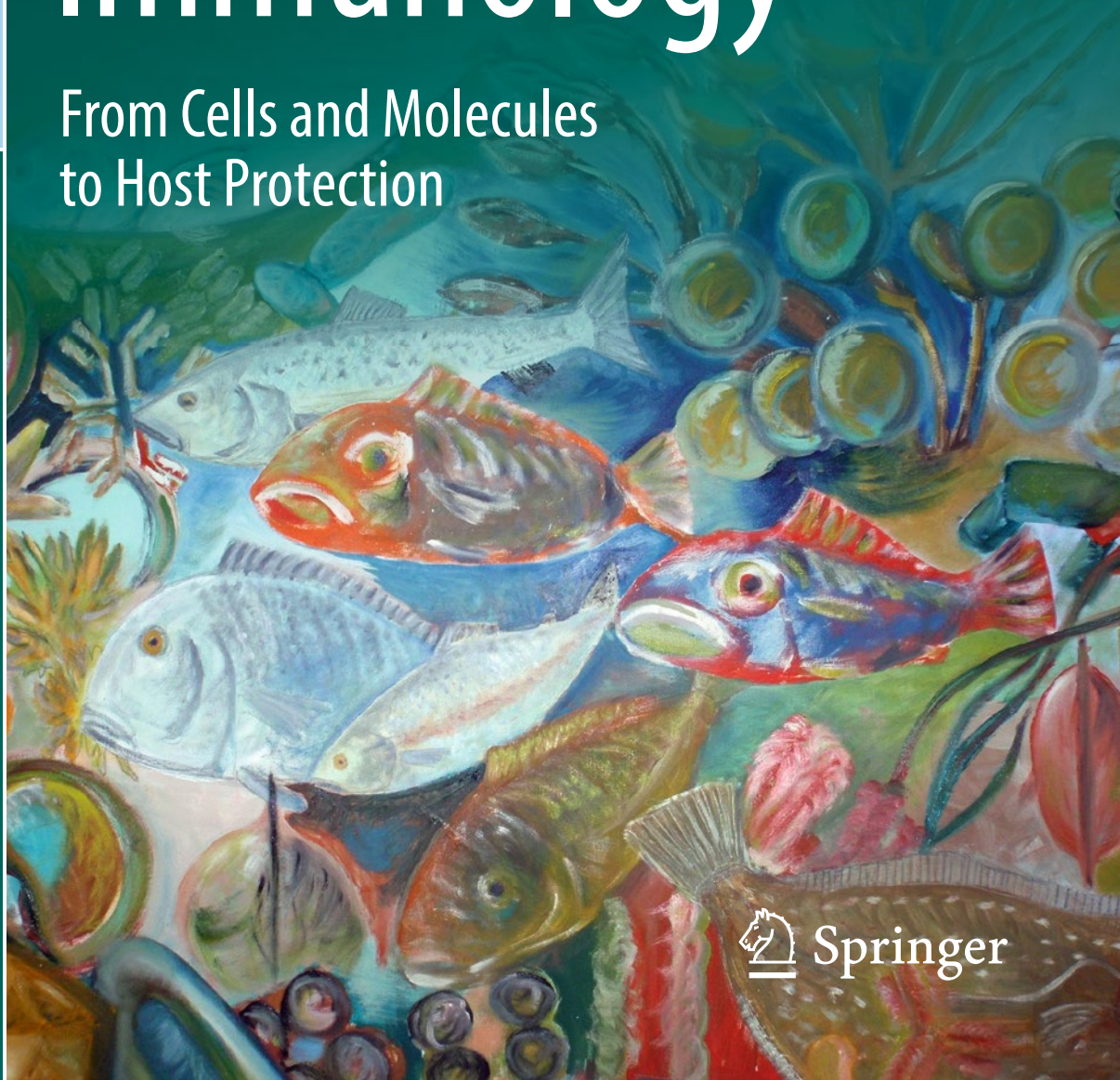Kurt Buchmann
Christopher J. Secombes
Editors

Principles of Fish Immunology

From Cells and Molecules to Host Protection

Springer

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From Cells and Molecules to Host Protection

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Preface

All organisms face exposure to potential pathogens seeking to invade and exploit resources of the host. During the evolution of life, since the first unicellular forms appeared on Earth, parasitism has proved to be a successful strategy and life form. This makes the number of parasitic organisms (viruses, bacteria, protozoans, metazoans) far higher than the number of free-living hosts. Even parasites may carry their own hyper-parasites (viruses, bacteria and/or protozoans and metazoans). In order to resist the colonization and invasion by foreign potential pathogens, a wide array of protective mechanisms have appeared in both aquatic and terrestrials organisms. The accepted term for keeping the host free from foreign invaders is "immunity" originating from the Latin word "immunis", meaning "released from a burden". With the advent of the early teleost fishes more than 450 million years ago, which possessed a relatively advanced immune system in common with all jawed vertebrates, many variations of this system are what we find today in extant teleosts and are the focus of this book. The study of fish immune systems and their evolutionary relationship to the armament in invertebrates and other vertebrates may add to our general understanding of evolutionary processes, especially with the incredible diversity seen in modern teleosts (~29,000 species of the 33,000 fish species listed in Fishbase) that represent half of all living vertebrates. However, fish immunology is not only of academic interest. Diseases in fish caused by viruses, bacteria, protozoans and metazoan parasites are continuously challenging not only commercial capture fisheries but also, and in particular, aquacultured fish. During the last 60 years, world aquaculture production has increased from low levels to more than 80 million metric tonnes annually. In addition, from a previous focus on a few fish species more than 400 species are now cultured. This expansion has enabled aquaculture to provide more than 50% of the fish products generated for human consumption. The nutritional and health promoting value of fish products will likely drive an even higher expansion of farmed fish production in the future. In order to secure this production, the health and welfare of the production animals is a prerequisite. Indeed, the economical and ecological sustainability of aquaculture enterprises may be questioned if production is dependent upon treatment with chemotherapeutants (antibacterial and antiparasitic chemicals and drugs). Increased knowledge on fish

immunology can make a significant contribution to the sustainable development of this important supply of animal protein in the world. The information can be translated into immunoprophylactic measures to improve fish health, from vaccination of fish against specific pathogens and methods to elevate generally the innate immune status, to management of the fish host and, last but not least, genetic improvement of the fish populations used in aquaculture. With this in mind, during the last 20 years research efforts within the field have increased and accelerated markedly. The advent of new technology has made it possible to explore the fish immune system to depths not hitherto seen, and the publication activity in a wide range of important journals has been intense and extensive. As a consequence, older textbooks on fish immunology, which have served the community well, no longer suffice as a means to allow new generations of scientists to stay updated. This book *Principles of Fish Immunology: From Cells and Molecules to Protection* provides a description of the recent findings and their importance for key topics within fish immunology. The editors are grateful for the contributions from leading experts within the field who have kindly written these updated chapters on their specialist research areas.

This textbook covers the anatomy of immune organs in fish, innate immunity, adaptive immunity, cellular immune responses, lymphocytes in fish, macrophages in fish, immunoglobulins, teleost fish B cells and their antibody repertoires, complement in fish, cytokines, major histocompatibility complex (MHC) in fish, mucosal immunity in fish, antiviral immune responses, antibacterial immune responses, antiparasitic immune responses, immunopathology, vaccination of fish, development of immune-competence in fish and finally the relation between stress and immunity in fish.

It is the hope of the editors that this work will be of use for older and new students of fish immunology, researchers, practitioners and health science workers. It is our aim that the text will stimulate new ideas and theories within both basic and applied fish sciences.

Frederiksberg C, Denmark   Kurt Buchmann
Aberdeen, UK   Christopher J. Secombes
March 2021

Contents

Anatomy of Teleost Fish Immune Structures and Organs

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Håvard Bjørgen and Erling Olaf Koppang

Abstract

The understanding of the functions of the immune system needs a fundament of solid anatomical knowledge. The construction and arrangement of the different components of the immune system determine where and how different immune responses may occur. Even though substantial efforts through the years have been invested in anatomical research of the teleost immune system, surprising discoveries are still made. Structures observable to the naked eye have recently been discovered and described as important immune organs. The application of improved morphological techniques, in particular in situ hybridization, has enabled research combing molecular biology and medicine with morphological investigations. The variety within fish species concerning the anatomical construction of the immune system is substantial. In this chapter, our emphasis is on salmonids where recent discoveries of teleost immune structures have been made. We clarify anatomical definitions, which are premises for the further parts of the chapter before we present our understanding of the anatomical construction of the immune system of teleost fish.

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Abbreviations

AID	activation-induced cytidine deaminase
AIRE	AutoImmune REgulator
CD	cluster of differentiation
cTECs	cortical thymic epithelial cells
GIALT	gill-associated lymphoid tissue
Ig	immunoglobulin
ILT	interbranchial lymphoid tissue
ISA	infectious salmonid anaemia
MALT	mucosa-associated lymphoid tissues
Mhc	major histocompatibility complex
mTECs	medullary thymic epithelial cells
NALT	nose-associated lymphoid tissue
RAG	recombination-activating genes
SHK	salmon head kidney
STC	stanniocalcin
TCR	T-cell receptor

1.1 Introduction

1.1.1 Anatomy in General

"Anatomy" is a Greek word, which literally means "cutting apart". In daily speaking, we use the term to encompass the knowledge concerning the form, the disposition and the structure of the tissues and organs that compose the body. This discipline may be divided into gross anatomy, which comprises structures observed with the naked eye in contrast to microscopic anatomy, which comprises structures only observable by microscopy. Further, a discipline known as developmental anatomy comprises the organism's changes from conception of birth, youth, maturity and onwards to old age. Embryology is thus a part of developmental anatomy (Dyce et al. 2010). Anatomy is one of the oldest disciplines in medicine (*anatomia fundamentum medicinae est*). If the understanding of anatomy fails, it is impossible to offer opinions regarding immunological responses. Thus, in this chapter, we first provide important anatomical definitions, which are premises for the rest of the chapter, before we present the current understanding of the anatomical construction of the immune system in teleost fish.

1.1.2   Definitions: Organs, Tissues and Structures

To define the term "anatomy", we have already used two other terms that need explaining. These are "tissues" and "organs". It seems easier to start with the term "organ". Most of the readers will have an impression of what an organ is, but they may find it more difficult to make a definition, and rightly so. Unfortunately, one will find several different definitions of the term "organ" by different authors. One definition is "an organ is a somewhat independent body part that performs a specific function or functions" (Studdert et al. 2012). Pabst definition of an organ is "a structured assembly of tissues forming a functional unit" (Pabst 2007). Importantly, both definitions imply that an organ may not only consist of a single tissue. As we first are into definitions regarding morphology, we take the opportunity to comment on the wrong use of the term "pathology", which we frequently observe in different works and reports. "Pathology" means "the science of the causes and effects of diseases". Stating that "there was no pathology in a tissue" or "no pathology was observed" is a violation of the medical terminology. Instead, "there were no pathological changes in the tissue" exemplifies correct use of this word.

A tissue is "a group or layer of similarly specialized cells that together perform certain special functions" (Studdert et al. 2012). A prime example of an organ consisting of different tissues is the eye. The eye is defined by its outer layer, the *tunica fibrosa*, which detaches the organ from surrounding structures. The organ itself consists of different tissues, for instance nervous tissue in the retina and modified epithelial tissue in the lens. The organ has a well-defined purpose, which is to record and transmit light signals. Not many organs are as specialized as the eye because they usually serve several different functions. Due to its high degree of specialization, the eye can be classified as a sensory organ.

When organs have multiple functions, it may not be straightforward to classify them. A fish immunologist will naturally classify the head kidney of fish as an "immune organ". A fish endocrinologist may not. In addition to containing lymphoid cells, the head kidney of fishes also harbours considerable amounts of endocrine cells arranged in clusters. So, it could be just as right to classify the head kidney as an endocrine organ. The correct way of classification seems to be "an organ consisting of lymphoid and endocrine tissues and properties". Therefore, to give an overview of immune organs in fish may not be as straightforward as it at first glance seems. When anatomists are uncertain of definitions, they frequently use the term "structure". This is an alternative escape route for potentially great terminological problems. A "structure" in medical terminology is used to describe "the components and their arrangement in constituting a whole unit" (Studdert et al. 2012). The reader will observe that this term will be used in this chapter.

1.1.3 Overview of Immune Organs and Cells

Fish organs, tissues and structures have been studied for centuries, first reported by Aristotle (384–322 B.C.) (Gudger 1934). It may therefore appear surprising that we still today can encounter new structures, possibly organs, which are macroscopically observable but have remained undescribed up until now. A good example is the salmonid bursa (Løken et al. 2020), which will be addressed later in this chapter. The classification of already described structures may also frequently be subjected to discussions.

The immune system of modern bony fishes or teleosts is constructed to facilitate both innate and adaptive immune responses. The populations of immune cells found in fish are not very different from those of mammals. Immune cells in teleost fish include lymphocytes, monocytes, macrophages, granulocytes, thrombocytes, mast cells, non-specific cytotoxic cells and possibly dendritic cells (Ellis 1977; Press and Evensen 1999; Fischer et al. 2013; Flajnik 2018). Striking exceptions include the melano-macrophages and rodlet cells, which both seem to be special immune cells found in lower vertebrates (Sichel et al. 2002; Reite and Evensen 2006). The melano-macrophages may be found in immune organs (Agius and Roberts 2003; Thorsen et al. 2006), whereas the rodlet cells are predominately found at mucosal surfaces but with a considerable species variation (Reite and Evensen 2006). The rationale and function for both melano-macrophages and rodlet cells remain obscure. Nevertheless, bony fishes possess an adaptive immune system based on molecules of the immunoglobulin superfamily (Flajnik 2018), and T and B cells and macrophages are found throughout different tissues. At the level of histological organization, the differences between fish and mammals are more apparent. Strikingly, B cells do not proliferate in follicles or germinal centres (Flajnik 2018). It has been speculated that melano-macrophage centres in fish spleen and head kidney may have similar functions, but so far, this is an unresolved matter (Steinel and Bolnick 2017). Anyhow, the organization of B cells proliferating in confined groups surrounded by T cells, as in mammals, has not been reported in fish.

