor suppression (Slee et al. 2004). Substantial discoveries concerning p53 homologs p63 and p73 have contributed new insights and revealed puzzles and some answers that challenge our understanding of the iconic p53 tumor suppressor. Yang et al. (2002) proposed that, despite their seemingly separate functions, there are clues suggesting that p53 family members collaborate but that they might also interfere with each other, thus increasing the obscurity of their functions. From such murkiness, a challenging question arise: Did these genes evolve to function independently, or did their familial ties bind them in pathways of cell proliferation, death, and tumorigenesis (Yang et al. 2002)?

Understanding p53

Unicellular Animals

The p53 superfamily's origins predate animal evolution and first appear in unicellular flagellates. Invertebrate p53 superfamily members appear to have a p63-like domain structure, which suggests an ancient origin. The radiation into p53, p63, and p73 proteins is a vertebrate invention. There is also emerging evidence revealing that invertebrate p53 superfamily proteins possess functions unrelated to apoptosis, such as DNA repair, cell cycle checkpoint responses, compensatory proliferation, aging autophagy, and innate immunity (Rutkowski et al. 2010). Taken together, these results contribute significantly to our understanding of the evolution of cancer as controlled by suppressor genes. According to Suh et al. (2006), debate persists as to whether invertebrate p53 superfamily proteins are phylogenetically more related to vertebrate p53 or p63. Agreeing with previous reports, phylogenetic analysis supports two main conclusions: a p63-like domain structure is evolutionarily more ancient, and apparently a protein with a p63-like domain structure originally evolved and was involved in mediating the apoptosis of damaged cells. In vertebrates, this earlier role of p53-like proteins is performed by p53, and p63 retains the ancient role of apoptosis in the female germline (Suh et al. 2006; Bosch 2014).

Multicellular Organisms: *Hydra* and Sea Anemone

Naturally occurring tumors in the basal metazoan *Hydra* is a recent major finding. The molecular nature of tumors is well analyzed in vertebrates, although their evolutionary origin remains unknown. Bosch's team asserts that there is no evidence for naturally occurring tumors in prebilaterian animals, for example, sponges and cnidarians. This is surprising and unclear since recent computational studies predicted that most metazoans may develop tumors. Cellular and molecular data reveal that tumors may be transplanted and originate by differentiation arrest of female gametes. Growth of tumor cells is independent of the cellular environment, and tumor-bearing polyps possess significantly reduced fitness. *Hydra* tumors significantly altered the transcriptome that mimics expression shifts in vertebrate cancers. Spontaneous tumors also possess extensive evolutionary roots, and continued analysis may be informative and reveal the fundamental basis of tumorigenesis (Domazet-Lošo et al. 2014). There seems to be no influence attributed to p53.

According to Belyi et al. (2010), a common ancestor of the three p53 family members of human genes p53, p63, and p73 was detected first by examining the evolution of modern-day sea anemones. These genes seem to protect the germline from genomic instabilities in response to stresses. This p63/p73 common ancestor gene is found in almost all invertebrates and first duplicates to produce a p53 gene

and a p63/p73 ancestor in cartilaginous fish. Bony fish contain all three genes, p53, p63, and p73, and the functions of these three transcription factors diversify in higher vertebrates. This gene family has preserved its structural features and functional activities for more than one billion years of evolution (Belyi et al. 2010).