Some additional comments should be attributed to the melano-macrophages. Melano-macrophages are predominantly present in kidney tissue and the spleen (Agius and Roberts 2003; Thorsen et al. 2006). Their presence in inflammatory changes in white muscle has severe consequences for fillet production of Atlantic salmon (*Salmo salar*) (Bjørgen et al. 2019a). Today, such changes are the costliest quality problem for the Norwegian aquaculture industry. Experiments in a fish cell culture have shown that such cells possess the machinery for melanin synthesis while simultaneously having properties of macrophages (Haugarvoll et al. 2006; Thorsen et al. 2006). This is in line with the proposed classification of melanocytes (Sichel et al. 2002), also including leucocyte populations of ectothermic vertebrates. The rationale for melanin synthesis in such cells is not understood. In cell line experiments, Larsen et al. (2013) showed that melanin production could be influenced by temperature, but not by antigen stimulation.

It is, however, at the gross anatomical level that the construction of the immune system of teleosts differs most apparently from that of mammals (Fig. 1.1). Till date, there are no
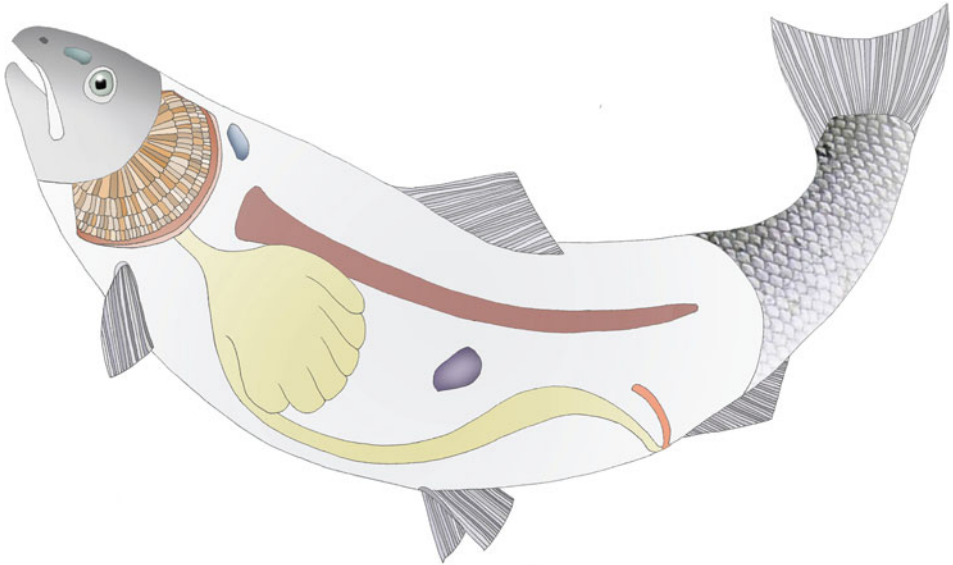
Fig. 1.1 A schematic illustration of the placement of important lymphoid organs and structures in fish. In a cranial to caudal sequence: nose-associated lymphoid tissue (green), gills (brown), thymus (blue), kidney (dark red), spleen (purple), bursa (orange). The gastrointestinal tract is marked in yellow

observations of lymph nodes in fish. Lymph nodes first occur in endothermic vertebrates (Zapata and Amemiya 2000). So far, no observation of lymphoid structures in the intestinal tract of fish has been observed. Rather, more diffusely occurring lymphoid aggregates have been reported located to the lamina propria and thus not as isolated organs (Zapata and Amemiya 2000)—like tonsils or Peyer's patches as found in mammals. The possible existence of lymphatic vessels is subjected to discussions. Obviously, fish have a secondary vascular system in several organs, but these vessels are not lymphatic vessels and their purpose is unknown. In contrast to lymphatic vessels, which have blunt ends, the secondary vascular system originates from arteries (Vogel and Claviez 1981; Vogel 2010; Rasmussen et al. 2013). However, several authors argue for the existence of lymphatic vessels. How these, if so present, interact with or function together with the secondary vascular system is not understood and seems never to have been discussed by authors addressing assumed lymphatic vessels or cells, particularly in the zebrafish. For instance, van Lessen et al. (2017) assumed that they identified brain lymphatic endothelial cells in the zebrafish, but the authors did not address the possibility that these cells might belong to the secondary vascular system. A rare exception in this literature is a paper by Hellberg et al. (2013) where available information is discussed in the context of the identification of vascular structures in the intestines of the common wolffish, *Anarhichas lupus* L. These authors did not dispute the existence of a secondary vascular system but argued that the observed structures indeed were lymphatic vessels. Nevertheless, the general negligence of the

existence of the teleost secondary vascular system, among in particular researchers using zebrafish as a model organism, is both sad and stunning and raises the question of the validity of such research. The questions regarding the vascular system of teleosts are indeed of fundamental importance for our perception of the teleost immune system and should be an obvious focus in future studies. A comprehensive study addressing the secondary vascular system in the context of the proposed presence of a lymphatic system is overdue.

Unfortunately, the terms lymphoid organs and lymphoid tissues are frequently mixed up in the scientific literature (Pabst 2007). Lymphoid tissues consist of reticular cells and leucocytes (Pabst 2007). However, if such a tissue is arranged in a functional unit, it may be called a lymphatic organ. The immune organs are especially dedicated to house immune cells. Traditionally, lymphoid organs are divided into primary and secondary lymphoid organs. The purpose of the primary lymphoid organs is the production of precursor leucocytes and the receptor-specific production of T and B cells. In mammals, the primary lymphoid organs comprise the bone marrow and the thymus (Pabst 2007). In birds, the bursa of Fabricius is regarded both as a primary organ and as a secondary lymphoid organ (Ekino and Sonoda 2014). The purpose of secondary lymphoid organs is to expand the number of lymphocytes following antigen exposure. In mammals, the lymph nodes, the spleen, the tonsils and Peyer's patches are regarded as secondary lymphoid organs. The two latter do not have a real capsule but are characterized by a specialized epithelium in the pharyngeal region and in the intestine, respectively. Rodents do not have tonsils, but they have accumulations of lymphoid tissue at the dorsal base of the nose called nose-associated lymphoid tissue (NALT) and considered as a "lymphoid structure". In humans, when present, the NALT is disseminated in the nasal mucosa and not concentrated as in rodents (Pabst 2007).

In their review from 2000, Zapata and Amemiya outlined excellently the till then current knowledge of fish immune structures and organs. There is conflicting evidence on which was the first lymphatic organ to evolve in fish. This fact may point to species variation. As reviewed by Zapata (1996), the yolk sack seems to be the earliest structure to exhibit certain haematopoietic capacities. However, the thymus seems to be the first organ to be lymphoid. But importantly, presumptive lymphohaemopoietic stem cells appear in the kidney before the onset of lymphoid differentiation in the thymus. Chilmonczyk (1992) noted that the origin of the thymic stem cells remained unresolved. Thus, primary immune organs comprise the thymus and the head kidney or the permanent pronephros/metanephros, which is regarded not only as the bone marrow equivalent in fish, but also as a secondary immune organ. The spleen is characterized a secondary immune organ in fish (Zapata and Amemiya 2000; Flajnik 2018). But in recent years, previously undescribed lymphoid tissues and structures have been described, mainly in salmonids. They include the NALT (Tacchi et al. 2014), the interbranchial lymphoid tissue (ILT) (Haugarvoll et al. 2008) and the salmonid bursa, a structural analogue to the avian bursa of Fabricius (Løken et al. 2020). Common for the two latter is that they are not encapsulated and contain abundant amounts of immune cells embedded in a matrix consisting of reticulated epithelial cells and are exposed to the surrounding environment. Thus, they share common features with the

mammalian tonsils and Peyer's patches, which are defined as secondary lymphoid organs. Due to these features, we regard them as lymphoid organs. Notably, the ILT and the salmonid bursa are strictly intraepithelial and contain no blood vessels. Teleost NALT is described as diffusely distributed in the fish olfactory epithelium. However, lymphoid aggregates have been shown in the African lungfish (*Protopterus dolloi*) (Sepahi et al. 2016). These properties do not warrant the classification as a lymphoid organ.

Non-lymphoid organs normally contain different amounts of lymphoid cells. Although not the scope of this chapter, we will briefly mention the distribution of such cells in organs throughout the organism. Of note, different organs have different potential to mount inflammatory responses. The term immune privilege implies that there is a high threshold for mounting immune reactions or that immune reactions may be muted, and this phenomenon occurs at sites where inflammatory responses may cause serious consequences, such as in parts of the eye and brain or in the testes. In an aquaculture context, the ability of the organism to downregulate immune responses in the gills and gastrointestinal tract is of vital importance to avoid aberrant responses to non-pathogenic antigens. The borders between immune privilege and tolerance are thus not clear-cut. But it seems as a correct observation that the degree of immune privilege differs from organ to organ.

1.1.4 Kidney

As in mammals, the fish kidney has both excretory and hormone-producing functions. But unlike mammals, the kidney of fish cannot concentrate urine in an efficient way. The reason for this is that the kidney tissue is not divided into a cortex and a medulla as the nephrons of the fish are not orientated. Rather, the fish kidney is divided into an anterior or head kidney and a posterior or trunk kidney. The origin of this division is found in the morphogenesis of the organ, which is quite intriguing. The kidney of vertebrates develops in generations. These generations are called the pronephros and the mesonephros, and in higher vertebrates, a third generation called the metanephros is formed as well. These generations develop in a successive anterior–posterior wave of cellular differentiation of the nephrogenic cord, which is a part of the urogenital plate. The urogenital plates are located along the dorsolateral aspect of the abdomen, one on each side. Thus, there are two anlagen for the kidneys, and the resulting fish kidney is a paired organ although it may appear fused and located ventral to the spinal cord in most species. Two ducts, termed the mesonephric ducts or the Wolffian ducts, form in these anlagen. They persist in fish as ureters (Hyttel et al. 2009). In mammals, the pronephros and mesonephros undergo atrophy, and only the third kidney generation, the metanephros, remains. In fish, where the metanephros is not formed, the pronephros and mesonephros persist together as the cephalic or head kidney and the exocrine or trunk kidney, respectively (Fernandes et al. 2019). The ureters appear as prominent tubular structures in both the right and left parts of the organ. Caudally, the ureters fuse in the urinary bladder.
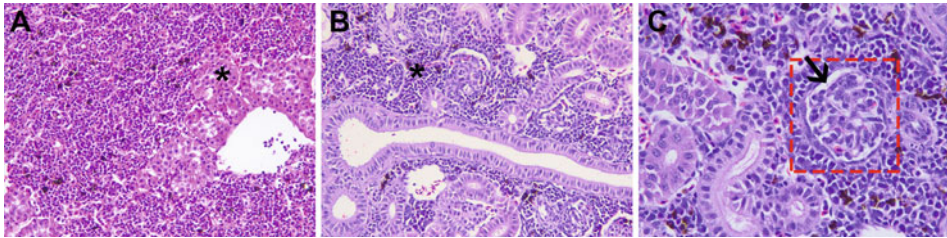
Fig. 1.2 Kidney tissue of Atlantic salmon, HE-stained. (**a**) Head kidney tissue with scattered melano-macrophages (black) dispersed throughout haematopoietic/lymphoid tissue. Note endocrine cells of the teleost adrenal counterpart arranged in clusters (asterisk). (**b**) Trunk kidney tissue with haematopoietic/lymphoid tissue (asterisks) dispersed between structures of the exocrine system including glomeruli. (**c**) Higher magnification showing a glomerulus (boxed) surrounded by Bowman's space (arrow)

The head kidney (Fig. 1.2a), placed in the very anterior end of the organ, contains haematopoietic tissue, endocrine cells and immune cells and no nephrons. The trunk kidney is formed by the remaining posterior part of the organ where the nephrons are located and is thus regarded as a predominantly urine-producing structure. Nonetheless, haematopoietic tissue is present between the nephrons. Nephrons with glomeruli and urinary canals seem to be haphazardly distributed in this tissue (Fig. 1.2b). An orientation of the loop of Henle, which is the premise for defining a medulla, is missing and consequently, the kidney cannot be divided into a medulla or a cortex as the mammalian counterpart (Demoll and Harder 1964). Thus, an osmotic gradient throughout the tissue cannot be established, and the fish's ability to concentrate urine is limited. Haematopoietic bone marrow has not been described in teleost fish. However, the kidney is thought to have this function (Zapata and Amemiya 2000; Zapata 1979).

Blood supply to the excretory part of the kidney is provided by *arteria renalis* originating from the *aorta dorsalis*, with its afferent arterioles supplying the glomeruli. From the glomeruli, blood drains via efferent arterioles to the sinusoids. The sinusoids are numerous in the kidney interstitium and are found both in the head kidney and in the trunk kidney. In addition to receiving blood from afferent arterioles, the sinusoids are supplied with venous blood from segmental veins from the lateral and dorsal musculature, thus constituting a renal portal system. The venous blood is drained through the *venae cardinales caudales*. Noteworthy, *v. cardinals caudales* sinister is often shorter and in many species rudimental, leaving *v. cardinales caudales dexter* as the primary draining vein of the kidney in salmonids (Harder and Sokoloff 1976). The amount of kidney tissue varies between species according to their habitats. In general, marine fish lose water through the gills and have reduced urine production and consequently reduced amounts of kidney tissue compared with freshwater species. Freshwater species absorb water through the gills and need to shed excess water through the kidneys.

In addition to being the bone marrow equivalent and thus a primary lymphoid organ, the head kidney also has antigen-sampling abilities, and antigen retention has been

demonstrated in several studies (Press and Evensen 1999). The Mhc molecules, which are essential in antigen presentation, are both highly expressed and regulated in the kidneys (Koppang et al. 1998a, b). Morphological studies of antigen-presenting cells showed that Mhc class II immunopositive cells could be found as single cells or in clusters, typically in association with melano-macrophages is the kidney tissue (Koppang et al. 1998a). Immunopositive cells seemed in most cases to be macrophages or macrophage-like cells. An exception was the melano-macrophages, which only occasionally were immunopositive.

The melano-macrophages are highly visible cells in the teleost kidney (Agius and Roberts 2003). Haugarvoll et al. (2006) discovered melanin synthesis in a cell line isolated from the head kidney of salmon (the SHK-1 cell line), which was previously classified as a macrophage-like cell line. Interestingly, the SHK-1 cells appear non-melanotic, but they produce melanosomes that seem to be released into the cell culture medium. Melanosomes are intracellular organelles that produce melanin. It is typical for cutaneous melanocytes to transfer melanosomes to recipient keratinocytes. Here, the melanosomes synthesize melanin using their membrane-embedded enzymes as encoded by the tyrosinase gene family. If a similar mechanism exists in the melanin-synthesizing macrophage-like cells in the salmonid head kidney, the melanin-containing cells that are observed histologically and termed melano-macrophages may not necessarily be the same cells that produce the melanosomes and express the genes of the tyrosinase gene family. Several interesting questions arise in this respect. The rationale for melanin synthesis is obscure, but several authors have suggested that melano-macrophage centres in fish lymphoid organs may represent germinal centres (Steinel and Bolnick 2017). A possible blueprint of the avian and mammalian germinal centres is, however, much obscured by the presence of the pigment-containing cells and our lack of understanding of their actions. A key premise for our conception of the actions in the melano-macrophage centres is to reveal the rationale for pigment production in fish macrophage-like cells.