Multicellular Organisms: Clams and Mussels

The human p53 tumor suppressor protein is inactivated, which permits the expression of many cancers; it is also a major component in apoptotic responses to cellular stress. Marine bivalves provide some of the most relevant and best understood models currently available for experimental analyses (Walker et al. 2011). Homologs for human p53 (Hsp53) and p73 (Hsp73) genes have been cloned and analyzed in soft-shell tissues from normal and leukemic soft-shell clams (*Mya arenaria*). Map 53 and Map 73 proteins serve functions similar to those of humans: First, they act as alternate splice variants of a p63/p73-like ancestral gene. Although not completely clear, in leukemic clam blood cells both proteins are not found in the nucleus. Here, this location suggests that a nonmutational p53/p73-dependent mechanism may be involved in clam diseases (Kelley et al. 2001). To support this view, we accept that the tumor suppressor p53 regulates genes involved in several well-known functions: progression through the cell cycle, DNA repair, and senescence, or apoptosis as a result of stress. Dysregulation of p53 can result in uncontrolled cellular proliferation. We may safely assume that mollusks are positioned at a critical juncture in the evolution of this gene family (Holbrook et al. 2009). Why there has been relatively no progress in annelids (e.g., earthworms) is a mystery since mollusks and annelids, as lophotrochozoans, share certain traits (Salzet et al. 2006).

Mussels are often employed as bioindicators. As such they are susceptible to a diverse group of environmental toxicants (as are earthworms). Despite this advantage, the *M. edulis* p53 (not *Mya arenaria*) sequence shows little similarity to other invertebrate p53-like sequences. However, if subjected to further analyses these cancer gene sequences will undoubtedly allow for the development of specific biomarkers of genotoxic damage (Ciocan and Rotchell 2005). Cancer has often been observed only in vertebrates, especially its relation with adaptive immunity. Invertebrates that possess innate immunity also develop tumors in response to environmental carcinogens. Analyzing cancer development in species possessing innate immunity is promising and may even reveal invertebrate adaptive functions (Kvell et al. 2007). Mussels are vulnerable to environmental toxicants and carcinogens. DNA sequence alignment of the *Mytilus edulis* homolog of vertebrate *ras* and p53 reveals significant evolutionary conservatism (Ciocan and Rotchell 2005). Transmissible sarcoma exists as caused by environmental carcinogens (i.e., chlordane) in the soft-shell clam *Mya arenaria* (Farley et al. 1991; Dungan et al. 2002).

Early Experiments on Transmission

McLaughlin et al. (1992) performed transmission experiments using adult soft-shell clams (*Mya arenaria*). They found that clam sarcomas are transmissible by hemolymph from neoplastic clams but not by cell-free ultrafiltrates. However, a lack of sarcomas in clams injected with the ultrafiltrate argues against viral etiology for the disease. There seemed to not have been a follow-up of these convincing experiments (McLaughlin et al. 1992).

Barber contributed much to this sporadic but promising discipline (Barber 2004). Still the area of molluscan neoplasms requires more active investigations that are well focused. We must exert careful scrutiny as we seek to define in comprehensive terms the nature of molluscan tumors. There is a need to clearly distinguish between (1) neoplasia, (2) hyperplasia, (3) response to injury, and (4) response to infection (Pauley 1969). This longstanding dilemma has been partially solved. Since Sindermann (1990) there have been broad definitions of tumors as "any swelling or abnormal tissue mass that has been derived from: (1) *hyperplasia* or non-neoplastic, controlled cell proliferation; (2) *hypertrophy* or non-neoplasic increase in cell size; (3) *neoplasia* uncontrolled cell proliferation" (Barber 2004). Barber (2004) distinguished between benign (nonfatal) and malignant (fatal) tumors and pointed out that certain examples of hemocytic sarcomas and germinomas possessed the criterion of malignancy and that they do occur in marine bivalves (Sparks 1985).

Moore et al. (1991) analyzed an alternate pathogenesis of systemic neoplasia in the bivalve mollusk *Mytilus*. The proliferative disease systemic neoplasia, also called hemic neoplasia or disseminated sarcoma, has been analyzed for Puget Sound, Washington, populations of bay mussels (*Mytilus* spp.). DAPI-stained hemolymph cells were measured using flow cytometry generating DNA content frequency histograms. Those cells that manifested systemic neoplasia existed as either of two separate types, characterized by G_0G_1 phase nuclear DNA contents of either 4.9 × haploid (pentaploid form) or 3.8 × haploid (tetraploid form). The two disease forms coexisted in all four populations, as 66% pentaploid, 29% tetraploid, and 5% showing both disease forms simultaneously. Results are convincing concerning multiple cell types found in molluscan neoplasia (Moore et al. 1991).