The head kidney also harbours mature T cells. Zapata and Amemiya (2000) noted that "the kidney contains hemopoietic stem cells before mature cells appear in the thymus, but mature lymphocytes are present in the kidney only after they are found in the thymus". Due to its function as a secondary lymphoid organ, a vast number of studies have used transcriptional data in the investigations of T-cell populations in the kidney tissue of fish, often measured as responses in connection with immune induction of different kinds as exemplified by Aas et al. (2014). These studies have shown that CD4 and CD8 transcripts are present as well as transcripts showing the presence of $\alpha\beta$ and $\Upsilon\delta$ T cells. Here, we will not review the vast and comprehensive information available from transcriptional studies, but concentrate on the morphological information at hand, which is not that much. Press et al. (1994) used some immune and enzyme histochemical markers to reveal lymphoid and non-lymphoid cells and their distribution throughout the head kidney including B cells. These studies were important for understanding the structure of the immune compartment of the tissue. The CD3 complex is a pan T-cell-specific molecule. Following the cloning, sequencing and production of antisera recognizing the salmonid CD3 complex, Koppang

et al. (2010) addressed the distribution of immunopositive cells in a range of tissues including the head kidney. These studies revealed large amounts of T cells in the tissue, corresponding to qRT-PCR results. Using in situ hybridization approaches, Løken et al. (2020) visualized the distribution of αβ and ϒδ T cells in addition to CD8-positive cells.

B cells are abundant in the kidney tissue. The kidney of teleosts appears to be the major source for B cells, and B-cell precursors are also present here (Zapata and Amemiya 2000). A maturation gradient of B cells was described by Zwollo et al. (2005). These authors showed that the head kidney contained mostly proliferating B-cell precursors and plasma cells, whereas the trunk kidney contained abundant B cells, some of which were activated, and in addition plasmablasts. Using in situ hybridization, IgM- and IgD-positive cells have been identified in kidney tissue of the Atlantic cod (*Gadus morhua*) (Stenvik et al. 2001), and IgM-, IgD- and some IgT-positive cells have been detected scattered within the salmon kidney tissue (Løken et al. 2020). In the cod, the organization of these cells seems more restricted compared with the seemingly haphazardly distribution in salmon.

In addition to being a centre for immune cells and immune reactions, the teleost kidney is also an important endocrine organ. In the head kidney, endocrine cells are found as thyroid cells arranged in follicles. These may be encountered within the renal tissue or in a pararenal location, depending on the species (Geven and Klaren 2017). Importantly, the fish adrenal counterpart is located in the head kidney, and these cells may be observed as islands between haematopoietic tissues (Di Lorenzo et al. 2020). In the trunk kidney, corpuscles of Stannius are seen as discrete structures containing cells producing stanniocalcin-1 (STC-1), which is the main calcium regulatory hormone of fish (Greenwood et al. 2009). Adrenal gland cells are innervated by the sympathetic nervous system (Gallo and Civinini 2003), although parasympathetic fibrae may also be found. In histological sections of kidney, it is therefore quite common to see thick peripheral nerves within the tissue.

The kidney is important in fish diagnostic work. Samples are frequently taken from this organ aiming at isolating pathogens, primarily virus or bacteria. It is common in diagnostic practice for all animals to investigate secondary lymphoid organs for pathogens. However, the head kidney is also a primary lymphoid organ with expression of recombination-activating genes (RAGs) and activation-induced cytidine deaminase (AID) as demonstrated by in situ hybridization in salmon (Løken et al. 2020). It is an organ with endocrine functions, and haematopoietic tissue is also located caudally in the trunk kidney tissue, which has excretory functions. With so many facets of functions, this complicated and intriguing organ deserves continuous attention in our quest in exploring the teleost immune system.

1.1.5 Thymus

It may be discussed whether the thymus or the kidney is the first lymphoid organ to appear during embryogenesis. As mentioned in the above sections, this seems to differ between

fish species—only a minority of them have been investigated in this respect. Nevertheless, the pharyngeal region, in which the thymus develops, seems to be of vital importance in the development of lymphoid tissues. In mammals, lymphoid tissues here, primarily tonsils and lingual follicles (*folliculi linguales*), form Waldeyer's ring (Matsunaga and Rahman 2001; Varga et al. 2008). In cyclostomata, which do not have an adaptive immune system involving the molecules of the immunoglobulin superfamily, a thymus analogue or tissue with some thymic properties was discovered in the gill tips (Bajoghli et al. 2011). This underlies the importance of the pharyngeal region in the development of immune organs. It has been noted by Nagy and Oláh (2010) that regions where ectoderm and endoderm are juxtaposed seem to have been important for the development of lymphoid tissues, and this event occurs in gill arches of fish (Gillis and Tidswell 2017). Until recently, it was thought that the only lymphoid structure developing in this region in teleost fish was the thymus and that other structures, such as tonsils, were restricted to higher vertebrates. With the identification of the interbranchial lymphoid tissue in the gills by Haugarvoll et al. (2008), this assumption had to be revised.

The thymus develops from endodermal part of the pharyngeal pouches, located between the gill arches. In sharks, the organ emerges from several pharyngeal arches (Lloyd-Evans 1993), whereas in teleosts, the third pharyngeal pouch is the main site for the organogenesis (Zapata et al. 2006). In all cases, the anlage is duplicated as it is found on each side of the body axis. Subsequently, lymphoblasts infiltrate this epithelium and start proliferating. As mentioned before, the origin of these cells is not established; they have been suggested to migrate from both kidney tissue and from the yolk sac (Zapata 1996; Zapata and Amemiya 2000; Chilmonczyk 1992; Bowden et al. 2005; Barraza et al. 2020). Thus, the thymus forms as a paired organ located in the dorsal projection in the epithelium of the opercular cavity and remains at this location through the life span of the fish. In contrast, the thymic anlage in mammals migrates ventrally to fuse into a single organ.

When the lymphoblasts start infiltrating the epithelium, the epithelial cells undergo a transformation and appear reticulated as they form niches where the immune cells proliferate. Thus, the tissue has distinct stromal and haematopoietic components. However, a portion of the pharyngeal epithelium remains flattened and covers the structure, forming an effective barrier towards the external milieu (Chilmonczyk 1983; Castillo et al. 1998). Castillo et al. (1998) concluded that the pharyngeal epithelium covering the thymus lacks antigen uptake capacity and that the underlying tissue was well protected from the entry of water-borne antigens with the exception of very young fish (4-day-old rainbow trout, *Oncorhynchus mykiss*, fry). In these experiments, it was, however, noted antigen uptake in the gill epithelium.

After the lymphoepithelium has been formed, blood vessels infiltrate the developing thymus, which may be divided into several different layers. The basement membrane disappears in this process. An epithelium is per definition avascular, and it is separated from the underling tissue by the basement membrane (Chilmonczyk 1983). Once this barrier is broken and vessels appear in the lymphatic tissue, it is no longer a strictly intraepithelial lymphatic structure—this in contrast to the ILT and the bursal lymphoid

tissue. The blood supply of the teleost thymus originates from vasculature penetrating the organ from the basal connective layer and subsequently branching out associated with thymic septa, which also consist of connective tissue (Chilmonczyk 1983). Transverse sections of such septae may easily be interpreted as Hassal's corpuscles that are structures found in the thymic medulla of higher vertebrates containing granular cells at the centre and are surrounded by layers of modified epithelial cells. Their presence in teleost thymus is therefore disputed (Chilmonczyk 1992). The absence of Hassal's corpuscles in the teleost thymus was also emphasized by Zapata and Amemiya (2000), who noted that these structures first appear phylogenetically in birds. However, as described in trout, tubular structures containing an amorphous PAS-positive material can sometimes be observed (Chilmonczyk 1983). These structures may also be observed in salmon (Fig. 1.3). The function of these structures is unknown. From the septae, the blood vessels branch out and divide into capillaries with a fenestrated endothelium, surrounded by a discontinuous layer of epithelial cells (Chilmonczyk 1983, 1992).
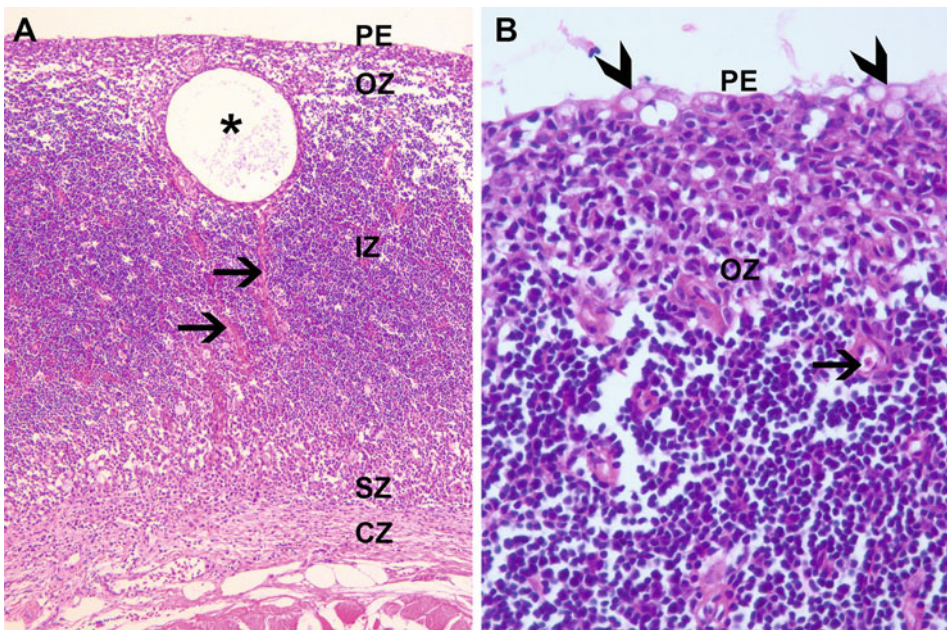
Fig. 1.3 Thymus, Atlantic salmon, HE-stained. (**a**) Low magnification shows the different layers of the thymus including an outer capsular zone (CZ) followed by a subcapsular zone (SZ), an inner zone (IZ), an outer zone (OZ) and finally the pharyngeal epithelium (PE). Note the trabeculae (arrows) and a room containing an amorphous material (asterisk). (**b**) High magnification of the pharyngeal epithelium (PE) and the underlying outer zone (OZ). Note small blood vessels in the outer zone (arrow), which predominantly consists of lymphocytes and some structural cells. The pharyngeal epithelium contains several goblet cells (arrowheads) and a meshwork of epithelial cells in which some lymphocytes are embedded

Not only vessels proliferate into the thymic tissue. Like the situation in mammals, the teleost thymus is innervated. Investigations on the interactions show some complexity, but this line of research is still in its infancy. In mammals, the thymic innervation is both sympathetic and parasympathetic, and both nerve fibres and neuropeptides are important for the generation of a functional microenvironment (Mignini et al. 2014). In fish, it seems as if only sympathetic innervation has been identified so far. In those few fish species, which have been investigated in this respect, nervous tissue originates from the fourth or fifth sympathetic ganglion (Chilmonczyk 1992).

When matured, the fish thymus consists of several layers arranged beneath the pharyngeal epithelium (Fig. 1.3). Flajnik (2018) noted that thymic organization in gnathostomes (including teleosts) usually follows the classic cortex/medulla organization as known from mammals. A recent review (Barraza et al. 2020) summarizes nicely our current understanding of the events in the teleost thymus, but with respect to more fundamental aspects, of which little is known in fish, we will in the following refer to results of investigations in different mammals. Much of this seems to be in line with what we so far know in fish (Barraza et al. 2020). In the thymic cortex, αβ T cells undergo a random rearrangement of their T-cell receptor (TCR) resulting in a repertoire of more than 10^{10} antigen specificities (Bhakta and Lewis 2005). This event is subsequently followed by the *positive selection*. Here, the thymocytes with the newly formed TCR search for peptide-MHC complexes on the cortical thymic epithelial cells (cTECs), and given an interaction, the thymocytes may proceed in their maturation process. Only a small number of cells are in this way positively selected, and at this stage, these emerging CD4- or CD8-positive thymocytes increase their chemokine receptor CCR7 expression, allowing them to migrate from the cortex to the medulla. In the medulla, medullary thymic epithelial cells (mTECs) and endothelial venules may express the chemokine CCL19, which is a ligand for CCR7. A subset of mTECs also express the chemokine CCL22, also a ligand for CCR7 (Dzhagalov and Phee 2012). This expression drives the migration from the cortex to the medulla. In the medulla, the thymocytes undergo *negative selection*. The negative selection implies that self-reactive T cells, that if released from the thymus could cause autoimmune disease, are eliminated. In the medulla, positively selected thymocytes are presented for self-antigens and this process can occur in a number of different ways (Dzhagalov and Phee 2012). However, much focus has been on the negative selection mediated by the AutoImmune REgulator (Aire), which is expressed by approximately 30% of the mammalian mTECs. Aire drives the stochastic expression of genes considered to be strictly restricted to peripheral tissues. Thymocytes reacting with these expressed genes are either eliminated or tolerance is elicited (Klein et al. 2014; Takaba and Takayanagi 2017). Following positive and negative selection, thymocytes may leave the thymus, the so-called thymic egress. This occurs as the thymocytes start expressing CE62L and the chemokine receptor $S1P_1$, enabling them to be attracted to thymic blood vessels, which they can penetrate to be released in the peripheral blood flow (Dzhagalov and Phee 2012). Υδ T cells are also produced in the thymus, but the selection mechanism for these cells is at the best illusive (Takaba and Takayanagi 2017).

In fish, most of this detailed information is not at hand. For instance, it has been challenging to establish the distinction between medulla and cortex in salmonids. As noted in their review from 2005, Bowden et al. (2005) showed to a great variation in reported thymus morphology both between fish species and within species. In salmonids, it has been difficult to identify any medullary and cortical organization. Rather, the trout thymus has been divided into an outer capsular zone (C) followed by a subcapsular zone (SZ), an inner zone (IZ), an outer zone (OZ) and finally the pharyngeal epithelium (PE) (Chilmonczyk 1983; Castillo et al. 1990) (Fig. 1.3). For a long time, histological markers for fish T cells were not at hand. However, this was changed in 2010. With the generation of antisera recognizing the CD3 epsilon chain, the first distribution of the distribution of T cells in a range of lymphoid and non-lymphoid tissues in a fish species was published (Koppang et al. 2010), and the salmonid thymus proved to be rich in CD3 epsilon-positive cells, particularly in the subcapsular zone. This suggests that this zone corresponds to the medulla as CD3 expression is lower in the cortex where T cells undergo recombination. In rainbow trout larvae and fry, Chettri et al. (2012) demonstrated abundant CD8-positive cells in the thymus, but at this developmental stage, there was no clear zone distinction. So far, a division of a salmonid thymic medulla and cortex, essential for the understanding of the dynamics of the organ, has not been established. Bjørgen et al. (2019c) addressed the expression pattern of the cytokine CCL19 in some salmon immune organs. In the mammalian thymus, CCL19 is highly expressed in cTECs in the junction between the cortex and medulla. In the salmon, positive cells were in particular confined to a layer between the outer zone and the inner zone. However, Mhc class II seemed most highly expressed in the thymic subcapsular zone (Koppang et al. 2003). To draw any conclusion regarding the organization of the salmonid medulla versus cortex based on these observations is not possible. However, it seems evident that the inner zone contains abundant amounts of small thymocytes, and it is therefore probable that this zone corresponds to the cortex. Applying enzyme histochemical staining, Castillo et al. (1990) investigated the properties of the rainbow trout thymic stroma cells and were able to differentiate seven different subpopulations. Based on these results, the authors could not draw any definitive conclusions regarding a possible homology between the different zones of trout and mammalian thymi. However, their studies allowed a description and charac-terization of the different zones present. Since this study was published, substantial efforts have been invested in the research of thymic epithelial cells of mammals (Abramson and Anderson 2017). With this information now at hand, it is an obvious task for future research to expand the efforts of Castillo et al. (1990) and establish additional properties of the different thymic epithelial cells.

However, the most useful method to establish the presence of the cortex seems to investigate the tissue for the expression of RAGs, so far not applied in salmonids. But as the recombination process occurs in the cortex, RAG expression should be confined to this portion of the thymus. The cortex/medulla distinction in the carp thymus was determined by Huttenhuis et al. (2005) on the basis of RAG-1 expression patterns using in situ hybridization approaches. Also in the zebrafish, the regionalization of cortex and medulla

was established using in situ hybridization for RAG-1 (Lam et al. 2002). Similar approaches were used to study the thymus of the common carp (*Cyprinus carpio*) (Huttenhuis et al. 2005) and flounder *Paralichthys olivaceus* (Wang et al. 2014). Other approaches have been used to identify the thymic organization of the European sea bass, *Dicentrarchus labrax* (Picchietti et al. 2015; Paiola et al. 2017). Mohammad et al. (2007) identified a similar distinction in the Australian lungfish, *Neoceratodus forsteri*, but this was not based on RAG expression.

The thymus is a dynamic organ, which develops at early age and in most fish species undergoes involution following sexual maturation (Bowden et al. 2005). In mammals, this event has been attributed to the onset of the production of sex hormones that bind to androgen/oestrogen receptors in thymic epithelial cells and this event induces involution of the organ. The normal involution is characterized by the loss of thymocytes and TECs and a breakdown of the cortical–medullary junction. Further, external stimuli including infection and different physiological stressors can induce thymic involution (Dyce et al. 2010).

Substantial efforts have been committed by several researchers to the studies of the thymus of fish. Stephan Chilmonczyk and Augustin Zapata and co-workers have been key contributors to get us where we are today. Recent novel results obtained from research in mammals show that there is great potential for expanding our efforts in the research of the thymus of fish. With its key role in the immune system, increased knowledge of the thymus will benefit our understanding on how the fish immune system functions.

1.1.6 Spleen

In mammals, the spleen is a plastic organ placed in the abdominal cavity. It may expand and contract several fold and is therefore an effective reservoir from which the cell content of the circulation may be recruited in times of stress (Dyce et al. 2010). This phenomenon has also been observed in fish with consequences for rise and fall of haematocrit values. But perhaps more importantly, the spleen is known to the fish pathologist for its variation in size as a response to a number of different infections. Together with the kidney, the spleen is a major organ in the filtering process of blood (Noga 2006). It also removes damaged blood cells (Lewis et al. 2019). When antigen is encountered, the splenic tissue may react. The spleen is regarded as the primordial secondary lymphoid organ, and almost all gnathostomes possess this organ in which adaptive immune responses are generated (Flajnik 2018). The filtration of blood through the spleen may be an even more crucial immune mechanism in fish than in mammals. This is because fish lack lymph nodes and possibly lymphatic vessels.

The primordium of the spleen arises from local mesenchyme derived from mesoderm, and a complex vasculature forms within this anlage. Subsequently, there is a development of the white pulp following leucocyte infiltration (Vejsted 2010). To filtrate peripheral blood, visceral arteries supply the organ, whereas the draining splenic veins join the hepatic portal system leading to the liver. The teleost spleen is innervated by sympathetic fibres,

and there are no indications of vagal innervation (Fänge and Nilsson 1985). This corresponds with the situation in mammals, where, so far, no innervation related to the parasympathetic nervous system has been identified (Verlinden et al. 2019).

The arteries branch out in the splenic tissue where they terminate in ellipsoids. Their structure varies between different fish species (Fänge and Nilsson 1985), but the construction in trout seems to be representative. Here, the ellipsoids consist of cubical endothelial cells, contrasting the more flattened ellipsoidal endothelial cells in mammals. There are gaps between the endothelial cells, and the underlying basal lamina is disrupted. This allows an effective filtration through the structures. The basal lamina is surrounded by reticular cells and macrophages (Fig. 1.4). Together, these cells and structures form the splenic *white pulp* (Espenes et al. 1995a, b). This construction is principally similar in both mammals and fish. Filtration occurs through sheets of leucocytes of the white pulp, and the filtrate is finally released in the red pulp, which just consists of peripheral blood surrounding the white pulp.

In mammals, the white pulp may be divided into distinct layers dependent on their content of different leucocytes including T-cell subpopulations, B cells, plasma cells and macrophages (Cesta 2006). In fish, similar investigations have not been conducted. However, Mhc class II-positive cells, most likely macrophages, and T cells have been shown to be present in the white pulp (Koppang et al. 2003, 2010), but any clear distribution of such cells within this compartment has not been discussed. Press et al. (1994) reported that immunoglobulin-positive cells could be detected in clusters or as solitary cells throughout the spleen, but with a clear association with melano-macrophages, and alkaline
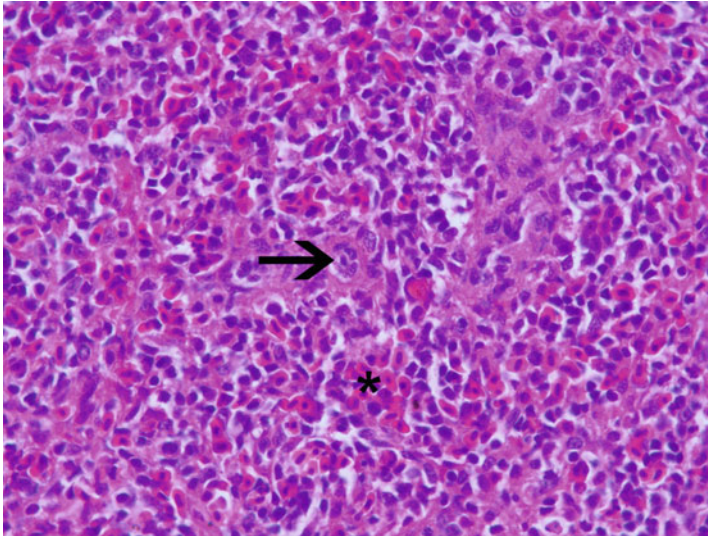
Fig. 1.4 Spleen, Atlantic salmon, HE-stained. Note ellipsoids (arrow) surrounded by white pulp and red pulp (asterisk)

phosphatase-positive arterioles and non-specific esterase-positive venous sinusoids. Several authors have throughout the years speculated if splenic melano-macrophage centres may represent phylogenetically early arrangements of germinal centres, but no definitive conclusion regarding this matter has so far been reached. With the identification of IgT in fish, Zhang et al. (2010) reported scattered immunopositive cells in the trout spleen.

In contrast to mammals (Lewis et al. 2019), the fish *red pulp* (Fig. 1.4) has not been much studied. In addition, a marginal zone (Lewis et al. 2019) between the white pulp and red pulp as described in mammals has not been reported in fish. Rather, fish immunologists and pathologists have concentrated on the melano-macrophages that depending on species can be arranged either in clusters or more loosely dispersed within the white pulp (Agius and Roberts 2003). It has been thought that such sites serve as "dumping sites" for discarded material of different nature, which has been supported by several studies addressing antigen retention (Press and Evensen 1999; Agius and Roberts 2003). Genes involved in the melanogenesis pathway have been shown to be expressed in the spleen (Thorsen et al. 2006), suggesting *de novo* synthesis of melanin in the organ. This is an obscure event, but given the involvement of the spleen in erythrocyte removal and subsequent iron exposure, the actions of melanin as a quencher of free radicals may explain this production. How this should be seen in context with the above-mentioned speculations regarding the melano-macrophage centres as analogues to the germinal centres of mammals is yet to be understood.

A number of different infections can cause enlargement of the spleen (splenomegaly) (Noga 2006), reflecting its function as a secondary lymphoid organ. In principle, splenomegaly can occur as a consequence of proliferation of cells in the white pulp (hyperplasia) or retention of peripheral blood in the red pulp or both. Fortunately, no lymphatic vessels are present in the mammalian spleen as this structure is considered as the "lymph node of the blood". Consequently, we can happily leave any speculations regarding the possible existence of a lymphatic vessel system in the fish spleen.

1.1.7 Interbranchial lymphoid Tissue—ILT

The functional parts of the fish gills are constructed as secondary lamellae attached to primary lamellae. The secondary lamellae have large surfaces consisting of respiratory epithelium exposed towards the external milieu. Here, the epithelium forms a selective barrier allowing the exchange of gasses, water and ions, while simultaneously striving to block the entrance of potential pathogens from infecting the organism (Koppang et al. 2015). In the primary and secondary lamellae, scattered leucocytes may be found, and they are often referred to as the gill-associated lymphoid tissue (GIALT) (Salinas and Miller 2015). During ontogenesis, pharyngeal arches develop into gills, and their medial epithelium originates from endoderm, whereas their lateral epithelium originates from ectoderm (Gillis and Tidswell 2017). In mammals and birds, sites of juxtaposed endoderm and ectoderm including the pharyngeal and anal regions form sites from which a range of

lymphoid structures, including the avian bursa of Fabricius, thymus, tonsils and other secondary lymphoid structures commonly known as Waldeyer's lymphatic ring, evolve (Varga et al. 2008; Nagy and Oláh 2010). In fish, tonsils have not been described. However, Haugarvoll et al. (2008) discovered a lymphoid structure at the terminal portion of the Atlantic salmon interbranchial septum, which connects the proximal third of the primary lamellae (Fig. 1.5). Later, Koppang et al. (2010) expanded these investigations also to include the rainbow trout and termed the tissue, found in both species, the interbranchial lymphoid tissue or the ILT. Thus, the ILT develops in the pharyngeal region where ectoderm and endoderm are juxtaposed. In larvae, with a well-developed thymus, the structure is not recognizable. But over time, it develops into a large lymphoid structure containing proliferating T cells, clearly visible in sexually maturated fish (Koppang et al. 2010; Dalum et al. 2016). Thus, the development of the ILT seems to follow the same pattern, though delayed, as the thymus, with immigration of lymphocytes into the epithelium at an early age and with a subsequent proliferation. However, further similarities seem to stop here.

The initial studies of the ILT (Haugarvoll et al. 2008; Koppang et al. 2010) discovered that the ILT consists of a lymphoepithelium consisting of lymphocytes, predominantly T cells, embedded in stromal cells. Few and scattered B cells and some strongly Mhc class II^{+} cells were also detected. The presence of ILT has in addition to salmonids also been described in the common carp (Dalum 2017). Rességuier et al. (2020) investigated a number of different species for the presence of ILT and reported the "taxonomic rule" seems to determine if the ILT is present or not. A major transmission seems to have
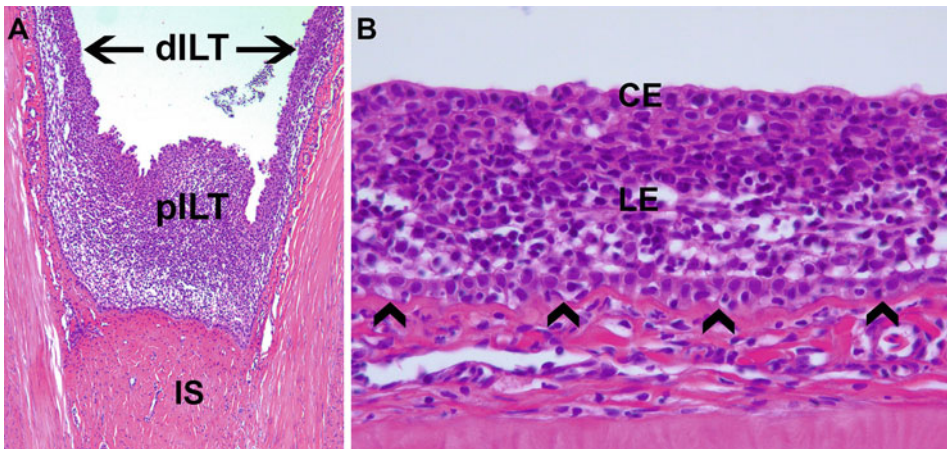
Fig. 1.5 Interbranchial lymphoid tissue (ILT), Atlantic salmon, HE-stained. (**a**) The ILT may be divided into a proximal part (pILT) placed on the terminal extension of the interbranchial septum (IS) of the gill and a distal portion (dILT), which extends along the primary lamellae. (**b**) Higher magnification of the dILT showing a prominent basal membrane (arrowheads) with an epithelial *stratum basale*. A prominent lymphoepithelium (LE) is placed between the *stratum basale* and covering flattened epithelial cells (covering epithelium, CE)

occurred at the basis of Percomorphs. In other words, this implies that in modern teleosts, where the interbranchial septum is rudimentary or absent, an identifiable ILT is not present. Following its initial discovery, the ILT was subsequently divided into a proximal part (pILT), or the original described lymphoid tissue, and a distal part (dILT), extending along the trailing edges of the primary lamellae (Dalum et al. 2015) (Fig. 1.5). All the morphological investigations in salmonids have described the ILT as strictly intraepithelial. It is separated from the underlying tissue by a thick basal membrane. Therefore, in contrast to the thymus, there is at no developmental stages any vascularization of the structure, and most probably no innervation. It is puzzling that such a large and cell-rich structure can exist without any vascularization in salmonids. In carp, it appears as if the situation is somewhat different. Here, vessels have been identified within parts of the ILT, which in addition seems to be compartmentalized (Dalum 2017). Thus, compared to salmonids, the anatomy of the carp ILT appears more complicated.