Drosophila Mutations and Malignant Growth

The work of Tipping and Perrimon (2014) strongly suggests that *Drosophila* possesses truly representative cancer traits. Characteristics include (1) evasion of apoptosis, (2) constant proliferation, (3) metastasis, (4) prolonged survival, (5) genome instability, and (6) metabolic reprogramming. For investigators this enables analysis of context-dependent tumorigenesis (Tipping and Perrimon 2014). *Drosophila* offers a unique model for identifying and characterizing tumor suppressor genes and mammalian homologs. Genome-wide microarray analysis of *Drosophila* brain

tumor caused by dysfunction of the *Brat* tumor suppressor gene reveals over 300 associated genes, and 60 sequences show homology to mammalian genes involved in tumor development (Loop et al. 2004; Bryant et al. 1993; Gateff et al. 1996; Watson et al. 1994).

Moreover, inactivation of any of the germline genes—*nanos, vasa*, piwi, or *aubergine*—suppressed l(3)mbt malignant growth. Thus germline traits are necessary for tumor growth in this *Drosophila* model. Inactivation of germline genes may exert tumor-suppressing effects in other species (Janic et al. 2010). This is a relevant relationship since the innate immune system of *Drosophila*, like other invertebrates, is germline, not somatic, capable of gene rearrangement (Cooper et al. 2002). The immune system of *Drosophila* is not deficient when cancer appearance or lack of it is the question.

Endocytosis: Role in Inflammation and Tumor Growth

As a component of the inflammatory process (a stage of germline innate immunity) endocytosis plays an important role in regulating tumor growth and metastasis. In *Drosophila,* several endocytic neoplastic tumor-suppressor genes have been identified; when mutated they cause epithelial disruption and overproliferation. The *Drosophila* model also suggests that inactivation of germline genes may exert tumor-suppressing effects in other species (Thomas and Strutt 2014). Experiments in mice and in humans reveal a role for the immune system in preventing tumor growth. Pastor-Pareja et al. (2008) found that during an innate immune response against tumors in *Drosophila melanogaster*, circulating blood cells or hemocytes adhere to tumors after they have detected disruption of the basement membrane; this counters their growth. Tissue damage also activates JNK (c-Jun N-terminal kinases) signaling in both tumors and aseptic wounds, causing expression of JAK/STAT (Janus kinase/signal transducer and activator of transcription) activating cytokines. This recalls one of the hallmarks of the danger hypothesis, that is, danger that is not nonself can induce an immune response (Pradeu and Cooper 2012).

According to Ohsawa et al. (2011), a newly emerged oncogenic cell in the epithelial population must confront antitumor selective pressures formed in host tissue. Yet the mechanisms by which surrounding normal tissue exerts antitumor effects against transformed oncogenic cells are poorly understood, with at least one exception. First, surrounding normal cells activate nonapoptotic JNK signaling to developing oncogenic mutant cells. Second, JNK activation precedes upregulation of PVR, the PDGF/VEGF receptor of *Drosophila*. Genetic and time lapse reveal that PVR expression in surrounding cells activates the ELMO/Mbc-mediated phagocytic pathway; this eliminates oncogenic neighboring cells by engulfment. Third, JNK-mediated cell engulfment may be a conserved byproduct of evolution. It may also be an intrinsic tumor-suppression mechanism programmed to eliminate premalignant cells from epithelia (Fig. 2) (Ohsawa et al. 2011).

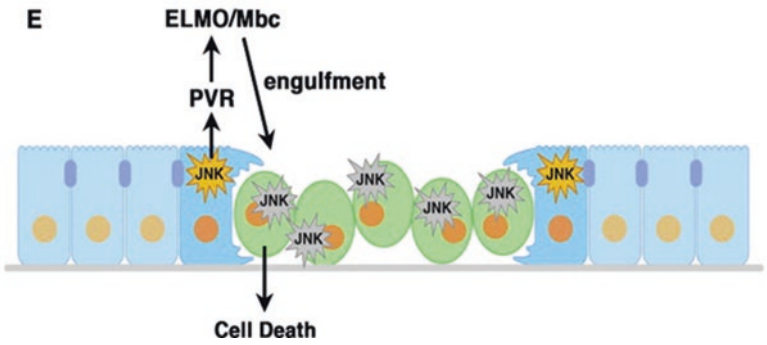

Fig. 2 A model for intrinsic tumor suppression caused by dual functions of JNK signaling in *Drosophila* imaginal epithelia. JNK promotes cell death in neoplastic tumor-suppressor mutant clones, whereas in surrounding cells JNK promotes the elimination of neighboring mutant cells through the PVR-ELMO/Mbc engulfment pathway. Whether these two JNK-activation processes are linked in a single pathway or act in parallel pathways, to our knowledge, is currently unknown. (Ohsawa et al. 2011)

Modes of Reproduction: Earthworms and Clonality

Parthenogenesis Ensures Genetic Diversity

Most earthworms are hermaphrodites that usually reproduce via cross-fertilization; certain species reproduce by parthenogenesis. Parthenogenetic reproduction creates an unexpected source of genetic diversity; the resulting offspring are hardier in changing environments (Díaz Cosín et al. 2011). According to Terhivuo and Saura (2006), over a dozen species reproduce parthenogenetically. All are polyploid and only one earthworm is necessary for a new population. By analyzing clonal trends and patterns of several species around the globe, local factors have been found everywhere to be responsible for altering genotype (Terhivuo and Saura 2006). Terhivuo and Saura (2003) also suggest that geographic distance leaves the parthenogenetic *Octolasion cyanem* relatively clonally uniform; for most of Europe to Australia, this generalization holds true. In Switzerland, however, an unusually heterogeneous population is present. More variable gene pools inhabit southern versus northern locales, a trend that may be due to the founder effect (Terhivuo and Saura 2003).

A Source of Stem Cells During Agametic Reproduction

Enchytraeus japonensis is unique. It is a small oligochaete annelid that reproduces primarily asexually by fragmentation (autotomy) and by regeneration; sexual reproduction can also be induced (Sugio et al. 2012). *E. japonensis* is capable of agametic reproduction: gonads arise from any segment of the body. Agametic offspring possess widespread specialized cells that express the piwi-homolog *Ej-piwi*. These

cells differ from neoblasts that give rise to somatic cells; germ and somatic cell lines are segregated during agametic development; they behave differently during regeneration (Tadokoro et al. 2006). The origin and development of germline stem cells and neoblasts are independent (Yoshida-Noro and Tochinai 2010). *Enchytraeus buchholzi* reproduces only sexually, and it is likely that its neoblasts are even more critical as stem cell sources during agametic reproduction than the need for regeneration after injury (Myohara 2012).