Several attempts have been made to classify the ILT. Bjørgen et al. (2019c) investigated CCL19 expression by in situ hybridization in the ILT and argued that the ILT should be considered as a lymphoid organ in its own right and not be regarded as a part of the GIALT, because the expression of this cytokine is restricted to primary and secondary lymphoid organs in mammals. The ILT is clearly identifiable as a discrete structure. Its construction has apparent similarities with the thymic outer zone (Bjørgen et al. 2019c). However, Aas et al. (2014) detected no RAG-1 or RAG-2 expression in samples from the ILT. Dalum et al. (2016) concluded from their morphological and transcriptional studies including investigations of RAG expression that there seemed to be no functional relationship between the thymus and the ILT. However, these authors observed T-cell proliferation within certain locations (growth zones) of the organ. As the thymic medulla expresses AIRE, Aas et al. (2017) cloned and sequenced this gene in the Atlantic salmon and investigated its expression in the ILT. Only thymic tissue proved positive, and these investigations thus gave no indications of possible negative selection events induced by ILT thymic stroma activity. Here, lack of RAG expression in the ILT was again observed. However, these studies revealed expression of factors that may indicate a function in peripheral immune tolerance. Observations of antigens within the ILT have not been reported. Austbø et al. (2014) infected fish with ISA (infectious salmonid anaemia) virus and observed transcriptional effects related to innate immune responses in the ILT, but no indications of virus presence. The ILT appeared un-infected in fish infected with *Aeromonas salmonicida* ssp. *salmonicida* (E.O. Koppang, personal observation). Further, in bath experiments with *Yersinia ruckeri* bacterin, the respiratory epithelium of the gills was active in antigen uptake, but this was not noted in the epithelium of the ILT (Ohtani et al. 2015). In their experiments addressing antigen uptake in the gills, Kato et al. (2018) observed no such events in the ILT, this being in stark contrast to the respiratory gill epithelium, where antigen uptake occurred. If antigen presentation occurs in the ILT, it seems not to be in the context of antigen processing at site, which is a key feature of MALTs (Smith et al. 2013). However, when bath-challenged with the ISA virus infection, a reduction in the structure's physical size was observed (Aas et al. 2014). This response

might indicate T-cell exodus from the ILT. This has also been indicated in studies of rainbow trout (Olsen et al. 2011). A secondary immune organ would be expected to react with an induction of immune cell proliferation, but no such events were observed. This spectacular finding needs confirmation by additional experiments. Finally, in transcriptome profiling experiments, Aas (2018) reported that the cellular pathways overrepresented in the ILT compared with thymus, head kidney and gill were related to cell adhesion molecules and cytokine–cytokine receptor interactions. In addition, genes related to the T-cell receptor signalling pathway were highly expressed. These investigations indicated that the ILT had transcriptional characteristics reminiscent of mammalian lymphoid secondary organ T-cell zones. However, no conclusions with respect to actual functions could be drawn.

So far, we thus have no indications of the ILT as a primary lymphoid organ. In contrast to the thymus, the organ increases in size over time and has a very prominent appearance in sexually mature salmon. It develops in the pharyngeal region just like the thymus, and most probably through lymphocyte seeding of the epithelium. But it contains no vessels and shares no key properties with a primary lymphoid organ. It has certain characteristics in common with secondary lymphoid organs, but efforts to provide functional evidences for a conclusive classification have so far not been successful. Not only does the ILT have anatomical properties unlike any other known immune organs (intraepithelial and no vessels), but it also responds as never reported in previously investigated lymphoid organs. The ILT thus does not seem to fall into any classifications of previously reported lymphoid organs.

Based on the investigations so far, we believe that the ILT should not be regarded as part of the GIALT, but as an organ in its own right. The structure is detachable from the rest of the gill tissue and possesses unique features not shared by other sites in the gills. So far, the focus of research on the ILT has been restricted to salmonids with some exceptions. The recent publication by Rességuier et al. (2020) shows that also other species could be subjected to more detailed studies. We believe that future research will show that this organ not only has a unique anatomical construction comparable to no other lymphoid tissues but also possesses unique functions yet to be discovered.

1.1.8 The Salmonid Bursa

In birds, the bursa of Fabricius is a primary organ for B-cell development and gut-associated lymphoid tissue. In mammals, which do not have this structure, bursa of Fabricius analogues are thought to include lymphoid tissues in the tonsils, Peyer's patches and the colon appendix (Ekino and Sonoda 2014). The cloacal bursa of Fabricius was, however, thought to be exclusive in birds. Studies of this structure prompted the diversification of T and B lymphocytes, named after the thymus and the bursa of Fabricius, respectively (Cooper et al. 1966). The anal region of salmonid fish attracted previously some attention due to the existence of two channels, called the abdominal pores (*pori*

abdominales), which is a paired system located lateral to the anus (George et al. 1982). It is amazing that such a seemingly open connection between the abdominal cavity and the external milieu can exist without apparently being an important port of entry for massive infections. The functions of the abdominal channels are unknown. At intraperitoneal vaccination of salmonids, one may sometimes observe that vaccine is released in the anus region escaping through the abdominal pores. As reviewed by George et al. (1982), these structures attracted significant attention in the late 1800 and early 1900. Now, they are seemingly without much interest. In 2018, we initiated research aiming at discovering immune mechanisms related to the abdominal pores, but in these efforts, we discovered a structure previously not addressed. We termed this structure the "salmonid bursa" (Fig. 1.6) and undertook a series of investigations in different age groups (Løken et al. 2020).

In most books of immunology, the bursa of Fabricius is depicted as drawings, and only rarely, photographs of the structure are provided. In most of these drawings, the bursa of Fabricius is presented as a diverticulum of the intestine. These drawings are schematic, but unfortunately quite misleading. Very few presentations show the bursa of Fabricius as it actually appears, with an opening at the very caudal part of the cloaca and with the orifices of the urogenital apparatus (urogenital papilla) cranial to that of the bursa. This may have prompted the misunderstanding that the avian bursa is part of the gastrointestinal tract. However, it is not, as just recently, Nagy and Oláh (2010) demonstrated that it is of ectodermic origin and not mesodermal, as the intestines. When carefully comparing the
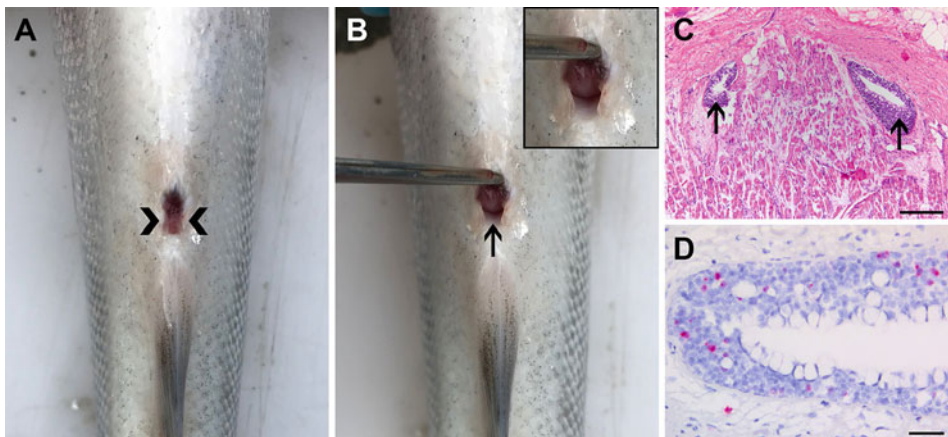
Fig. 1.6 Salmonid bursa, Atlantic salmon. (**a**) The anal region of a euthanized fish. Note the anal labia (arrowheads). At this stage, they are relaxed and the anus and urogenital papilla are exposed. In live fish, they appear frequently closed and the underlying structures are not exposed. In this way, they may form a compartment, which is similar to the avian cloaca. (**b**) The urogenital papilla is projected cranially, and the orifice to the salmonid bursa is exposed (arrow). (**c**) Transversal section, HE-stained, of the distal portion of the salmonid bursa. Here, the bursa is divided into two sacks, both with a very prominent lymphoepithelium (arrows). (**d**) In situ hybridization reveals γδ T cells (red staining) in the lymphoepithelium

anatomical features of the salmon and chicken anal regions, it became evident that we had discovered a bursa in salmon, placed caudal to the urogenital papilla and with astonishingly similar features as that of the early developmental stages of the avian bursa of Fabricius.

The investigations of the salmonid bursa are in their very infancy. However, in our initial investigation, we established that this structure is of ectodermic origin, and it develops a prominent lymphoepithelium containing immune cells with a distribution similar to that of early stages of the avian bursa of Fabricius. The expression of CCL19 seems to be a driving force in the formation of the lymphoepithelium. As CCL19 expression is restricted to lymphoid organs in mammals, and as the salmonid bursa comprises an enclosed and detachable structure, we will argue that this structure must be regarded as a lymphoid organ. No other purposes but to interact with the immune system seem at this stage of investigations to be possible. But in contrast to the avian bursa of Fabricius, few Ig-positive cells were present, and most lymphocytes seemed to be T cells. Naturally, no B-cell follicles were present, and even though a thick lymphoepithelium was formed, a microscope is required to observe this layer. So, until we performed histological examinations of this region that revealed the prominent lymphoepithelium, it may not be very surprising that this structure has not been noticed and described before.

We did not find any evidence for recombination or maturation in the salmonid bursal lymphoepithelium. This suggests that unlike birds, the salmonid bursa is not a primary lymphoid organ. However, the avian bursa of Fabricius is also a secondary lymphoid organ responding to environmental and enteric antigens. Indeed, active transport of such antigens from the avian cloaca up and into the bursal lumen prompted by musculature activity has been addressed in several studies (Ekino and Sonoda 2014). Antigen degradation, processing and presentation within the avian bursa seem to be of vital importance in the development of the immune system in birds. With its cloacal placement, we would expect similar events to occur in the salmonid bursa. The salmonid bursal epithelium involutes at sexual maturation and appears as scale-free truncal skin. The number of immune cells in its epithelium at this developmental stage is negligible. Thus, the structure follows the pattern of thymic involution at sexual maturation and distinguishes itself from the ILT, which only appears to increase in size following body growth. But just like the ILT, and in contrast to the thymus, no vasculature seems present in the lymphoepithelium of the salmonid bursa. The existence of any form of innervation is at this stage unknown. We have no information of any similar structures in other fish species but for brown trout, *Salmo trutta* (E.O. Koppang, personal observation), where the bursa seems to be very well-developed.

1.1.9 Occurrence of Immune Cells in Non-lymphoid Organs

Immune cells in fish are present in most non-lymphoid organs in various degrees. We will briefly mention some information here related to morphological but not transcriptional studies.

1.1.9.1 Immune Cells of Mucosal Organs

In mucosal organs including intestines, gills, skin and nasopharynx, it is commonplace to refer to the occurrence of immune cells at these locations as the mucosa-associated lymphoid tissues (MALT) (Salinas and Miller 2015). In fish, we understand the MALT as diffusely distributed lymphoid cells. However, the concept was originally introduced to "emphasize that solitary organized mucosa-associated lymphoid follicles and larger follicle aggregates have common features" (Smith et al. 2013). This concept seems difficult to adopt to the teleost immune system, and attempts have been made to clarify the matter by different authors. However, in our opinion, clearly distinguishable structures predominantly consisting of lymphoid and stromal cells in fish including the ILT or the salmonid bursa (and even the thymus, which no one disputes is an organ and which in fish develops and resides within the compartment of the skin) should be regarded as lymphoid organs and not as parts of the MALT.