Clonal Responses to Pathogens

Pathogens induce immune responses in specific cells. This is measured by a second challenge that may rapidly yield specific clones. To initiate immune responses, pattern recognition receptors (PRRs) (immune system components) evolved before features of adaptive immunity. PRRs are proteins derived from cells of the innate immune system that have been programmed to identify pathogen-associated molecular patterns (PAMPs). These are associated with microbial pathogens, cellular stress, and damage-associated molecular patterns (DAMPs), components released during cell damage. Cancer may not appear in some species, and its absence requires analysis. The appearance of cancer is likely due to its escape from suppression by an immune system, not by an immunologic deficit relative to evolutionary position, short lifespan, or combinations of these factors. Toll-like receptors (TLRs) are ubiquitous and play an important role in immune responses to pathogens in invertebrates. TLRs were first isolated from an oligochaete annelid, *Eisenia andrei* (EaTLR), revealing its expression pattern. Phylogenetic analysis has revealed the highest similarity of EaTLR with a polychaete annelid, *Capitella teleta*, and TLRs of mollusks and echinoderms. The highest constitutive expression of EaTLR occurs in the digestive tract. Supporting its role in signaling immunity, gene expression is significantly increased in coelomocytes of *E. andrei* following a challenge with Gram-positive bacteria (Škanta et al. 2013). For further confirmation an analysis of coelomocyte cell surface revealed Toll-like immune receptors (Francis et al. 2007; Davidson et al. 2008).

Earthworms: Nonpathogenic Antigens Induce Short-Lived Clones

Earthworms are oligochaetes (about 3000 known species), with a true coelom containing fluid and numerous coelomocytes, free leukocytes that react to certain pathogenic organisms and destroy nonpathogenic, allogeneic tissue grafts by immune rejection. Coelomocytes possess leukocyte surface markers that define function (Hostetter and Cooper 1974). Autografts (self) and xenografts (nonself) heal promptly within 24 h, though xenografts are always eventually destroyed. Allografts (between different individuals of the same species) are slowly rejected; autografts reveal self–nonself recognition by complete and permanent acceptance

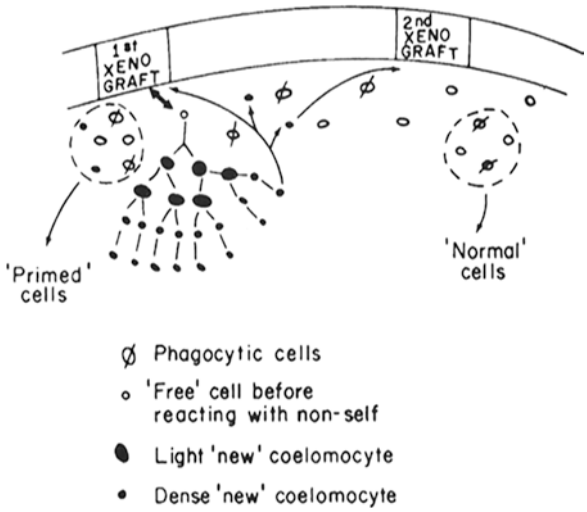

Fig. 3 Incorporation studies in earthworms indicate that ^{3}HTdR-labeled cells disappeared from the coelomic cavity in an exponential manner, with a half-life of 3.5 days. Ten days after grafting, a considerable concentration of labeled cells was still recovered from the coelomic cavity. Not only did these cells decrease slowly in number, but they were found throughout the coelomic cavity away from the xenografted region. These properties can be extremely useful for those cells able to recognize nonself (proliferation occurs only by xenogeneic stimulation, not by injury or autografting). With this capability and their migration throughout the earthworm's coelom, an effective surveillance system could be set in motion. (Lemmi and Cooper 1981)

(Cooper 1968). When second xenografts are transplanted 5 days after a first, rejection times of second grafts are accelerated (evidence of survival and primed immune clones).

Earthworm cellular immune responses react specifically against nonself antigens and retain some memory of past exposures (Cooper 1969; Hostetter and Cooper 1973). Clones could be derived locally or recruited from sites within the extensive coelomic cavity. Responses may also be diverted against danger signals emanating from the inflamed sites (Fig. 3) (Pradeu and Cooper 2012; Lemmi and Cooper 1981). Specificity is demonstrable and temporal. *Lumbricus terrestris* and *Eisenia foetida* have also been used experimentally to demonstrate that induction of immunocompetence can be artificially transferred between individuals, that is, host *Lumbricus* injected with coelomic fluid containing coelomocytes from other *Lumbricus* immunized earlier to *Eisenia* grafts. Recipients of sensitized (immune, primed) coelomocytes rejected test grafts more rapidly than controls, at times significantly shorter than earthworms injected with coelomocytes from unsensitized worms or only saline (Bailey et al. 1971). Since, therefore, memory is short-lived, second-set *Lumbricus* allografts from the same earthworm donor to the same recipient showed accelerated rejection only if the second set was transplanted less than 10 days after the first set of allografts (Figs. 4, 5, and 6) (Cooper and Roch 1986; Lemmi and Cooper 1981; Engelmann et al. 2011).

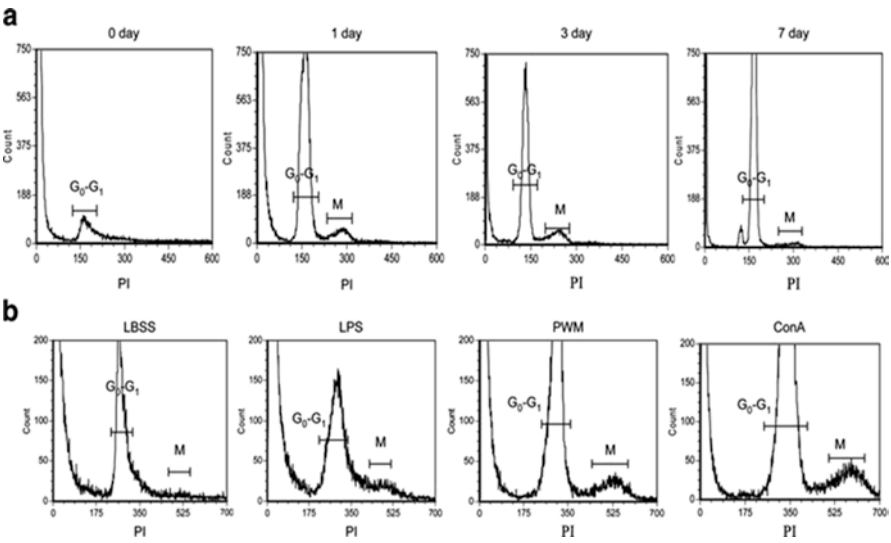

Fig. 4 A recent report reveals that isolated coelomocytes proliferate after cell depletion as analyzed by flow cytometry (Homa et al. 2008). In addition, several lines of evidence indicate mitotic activity of coelomocytes under stress conditions (Bilej et al. 1992) that could be triggered by lectins and transplantation antigens; however, cellular activation has not been analyzed at the molecular level. In our recent experiments, we observed an in vivo proliferative ability of free coelomocytes from *E. fetida* by flow cytometry. Freshly isolated coelomocytes were stained with propidium iodide for cell cycle analysis. We did repeated isolations from the same earthworms and allowed various resting times. Results revealed an increase in proliferating cell numbers that indicate mitotic division of coelomocytes. We also measured coelomocyte proliferation after using various stimuli. Our results clearly show the effects of different mitogens (lipopolysaccharide, pokeweed mitogen, concanavalin A). Lectin stimulations caused increased activation and proliferation. (Engelmann et al. 2011)

Primed Effector Clones Against Nonpathogens

Recruitment of descendant coelomocytes must occur to support observations of specificity. Coelomocyte proliferation is a component of earthworm xenograft rejection mechanisms. After ^{3}HTdR is introduced into coelomocytes, increased incorporation occurs after test xenografting. Coelomocytes propagate in response to foreign antigen exposure, evidenced by proliferation (Cooper and Roch 1984). By confronting host leukocytes with nonpathogens, autogeneic but not allogeneic coelomocytes kill the nonpathogenic mammalian tumors. Earthworm coelomocyte effectors react against a test tumor target, K562. The viability of effectors reveals the incorporation of [^{3}H]-thymidine as higher in autogeneic (A<==>A, self) than in allogeneic (A<==>B, nonself) coelomocytes. A<==>A showed significantly greater numbers in S, G2, and M phases. When A<==>A or A<==>B were cocultured, no significant nonpathogenic cell killing occurred in either, as measured in a 4-h 51Cr release assay. A<==>A but not A<==>B killed K562 target cells. Cytotoxicity is dependent upon membrane binding between small, electron-dense coelomocytes and targets.