The immune cells of the *intestines* vary in number, phenotype and microscopic location (for instance the occurrence of intraepithelial B cells seems to be different between different species) according to intestinal segment and species (Salinas and Parra 2015; Bjørgen et al. 2019b). Two phenotypes of antigen-sampling cells have been described in the fish intestines: one with M cell-like properties and one with macrophage-like appearance (Løkka and Koppang 2016). In the *gills*, lymphocytes and macrophages are present in the primary and secondary lamellae (Koppang et al. 2015). Antigen sampling may occur through macrophage-like cells and cells with M cell-like properties (Kato et al. 2018). The *skin* in fish shares key features of mammalian mucosal surfaces and harbours immune cells associated with mucosa-associated lymphoid tissue (Ángeles Esteban 2012; Esteban and Cerezuela 2015; Xu et al. 2013). Interestingly, elegant experiments have provided evidence for antigen entry at the fin bases (Harmache et al. 2006). Further, there is a clear differentiation of skin immune properties with respect to the location on the truncus (Leal et al. 2016). Different lymphocytes, IgT, IgD and IgM-positive cells, have been identified in the skin, but at low numbers. However, these numbers increase as a response to infection (Xu et al. 2013; Løken et al. 2020). In recent years, the epithelium of the *olfactory organ* has received much attention in immunological research. In fish, there is no connection between the olfactory and respiratory systems. The olfactory organ or the olfactory bulbs are placed in a left and right nasal cavity, each having two openings, allowing entry and outflow of water. The olfactory epithelium is located on the floor of the nasal cavity and is in most species arranged in the form of sheets or primary lamellae, which also may contain secondary lamellae (Kudo et al. 2009; Olivares and Schmachtenberg 2019). The immune cells in this epithelium are usually referred to as the nasopharynx-associated lymphoid tissue (NALT) and are found in two different immune compartments, i.e. the so-called mucosal tips and the neuroepithelium (Fig. 1.7), both with different immunological properties (Sepahi et al. 2016; Das and Salinas 2020). Tacchi et al. (2014) investigated four different families of teleost fish and reported diffusely distributed lymphoid cells in the tissue. Both local and systemic responses to nasal immunization and infections have been observed.
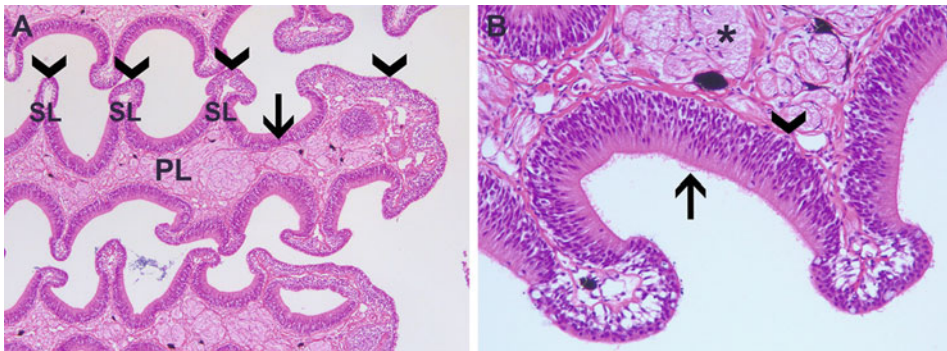
Fig. 1.7 Olfactory organ of sexually mature Atlantic salmon, with primary (PL) and secondary (SL) lamellae, HE stain. (**a**) Note the division of two mucosal compartments: tips (arrowheads) and neuroepithelium (arrow). (**b**) Higher magnification of the epithelium. Lymphocyte-like cells may be seen embedded in the tissue (arrowhead). Epithelial cells are ciliated (arrow). Beneath the epithelium, the tissue is dominated by the presence of nervous tissue (asterisk)

1.1.9.2 Immune Cells in Non-lymphoid and Non-mucosal Organs

In the *eye*, the distribution of immune cells seems to be restricted to the uveal tract, just like in mammals (Koppang et al. 2003; Koppang and Bjerkås 2006). There are no indications for any presence of immune cells in structures of the optical axis, and the cornea must thus be one of the few outer surfaces of the fish if not the only one with no content of immune cells. The *heart* of fishes seems to be immunologically a much more important organ compared with its mammalian counterpart. Here, the endocardial endothelial cells have been shown to be active in antigen uptake (Leknes 2011). In situ hybridization targeting Ig transcripts have revealed a prominent expression of IgM in the atrium of clinically healthy Atlantic salmon, but remarkably little in the ventricle. In the same study, scattered IgT transcripts were detected in both the atrium and the ventricle (Bakke et al. 2020). The *liver* has attracted some attention with respect to its content of immune cells. Both Castro et al. (2014) and Løken et al. (2020) screened liver tissue for the occurrence of a number of different immune cells and found scattered lymphocytes, B cells and macrophage-like cells. There seem to be no published studies with respect to the natural occurrence of immune cells in the *pancreas*. In red and white skeletal muscle, only very few and scattered immune cells have been encountered, with negligible amounts of Mhc class I-positive cells appearing scattered in the perimysium (Bjørgen et al. 2020).

1.2 Conclusions

It is important to invest efforts into continuous anatomical investigations of the teleost immune system. We lack substantial information with respect to essential questions, e.g. the construction of the secondary vascular system in the context of the likely existence of

lymphatic vessels. The dynamics of lymphocytes within the thymus is another key example. The recent discovery of the ILT and salmonid bursa also points to the fact that the days of macroscopic discoveries are not over and need to be further explored. To better understand immune and pathological responses in the fish, the basics must be in place—and true in ancient times and now: *anatomia fundamentum medicinae est*.

Note: We recently published a review paper (Bjørgen and Koppang 2021) addressing the immune structures of fish with a narrower scope compared with this book chapter.

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Innate Immunity

Roy Ambli Dalmo and Jarl Bøgwald

Abstract

Research on the innate immunity has accelerated over the last decades. The main reason for this has been the discovery of receptors recognizing danger molecules from pathogens. This has been facilitated through genome and transcriptome sequencing of different fish species. Also, endogenous host molecules from sterile physiological insults may also bind to certain receptors and induce immunological processes. The magnitude and quality of adaptive immunity are known to be dependent on the instructions the innate response gives. This chapter gives an overview of selected innate immune organs/tissues, factors, and processes that have been suggested to possess important roles during innate immune response in fish.

Keywords

Innate immunity · Fish · TLR · Complement · Cytokines · Acute-phase proteins · Antimicrobial peptides · Chemokines · Epigenetics

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Abbreviations

AMP	Antimicrobial peptide
APC	Antigen-presenting cell
APP	Acute-phase proteins. Resulting from acute inflammation
c1, c2, c3, c4, c5, c6, c7, c8, and c9	Complement components
c1q	A protein complex involved in the complement system
Cathl1	Cathelicidin gene 1. Antimicrobial peptide
Cathl2	Cathelicidin gene 2
Ccl19	Chemokine (C–C motif) ligand 19. A regulator of the induction of T-cell activation, immune tolerance, and inflammatory responses during continuous immune surveillance, homeostasis, and development
Cd4	Cluster of differentiation 4
Chemokine	A protein that can attract cells, toward a chemical gradient, having the specific receptor, and promote differentiation and multiplication of leukocytes, and cause tissue extravasation
CircRNA	Is a type of single-stranded RNA, which, unlike linear RNA, forms a covalently closed continuous loop. Can be protein coding and noncoding
CpG DNA	DNA that contains methylated nucleotides (CpG islands). Normally found in promoter regions, which modulate gene expression
CRP	C-reactive protein. An acute-phase protein
Cxcl13	Chemokine (C-X-C motif) ligand 13. Involved in chemotaxis of B lymphocytes
Cxcl9	Chemokine (C-X-C motif) ligand 9 is a small cytokine belonging to the CXC chemokine family. Plays role in chemotaxis
DAMPs	Damage-associated molecular patterns. Molecules released in a sterile inflammation or damage
DC	Dendritic cell
DEGs	Differentially expressed genes. From RNAseq data and bioinformatics
DEPs	Differential expressed proteins
dsRNA	Double-stranded RNA
Epigenetics	Is the study of heritable phenotype changes that do not involve alterations in the DNA sequence

Factor B (c3 convertase)	Protein that is activated by cleavage, yielding Bb and Ba fragments. Factor B is cleaved only when it is bound to c3b
Factor D	A serine protease present in blood and tissue in an active sequence but self-inhibited conformation. The only known natural substrate of factor D is factor B. Alternative pathway of complement activation
Factor H	The main cofactor of factor I
Factor I	Protein of the complement system (c3b/c4b inactivator). Alternative pathway of complement activation
Foxp3	Forkhead box P3. Regulator of the regulatory pathway in the development and function of regulatory T cells
Galectins	Proteins that bind specifically to β-galactoside sugars
Hepcidin	An antimicrobial peptide
Histone modifications and DNA methylation	Both DNA methylation and histone modification are involved in establishing patterns of gene repression during development
HSP1	Heat shock protein I gene. Mitochondrial product
HSP90b	A chaperone protein that assists other proteins to fold properly, stabilizes proteins against heat stress, and aids in protein degradation
Ifn	Interferon (cytokine)
Ifnrel	Interferon-related
Ifng	Interferon-gamma
IL	Interleukin (cytokine)
IRF	Interferon regulatory factor
Isoforms (subtypes)	Alternatively spliced genes
KEGG	Kyoto Encyclopedia of Genes and Genomes
lncRNAs	A large and diverse class of transcribed RNA molecules with a length of more than 200 nucleotides that do not encode proteins
LPS	Bacterial lipopolysaccharide. A main constituent in gram-negative bacterial cell wall
MAP kinase 1	Mitogen-activated protein kinase I. Transcription factor
MAP	Mannan-binding lectin (MBL)-associated protein
MASPs	Serine proteases that function as a component of the lectin pathway of complement activation
MBL	Mannose-binding lectin (lectin pathway of complement activation)
MHC I and II	Major histocompatibility complex I and complex II. Function in antigen presentation

MicroRNAs	(miRNAs) are a family of small noncoding RNAs
MPO	Myeloperoxidase. An enzyme that catalyzes the formation of a number of reactive oxidant species
MyD88	Myeloid differentiation factor 88 (MyD88). A central component of the Toll-like receptor pathway
Mx	An interferon-induced GTP-binding protein
NOD-like receptors (NLRs) and C-type lectin receptors (CLRs)	Belongs to RIG-I and PPR family
PPRs	Pattern recognition receptors. Expressed on many types of cells, especially on antigen-presenting cells. Recognize repeating molecular patterns often found in pathogens
RNAseq	RNA sequencing
TLRs	Toll-like receptors (belongs to PPR family)
PAMPs	Pathogen-associated molecular patterns
Paraquat (PQ)	Toxic chemical that is widely used as an herbicide
Pentraxins	Family of acute-phase proteins produced during acute-phase response
Poly I:C	Polyinosinic:polycytidylic acid. Binds TLR3
QPCR	A real-time polymerase chain reaction, also known as quantitative polymerase chain reaction
RAG	Recombination-activating gene
RIG-I-like receptors (RLRs)	Retinoic acid-inducible gene I-like receptors. Belongs to RIG-I and PPR family
ROS	Reactive oxygen species
SAA	Serum amyloid A. A protein formed during acute-phase response
SETD3-OT	Has function on histidine methylation which belongs to epigenetic occurrence
ssRNA	Single-stranded RNA
STAT1	Signal transducer and activator of transcription 1. STAT1 can be activated by several ligands such as IFN-α, IFN-γ, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), IL-6, or IL-27
TGF-ß	Transforming growth factor-beta
Th1 cells	Pro-inflammatory T cells that are responsible for cell-mediated immunity and phagocyte-dependent protective responses
Tnf	Tumor necrosis factor
Tnfr	Tumor necrosis factor receptor

TRIF	TIR domain-containing adapter-inducing interferon-β. An adapter in responding to activation of Toll-like receptors (TLRs)
Type I interferon	A role in antiviral responses
Type II interferon	A role in adaptive and innate immunity
Viperin	Virus-inhibitory protein, endoplasmic reticulum-associated, interferon-inducible. An IFN-inducible gene

2.1   Introduction

2.1.1   Innate Immunity: The Concept

Innate immune defense is important for protecting the host from infection, not only in naïve fish but also in fish that have previously been infected. "Innate immunity has shed its older, disparaging title of 'non-specific immunity' and now stands as a proud partner with the adaptive immune system in protecting human hosts from infectious insults. For any who doubt the impressive protective capacity of the innate immune system, it is instructive to consider that only vertebrates boast the added benefits of an adaptive immune system, leaving most organisms on our planet to survive on innate immunity alone" (Turvey and Broide 2010). Indeed, this applies also to fish. The immune system of teleost fish is composed of two kinds of receptor types: The germline-encoded pattern recognition receptors (PRRs) and the antigen-specific receptors are made from gene arrangement after, e.g., pathogen infection. The latter consist of, e.g., antibodies, MHC I and MHC II, and T-cell receptors. In addition, numerous other receptors/molecules can take part in the innate immunity. The innate mechanisms can be divided into *constitutive* and *inducible*. The former represents rapid ongoing ligand binding to receptors and a quick response, while the inducible (e.g., many PPRs) acts slower—but with a higher magnitude (Paludan et al. 2020) (Figs. 2.1 and 2.2).

2.1.2   Innate Receptors

Innate and adaptive immunity can cooperate to clear the infections. Central receptors in the early innate responses are so-called Toll-like receptors (TLRs) and are vital for the communication between the innate and adaptive branches (Rivera et al. 2016). The germline-encoded pattern recognition receptors (PRRs) are central in the recognition of microbial components and for the activation of innate immunity, which may induce inflammatory response to eliminate pathogens. The PRRs, expressed in innate immune cells, include receptors such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs). Upon recognition of microbial components known as pathogen-associated molecular patterns (PAMPs), PRRs
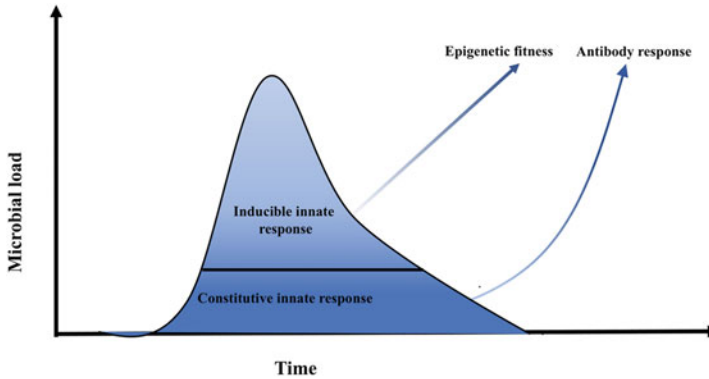
Fig. 2.1 Simplified illustration shows how inducible innate immunity changes over time, whereas the constitutive is stable vs. time. To get complete sterilization and resolution, both inducible and constitutive innate immune responses plus antibodies are often needed. Epigenetic changes may contribute to better fitness/increased protection when fish are exposed to a second infection. Targeted gene expression surveys or transcriptomics has focused primarily on describing or identifying inducible genes (e.g., DEGs), while in contrast, factors contributing to the constitutive arm have been poorly described
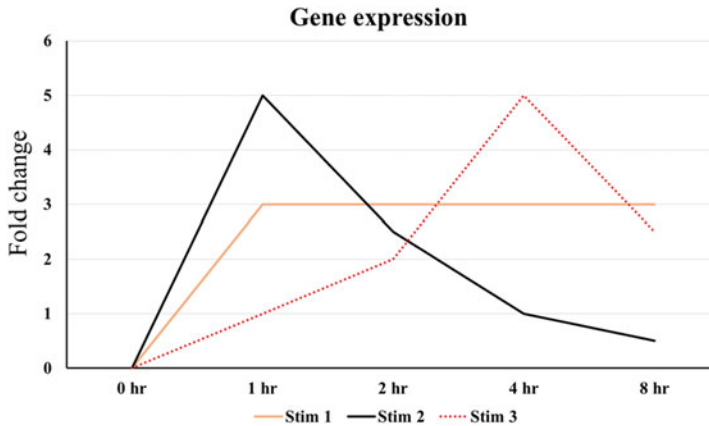
Fig. 2.2 Hypothetical time-course study of a gene (qPCR) expression. The orange line represents a gene with constitutive expression upon treatment with stimulant 1, and the black line demonstrates an early expression of the same gene after the fish were treated with stimulant 2, whereas the dotted red line represents a delayed expression of this gene after the fish were treated by stimulant 3. A specific gene may be induced by a certain stimulant and not by others, or there may be a stable, rapid, or delayed induction. The magnitude of induction may likely be dependent on number of specific receptors on cells or/and the number of cells that harbor specific receptors

induce intracellular signaling networks to activate transcription factors that regulate genes involved in inflammatory responses. Importantly, these innate immune signals also trigger dynamic chromatin changes. Such changes may in turn induce modulated gene-specific expression patterns resulting in even more pathogen elimination.