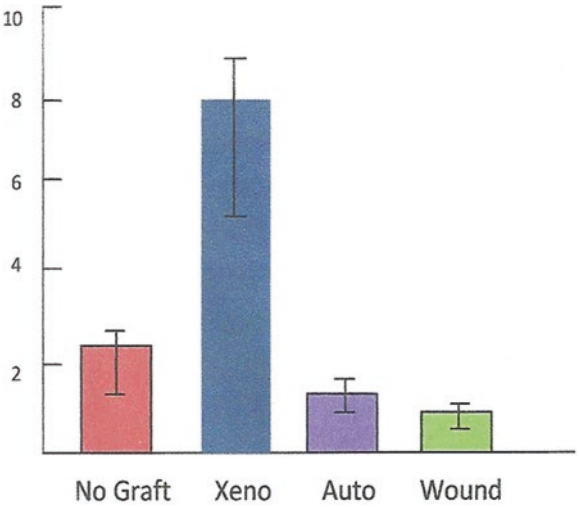

Fig. 5 Histogram representing incorporation of tritiated thymidine (^{3}HTdR) into *Lumbricus terrestris* coelomocytes 48 h after various operative procedures. Values are counts per minute (cpm) in thousands, per million cells ± SE. (Lemmi and Cooper 1981)

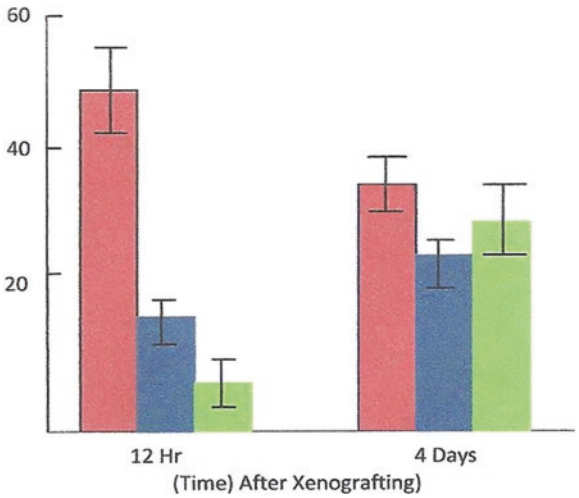

Fig. 6 Histogram representing percentage distribution of "free," nonphagocytic, labeled coelomocytes 12 and 96 h (4 days) after xenografting. ^{3}HTdR was injected into coelomic cavity at the time of grafting. Red bars: percentage of low-density (<1.031) coelomocytes; blue bars: cells of medium density (1.062); green bars: percentage distribution of high-density coelomocytes (<1.093); all values are percentage ± SE. (Lemmi and Cooper 1981)

Heat-labile supernatants from A<==>A but not from A<==>B killed K562 (non-pathogenic cancer) targets. Recognition of, binding to, and killing of foreign cells in a natural killer cell-like reaction reflects natural immunity (Cooper et al. 1995). The difference in responses between autogeneic and allogeneic effector cells reflects the capacity for interclonal *immunologic rivalry* between incompatible effectors.