2.2 Cells in the Innate Immune Response

The traditional view that the adaptive and innate immune defense is divided into two compartments is now more or less history. The fish innate immune cells comprise not only the "traditional" innate cells such as macrophages (Kordon et al. 2018; Rieger and Barreda 2011; Grayfer et al. 2014, 2018) and granulocytes (Pijanowski et al. 2013; Schmidt 1905) but also red blood cells (Puente-Marin et al. 2018, 2019b; Dahle et al. 2015; Wessel et al. 2015), thrombocytes (Stosik et al. 2019), B cells (Wu et al. 2020), and subtypes of T cells (Scapigliati et al. 2018).

2.2.1 Monocytes/Macrophages

Monocytes are large mononuclear circulating leukocytes, which become macrophages when they settle tissues and organs. The nucleus may display different oval-, kidney-, or bean-shaped conformations, while the cytoplasm is usually pale and agranular, with varying amounts of vesicles and lysosomes. Macrophages are one of the main immune cells performing phagocytosis, the other presumably being the neutrophilic granulocytes. Phagocytosis is a multistage process for removal and cellular ingestion and destruction by intracellular enzymes and other substances (Grayfer et al. 2014; Hodgkinson et al. 2015). In addition to being professional phagocytes, macrophages can also function as professional APCs by presenting antigen to T cells on MHC class II. Such functions have also been suggested from studies on fish monocytes/macrophages (Sugamata et al. 2009; Wittamer et al. 2011). It has been suggested that fish display at least three different phenotypes of macrophages, based on their activation processes: innately, classically, and alternatively activated macrophages (Wentzel et al. 2020; Hodgkinson et al. 2015). Innate activation occurs when a macrophage receives a stimulus from the recognition of a microbial substance (e.g., PAMP) through cell receptor(s) without any need for any co-stimulation. Classical activation, however, occurs with the combination of such a stimulus and the cytokine interferon-gamma (IFN-gamma). Both innate activation and classical activation typically lead to increased pro-inflammatory response as opposed to alternative activation. A presence of cytokines (interleukin 4 (IL4) and/or interleukin 13 (IL13)) induces a macrophage phenotype with a resolving function (wound healing and tissue repair). There is also a suggestion for a fourth type of macrophages, namely regulatory macrophage. Regulatory activation is associated with the cytokine interleukin 10 (IL10) and important for downregulation of the inflammatory process (Wiegertjes et al.

2016). However, macrophages are apparently able to change between different phenotypes, and there is still some uncertainty whether all these activation pathways perform the same way in fish, as in mammals (Forlenza et al. 2011). Please see Chap. 6 for a more thorough overview of macrophages in fish.

2.2.2 Dendritic Cells

Dendritic cells (DCs) are categorized as a professional APCs found within several different tissues and are very effective at initiating both innate and adaptive immune responses in mammals (Banchereau et al. 2000; Worbs et al. 2017). DCs are typically small cells, with several elongated, cytoplasmic processes (dendrites) that increase the cell surface area (Collin and Bigley 2018). Cell populations with DC-like morphology and functions have been reported from teleost fish (Shao et al. 2015; Bassity and Clark 2012; Haugland et al. 2012), but due to lack of specific markers it is currently unknown whether they are true homologs of the mammalian cell type.

2.2.3 Granulocytes

Granulocytes are leukocytes with cytoplasmic granules and often nucleus with varying shapes (lobes) (Flerova and Balabanova 2013). They are central pool of the innate immune cells (Lieschke and Trede 2009). The granulocytes have traditionally been grouped into neutrophilic granulocytes (neutrophils), eosinophilic granulocytes (eosinophils), and basophilic granulocytes (basophils), based on their staining characteristics with different dyes. However, this classification was originally developed for use in mammalian hematology and does not appear to always correlate well with characteristics of fish granulocytes (Kelenyi and Nemeth 1969; Drzewina 1909; Rombout et al. 2005).

2.2.3.1 Neutrophils

Neutrophils typically possess nucleus with varying degrees of lobulation and contain granules that usually do not display marked affinity for staining with basic or acid dyes (such as hematoxylin and eosin). Neutrophils are generally most abundant between the granulocytes. In mammals, neutrophils are very mobile and are usually among the first cells to infiltrate tissue during onset and early phases of inflammation (Rosales 2018). Similar cellular recruitment speed has also been reported from teleosts (Lamas and Ellis 1994; Katzenback and Belosevic 2009; Havixbeck and Barreda 2015). Neutrophils are armed with a diverse arsenal of cellular weapons, making them effective combatants against invading pathogens (Havixbeck and Barreda 2015). Like macrophages, they are able to degrade ingested microbes and particles through production and release of reactive oxygen species (ROS) and proteases (Katzenback and Belosevic 2009; Rieger and Barreda 2011). They can also release different granules, upon degranulation, containing antimicrobial

proteins and enzymes such as myeloperoxidase (MPO) (Lieschke and Trede 2009). In addition, neutrophils are able to form extracellular traps, which contain antimicrobial factors (Palic et al. 2007; Pijanowski et al. 2013; Chi and Sun 2016; Zhao et al. 2017; Van et al. 2020).

Eosinophils, or acidophils, are described to contain cytoplasmic granules that stain bright red with the acidic dye (eosin). However, cellular identification based solely on cytochemical and/or histochemical staining characteristics may lead to misinterpretation as basophils, eosinophilic granule cells, mast cells, some neutrophils (also called heterophils), and rodlet cells also are capable to be dyed to various degrees. Consequently, it has been suggested that mammalian terminology should be used whenever possible for describing these cell types (Watanabe et al. 1997; Suljevic et al. 2017). In mammals, the eosinophils have immunological roles regarding both immune regulation, defense against parasitic infections, and allergic inflammatory reactions (Hogan et al. 2008). Teleost eosinophils have been reported to be phagocytic (Watson et al. 1963), and they increase in cell numbers and increase the degranulation activity as a response to infection (Balla et al. 2010).

2.2.3.2 Basophils

Basophils are large granulocytes with staining of their cytoplasmic granules with a basic dye (hematoxylin). Basophils are rarely observed in teleost species (Tavares-Dias 2006). Their granules contain histamine, an inflammatory mediator, and basophils are associated with anaphylaxis, allergy, and hypersensitivity reactions (Chirumbolo 2012). As such, they are similar to the mast cells. Although not fully established, these granulocytes might also have other functions within the fish immune system (Odaka et al. 2018).

2.2.3.3 Eosinophilic Granule Cells

Eosinophilic granule cells (EGCs) and rodlet cells have been observed in fish (Reite and Evensen 2006). Such cells resemble the classical mast cells (Reite 1998). Teleost EGCs have been identified in several species, as part of the host inflammatory response to injected vaccines, bacterial infection, parasite infestations, or other types of noxious stimuli (Rombout et al. 2011).

2.2.4 Thrombocytes

Thrombocytes are oval-shaped, nucleated, and agranular cells located in fish. In some fish species, thrombocytes have been shown to be phagocytic and it has been discussed whether thrombocytes can function as APCs and/or is coupled to the innate immunity (Stosik et al. 2019; Passantino et al. 2005).

2.2.5 Red Blood Cells

From a RNAseq study on trout red blood cells exposed to either poly I:C, it was found that the cells expressed numerous transcripts of immune molecules—such as *ifna*, *tlr3*, *tlr9*, *mx*, and *ccl4* (Morera et al. 2011). Thus, the authors suggested that red blood cells indeed participate in innate immune response.

2.3   Epigenetic Control of Innate Immunity

2.3.1   Epigenetics: The Concept

Epigenetics involves heritable factors that regulate spatiotemporal genome expression, which may induce different phenotypes. Two of the molecular mechanisms, histone modifications and DNA methylation, regulate gene expression at the chromatin level. In contrast, microRNAs are molecules that affect gene expression at the posttranslational level. Epigenetic histone modification involves acetylation/deacetylation, methylation/demethylation, and phosphorylation/dephosphorylation of specific histone amino acids. Pathogens have evolved a variety of strategies to modify host epigenetics. For example, they can (1) directly modify host proteins and chromatin, (2) attenuate PRR binding and signaling pathways, and (3) modulate the expression of activators and repressors of innate immunity. Hosts can abrogate pathogen-induced epigenetic changes to maintain their innate defense characters (Zhang and Cao 2019). Analysis of posttranslational processes on immunity has not generally been well studied in fish. However, in one study, the impact of histone modification after infectious necrosis virus infection (IPNV) and temperature control has been shown (Boltana et al. 2018). In this study, IPNV-infected fish that preferred a given temperature showed histone modification, which could explain modulated expression of *il1*, *il2*, *ifng*, and *ifnrg* receptor. The pattern of histone modification was different from IPNV-infected fish kept at constant temperature. In another study, spring viremia of carp virus (SVCV) infection induced histone modification in zebrafish (*Danio rerio*). The authors indicated that the *ifn*, *tlr*, and C-reactive protein promoters were methylated postinfection; thus, these genes were upregulated compared to controls (Medina-Gali et al. 2018). Since epigenetic modification of the genome is a heritable trait, epigenetic programming of brood stockfish, by, e.g., immunostimulants, may be a viable approach to produce offspring with higher innate disease resistance (Zhang et al. 2019).

2.3.2   Micro RNA

MicroRNAs (miRNAs) are a family of small noncoding RNAs that play vital roles in modulating host immune response. Accumulating evidence demonstrates that host

miRNAs are involved as mediators in regulating viral replication and host antiviral immunity in mammals. In a miiuy croaker macrophages, miR-3570 that was upregulated after rhabdovirus infection interfered and led to downregulation of type I interferon in the cells. In turn, this downregulation caused increased virus replication in cells (Xu et al. 2018a). Binding to Toll-like receptors (TLRs) and subsequent intracellular signaling may also bring about production of microRNA. This may result in a positive or negative feedback loop system regulating immune response. More on this complex issue is described in a review authored by Zhou et al. (2018).

2.3.3 Long Noncoding RNA

lncRNAs have been demonstrated to play pivotal roles in various biological processes, especially gene expression regulations, including transcriptional regulation, posttranscriptional control, and epigenetic processes. The functional significance of lncRNA lags far behind what is the status on mammals. However, a novel lncRNA (*SETD3-OT*) in turbot (*Scophthalmus maximus*) has been identified. From the annotation of neighboring adjacent genes, *SETD3-OT* might be involved in the regulation of cell apoptosis and cycle, the immune cell development, and the immune response against infection. The expression pattern of *SETD3-OT* was similar to the majority of the neighboring genes following *Aeromonas salmonicida* challenge. The *SETD3-OT* expression was high levels in mucosal surfaces in controls fish (intestine, gill, and skin), but was downregulated following *Vibrio anguillarum* infection (Yang et al. 2020). In another study, Nodavirus infected European sea bass (*Dicentrarchus labrax*) displayed many putative lncRNA, suggested to possibly be involved in immune responses (Pereiro et al. 2020). Other studies have also suggested lncRNA to be involved in the regulation of immune responses (Boltana et al. 2016; Valenzuela-Miranda and Gallardo-Escarate 2016; Paneru et al. 2016; Valenzuela-Munoz et al. 2018, 2019).

2.3.4 Small Interfering RNA and Circular RNA

In addition to microRNA and lncRNA, the methylation of mRNA, occurrence of small interfering RNAs, and circular RNAs may all contribute to epigenetic modulation of gene expression in vertebrates, including fish (Wang et al. 2018a). Olive flounder (*Paralichthys olivaceus*) experimentally infected with *Edwardsiella tarda* showed differentially expressed circRNA. The authors suggested that these belonged to the circRNA-miRNA-mRNA network, where KEGG analysis indicated that they were part of the *Herpes simplex* infection and intestinal immune network for IgA production (Xiu et al. 2019). Another study showed that circRNAs are involved in mammalian antiviral immunity (Wang et al. 2017). KEGG (Kyoto Encyclopedia of Genes and Genomes; www.genome.jp), a huge database integrating genomic, chemical, and systemic functional information, is often used

to find what cellular networks/pathways the DEGs belong to. It refers to what is described in and annotated from human/mice systems.

2.4 Mucosal Innate Defense

2.4.1 Innate Immune Molecules of the Fish Skin

The skin of fishes protects fish from external pathogens. The outermost layer is mainly composed of epithelial cells, termed keratocytes. These cells cover scales and are highly phagocytic toward certain particles. They are also motile. The motility of fish keratocytes is studied in a number of fish species (Asbakk and Dalmo 1998; Tsuchida and Theriot 2013; Galbraith and Sheetz 1998; Jurado et al. 2005; Okimura et al. 2018; Ream et al. 2003). There has been limited research on the production of innate defense factors, but this topic deserves more attention. Whether the cells possess phagocytic receptors is not known, it seems that the cells are able to discriminate the uptake dependent on the kind of bacteria (Karlsen et al. 2012). The skin mucus contains an array of molecules enabling protection from pathogens. In a study on yellow catfish (*Pelteobagrus fulvidraco*), 133 differentially expressed proteins were found after bath infection with *Edwardsiella ictaluri*. A minority of these differentially expressed proteins were directly immune-related. Examples of the upregulated genes were complement component *c3*, *MAP kinase 1*, and *interferon-induced 35 kDa protein* (Xiong et al. 2020).

Among the antibacterial enzymes, the best studied in the fish skin is lysozyme. Lysozyme is a glycoside hydrolase that catalyzes the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, which is the major component of gram-positive bacterial cell wall. However, it seems that lysozyme-like enzymes have activity also against gram-negatives, parasites, and virus, as reviewed by Dash et al. (2018). This review also contains detailed description of other skin-related innate immune factors (Dash et al. 2018).