Effector Cell Markers

Induction of cytotoxicity at significantly high levels against other nonpathogenic natural killer (NK)-sensitive, human tumor cells, K562, and NK-resistant targets (U937, BSM, CEM) (clearly nonpathogenic) clarifies effectors more clearly. By

cytofluorimetric analyses using mouse antihuman monoclonal antibodies and by morphological evaluations, two coelomocyte types have been identified: (1) small (8–11 μ) electron-dense cells (SC): CD11a+, CD45RA+, CD45RO+, CDw49b+, CD54+, β2m+, and Thy-1+; (2) large (12–15 μ) electron-lucent cells (LC) that are negative for these markers. Both cell types are negative for other CD and MHC class I and class II markers. SCs are active during recognition, rapidly binding to targets; LCs are phagocytic. Results confirm for the first time: killers and scavengers, which supports an important evolutionary development; primitive NK-like distinct from universal phagocytosis activity appeared early in evolution (Engelmann et al. 2005; Homa et al. 2013). Monoclonal antibodies identify distinct earthworm leukocyte markers (Engelmann et al. 2005).

Nonpathogenic Antigens: Clonality and Immunologic Theory

Related to immunologic theory, nonpathogenic transplant antigens stimulated the production of specific leukocyte clones that are positive responders and nonresponders. This induction, if accelerated, assumes that those showing no acceleration are evidence of negative memory, clonal energy, or clonal deletion. By varying intervals to test for a second response, longer intervals allowed the death of original specific clones or their descendants. The source of *en masse* coelomocyte appearance in response to second grafts as manifested by the rapid appearance of new specific clones is not clear. Three plausible views await clarification: (1) proximal proliferation, (2) distal proliferation, (3) proximal and distal proliferation of recruits.

Short lifespans (not the long-lived earthworms) and past questionable innate immune responses currently play no role that justifies an absence of cancer in short-lived invertebrates. As a normal life process or event there is currently no satisfying explanation for the lack of cancer in earthworms. We are left with having to discover the optimal formula for releasing or inactivating suppressors that maintain these ubiquitous terrestrial animals cancer free. Although their leukocytes lyse foreign cancer cells in vitro, this is a broad-based nonspecific response to what effectors recognize and react to as a pathogen. Finally, earthworms are no different from humans in harboring "peaceful" relations with pathogens. In fact, certain bacteria are essential, and the symbiotic relationship works!

Perhaps we can now say with relief that immunology has lost its innocence! Self/nonself no longer dominates the *raison d'être* for intellectual approaches to clarifying immune functions (Cooper 2010a, b). Since a few rebellious basic scientists interested in inclusiveness have joined Cooper (2012), additional animal models from the sea and the earth have aided this quest (Burnet 1976). Still, we are well on the path to certain amalgamation in biomedicine with immunology as one of the spotlights (Cooper and Overstreet 2014). Surely this approach, linking immunity to other questions, will draw us even closer to understanding the long history of cancer—a current plague of humanity (Mukherjee 2010, Cooper 2010c).

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With kind permission from Springer Science+Business Media: Earthworm Innate Immune System, chapter 14/article title, 24, 2011, 229–45, Engelmann P, Cooper EL, Opper B, Nemeth P Fig. 14.1. *Soil Biol*.

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Correction to: Echinodermata: The Complex Immune System in Echinoderms

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This chapter was inadvertently published with an incorrect spelling of the author's name as V. Arriza whereas it should be V. Arizza.

In addition to this the affiliation of one of the chapter authors Elisse Sutton was published incorrectly and it has now been corrected to read as Department of Biological Sciences, Macquarie University, Sydney, NSW, Australia.

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Earthworm

They face in opposite directions to mate.

What a miner, pistoning in slow
motion through the underworld of the earth,
engineering vents, channels, water flow,

converting death and dearth,
day in, night out. Each eyeless body
digesting the soil, nursing birth.

Cut in two, they double, breathe via marly
skin, a must for farm and garden: alfalfa,
spuds, spinach, carrots, cabbage, barley,

wasabi, wheat, gourds, rutabaga, papaya,
endive. You name it. Build them a shrine.
May these lowly laborers of Gaia

multiply, flourish, never decline,
stick with worm love, position 69.

A poem
By Greg Delanty

(originally published in The Atlantic, September 2017)

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