How the mucus is obtained, for concomitant analysis of factors, will inevitably decide which substances will be found during a screening process. As an example: If the mucus sample contains cells or scales, it is clear that the samples also contain cellular factors and most probably also immune factors normally localized in deeper layers (e.g., connective tissue and muscle). The most gentle and sensible protocol is to adsorb the mucus using a tissue paper. While a wiping method using tissue paper also gives a good protein yield, this method comes with some degradations. If the research requires a high mucus yield together with substances from the epithelial layer, the wiping method is preferable (Faeste et al. 2020). It is quite difficult to discriminate between substances normally found in mucus compared to what is intracellularly or extracellularly localized in epidermis, subdermis, and

connective tissue. Thus, many reports describe the presence of substances not (only) found in the mucus itself but also found in the underlaying tissue. As an example of the latter, the transcriptomic analysis of a skin sample (3 × 1 cm) from large yellow croaker (*Larimichthys crocea*) followed by *Cryptocaryon irritans* challenge revealed up to 1055 DEGs (differentially expressed genes) (96 h postinfection). Since many of the DEGs were clearly innate immune-related, it would have been interesting to see how many and which transcripts were from epithelial cells, connective tissue cells, blood cells, and muscle cells, respectively. Probably, a similar sampling protocol was followed by Liu et al. (2020a, b) where zebrafish were challenged with spring viremia of carp virus (SVCV)—causing skin lesions. This study revealed 320 DEPs (differentially expressed proteins) (48 h postinfection) and 181 DEPs (96 h postinfection). Sixteen of these were confirmed by means of QPCR analysis (Bai et al. 2020). DEPs often found are complement factors, and chemokines, heat shock proteins, MHC, cell adhesion molecules, TNF-induced protein, and many more were regulated (Liu et al. 2020b). In conclusion, analysis of skin innate defense mechanisms should discriminate between mucus itself, epidermis, subdermis, and connective tissue.

The epidermis consists of keratocytes, which are highly mobile cells and also possess (mostly overlooked) phagocytic activity (Asbakk and Dalmo 1998; Sveen et al. 2020). The immunological significance of their phagocytic ability is not yet fully understood. One theory is that they engulf as many particles they can before going into a cell death pathway and are sloughed off from the epidermis (Asbakk and Dalmo 1998). It is speculated that these cells possess some innate defense mechanisms (e.g., receptors) (Lindell et al. 2012). The epithelial layer of the fisheye cornea consists of cells that highly resemble skin keratocytes. These cells are not studied with respect to their innate defense abilities. We have preliminary results showing that these cells also engulf foreign particles (Fig. 2.3). For more details on mucosal immunity in fish, see Chap. 12.

Fig. 2.3 Corneal epithelial cells of Atlantic salmon (*Salmo salar*) possess phagocytic ability, as illustrated by the intracellular presence of (cyan) microbeads. Lysosome is stained by pink color. Courtesy: Dalmo, Wolfson, Kjølstad, Svartaas (UiT)
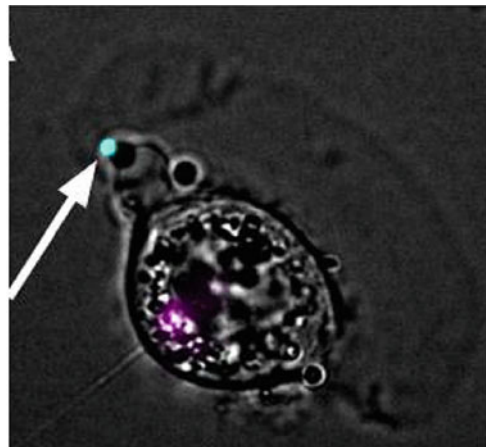
2.4.2 Nasopharynx-Associated Lymphoid Tissue (NALT)

NALT has been discovered to harbor lymphocytes, but also genes central in induction of innate immunity. These include *mx1*, *tlr3*, *il1r*, *il8*, *tnfr*, *myD88*, *c3*, *c4*, *c7–1*, *cxc9*, *cxcl9*, *cathl1*, *ccl19*, and *il6* (Tacchi et al. 2014; Yu et al. 2018). The significance of NALT-mediated innate response, compared to, e.g., skin or intestine, is not clear. Another assemblance of lymphoid cells can be found in the buccal cavity of rainbow trout (*Oncorhynchus mykiss*) that have been infected by *Flavobacterium columnare*. After infection, this buccal cavity lymphoid tissue was found to express innate factors such as *il8*, *il1b*, *chemokine like 19*, *cathl1* and *cathl2*, *rig1*, among other adaptive immune genes (Xu et al. 2020).

2.4.3 Gills in Innate Immunity

Gill-associated lymphoid tissue (GIALT) has been characterized in Atlantic salmon and different fish species (Resseguier et al. 2020; Haugarvoll et al. 2008). This tissue was, in Atlantic salmon, identified to express upregulated genes such as complement component *c3*, *il18*, *mx3*, *il20*, *ifn type II*, *viperin*, *rig1*, and *ifna* after ISAV challenge (Austbo et al. 2014; Valenzuela-Miranda et al. 2015). Pro-inflammatory (*il6*, *il17c1*) and anti-inflammatory (*il10*, *tgfb*) genes have been found, in rainbow trout gills, after Ich (*Ichthyophthirius multifiliis*) infection (Syahputra et al. 2019). Another study aimed at doing a transcriptomic survey of Atlantic salmon gills suffering from multifactorial pathologies. Genes that were differentially expressed were depicted to be involved in pathways such as cellular immune response (IL-17 signaling, IL-6 signaling, granzyme A signaling, crosstalk between dendritic cells and natural killer cells, granulocyte adhesion and diapedesis, and HMGB1 signaling), cytokine signaling (IL-17 signaling, IL-6 signaling, acute-phase response signaling, role of JAK family kinases in IL-6 type cytokine signaling, TNFR2 signaling, and HMGB1 signaling), and tissue damage and repair (Krol et al. 2020). Some of these genes possess central functions in innate immunity. More details on the gills' function in the immune response, please see Chap. 1.

2.4.4 Intestine in Innate Immunity

During the recent years, many excellent review articles describing the fish's intestinal immunity have been published (Dawood 2020; Nadal et al. 2020; Sitja-Bobadilla et al. 2016; Brugman 2016; Dezfuli et al. 2016; Scapigliati et al. 2018; Brinchmann et al. 2018). Recently, there have been many innovative approaches to better understand intestinal immunity. In one of these studies, proteomic and transcriptomic examination of *the intestinal mucus* in Tilapia infected with *Streptococcus agalactiae* showed that innate factors such as *c1r-like EGF domain*, *c1q-binding protein*, *hsp1*, *hsp90b*, *galectin*, and

membrane attack complex component/perforin domain, *conserved site*, *complement factor D*, *C-type lectin fold*, *il1*, *il1r*, and *foxp3* (Wu et al. 2016). Another study, in grass carp, did a transciptomic and proteomic examination of the intestine after oral DNA vaccination (Li et al. 2020). The study revealed 250 and 50 immune-related DEGs and DEPs, respectively, after the oral vaccination. KEGG enrichment analysis showed genes and proteins participating in the Toll-like receptor signaling pathway, MAPK signaling pathway, NOD-like receptor signaling pathway, and the complement cascade were present both in the mucous and tissue homogenates. It is obvious that the intestinal innate mechanisms are quite diverse. More research using modern omic technologies will inevitably give us more information about the significance of the various intestinal innate factors that have on disease resistance.

Recently, a lymphoid structure in the cloacal region was discovered in Atlantic salmon (Loken et al. 2020). This may be the same as Inami et al. (2009) described in Atlantic cod—although the work on cod did not specify the anatomical localization properly (Inami et al. 2009). Whether this collection of lymphatic cells has any function in innate defense is not clear. However, through gene expression studies, genes encoding *il1b*, *il8*, *il10*, *hepcidin*, and *ccl19* were found. These genes likely play roles in the innate immunity.

Future studies of intestinal mucus must be carefully planned and executed to avoid contaminant cells and blood. This may give false assumptions with regard to the actual presence of innate factors.

2.5 Innate Defense Mechanisms in Muscle Tissues

Skeletal muscles have been implicated in several atypical physiological processes including immune response, especially after pathogen challenge. When zebrafish were intramuscularly challenged by *Salmonella enterica*, pro-inflammatory *il1b* and *tnfa* were highly expressed in their skeletal muscle. Likewise, *hep* (hepcidin) and *il10* were also expressed (Chatterjee et al. 2016). The authors did not examine any presence of leukocytes in muscle tissue samples after the fish were challenged with *Salmonella*. Thus, it is likely that inflammatory leukocytes did contribute to the expression of the pro-inflammatory cytokines. The contribution of incoming leukocytes to the inflammatory event is discussed by Valenzuela et al. (2017) and Kaitetzidou et al. (2012). Similar to the induction of innate immune genes in skeletal muscle, Atlantic salmon infected with salmonid alphavirus and piscine reovirus showed altered gene expression in the heart tissue (Johansen et al. 2015). In this study, several innate genes were expressed in the heart muscle. The authors did not elaborate whether this contribution was caused by inflammatory cells or not.

2.6   Innate Defense Mechanisms in Kidney and Spleen

The kidney and spleen are hematopoietic organs capable of inducing and exerting innate immune responses (Uribe et al. 2011; Svingerud et al. 2012; Kumar et al. 2018). The head kidney is the principal immune organ responsible for phagocytosis, antigen processing, and formation of Igm and immune memory (Page et al. 2013; Stosik et al. 2018; Rauta et al. 2012; Rombout et al. 2005; Kim et al. 2017). Kidneys in fish are paired and have a Y shape along the body axis. The immune relevant part, the head kidney, is located anteriorly. The posterior is mostly the renal system. The form of the head kidney varies between species. In some species, there are two separate extensions in the most anterior part of the organ, while in salmonid species the kidney is present as a single organ (Press and Evensen 1999). It is acknowledged that the head kidneys' main function is hematopoiesis of lymphocytes, phagocytosis, antigen presentation, and maturation of lymphocytes. Its significance in innate immunity is not very well researched yet, although the head kidney leukocytes are armed with innate factors (Aballai et al. 2017; Gerdol et al. 2015; Cao et al. 2020; Hwang et al. 2017; Rozas-Serri et al. 2019; Zhou et al. 2019). It should be clear that cells in the posterior part of the kidney also have capability to express immune genes after pathogen challenge, as reported by Sudhagar et al. (2019).

2.7   Innate Defense Mechanisms in the Spleen

It is acknowledged that the main functions of the spleen are in hematopoiesis of lymphocytes, antigen trapping, and destruction of red blood cells (Press and Evensen 1999). However, from RNAseq analysis it is evident that the spleen cells contain and express numerous innate immune genes such as those involved in chemokine signaling, Toll-like receptor signaling, RIG-1, and NOD-mediated signaling and complement cascade (Ali et al. 2014).

2.8   Innate Defense Mechanisms in the Liver

The liver is acknowledged to produce acute-phase proteins, including complement components following infection or physiological insult. An array of innate defense factors has been found following a transcriptomic study of rainbow trout. This study revealed transcripts coding for genes important in acute-phase response, inflammatory response, genes coding for PAMP-binding receptors, and molecules central in chemotaxis (Martin et al. 2010). This finding suggests that the liver also has capacity to mount innate responses.

2.9   Receptors and Molecules of the Innate Immune Defense

Innate immunity is orchestrated by numerous molecules such as cytokines, complement factor, and receptors. Many molecules participate in both innate immunity and adaptive immunity. The following chapters describe the roles of selected innate molecules that have been ascribed to innate immunity—as central components.

2.9.1   Toll-Like Receptors (TLRs) as Pattern Recognition Receptors (PPRs)

The number of TLRs adds to other pattern recognition receptors (Tribouley et al. 1978) (including splice variants) such as different C-type lectin receptors, NOD-like (nucleotide-binding oligomerization domain-like) receptors (NLRs), RIG-1-like receptors, and scavenger receptors (Brubaker et al. 2015), and suggests that fish may very well be equipped with innate receptors that may likely be targets for innate immune training. TLRs are a family of pattern recognition receptors that bind pathogen-associated molecular patterns (PAMPs) (Pietretti & Wiegertjes, 2014). In addition, several TLRs are able to bind certain endogenous molecules called damage-associated molecular patterns (DAMPs) (following, e.g., trauma). TLRs are highly important since they represent a considerable diversity in their ligand-binding properties and thus facilitate responses against a wide array of pathogens. Genome duplication events in fishes during evolution have been attributed to the diversity of TLRs; therefore, differences with respect to the number of TLR loci exist between mammalian species and many fish species (Palti 2011). As an example: The genome of a mudskipper species (*Periophthalmodon schlosseri*) contains 11 copies of *tlr13* (You et al. 2014). Most vertebrate genomes are recognized to have at least one gene representing each of the seven major *tlr1*, *tlr2*, *tlr3*, *tlr4*, *tlr5*, *tlr7* and *tlr11* families (Roach et al. 2005). Within Osteichthyes, the large *tlr1* subfamily members include *tlr1*, *tlr2*, *tlr14*, *tlr18*, *tlr25*, *tlr27*, and *tlr28* (Nie et al. 2018). The *tlr3*, *tlr4*, and *tlr5* subfamilies recognize dsRNA, LPS, and bacterial flagellin. The *tlr7* subfamily ligands are nucleic acid motifs, whereas the *tlr11* family TLRs recognize an array of different molecules—from proteins to nucleic acids—reviewed by Nie et al. (2018). The ligand specificities for each TLR have not been very well studied in fish, though flagellin, synthetic triacetylated lipopeptide (Pam3CSK4), lipopeptides from gram-positive bacteria, and short double-stranded RNA (dsRNA) have been shown to interact with/bind to tlr1/2, tlr5, and tlr22, respectively (Nie et al. 2018). This means that fish immunologists assume that tlr localization and ligand specificities of fish tlrs are similar to mammalian counterparts. This is reviewed by Pietretti et al. (2014) and Kanwal et al. (2014). Tlrs are, in mice and humans, localized in the cell membrane and in the endoplasmic reticulum (ER), endosomes, and lysosomes (Fink et al. 2016). Tlr receptors 1, 2, 6, and 10 have, in human or mice models, been found to recognize a broad range of peptidoglycans and lipoproteins from, e.g., bacteria and parasites. These are located on the cell surface and, following engagement, there is intracellular signaling ending in NF-kB-dependent gene expression. NF-kB promotes expression of

pro-inflammatory cytokines. Viral recognition may be brought about by tlr3, tlr7, tlr8, and tlr9 where they potentially can bind dsRNA, single-stranded RNA (ssRNA), and CpG DNA. As found in zebrafish, the tlr22 may also bind dsRNA or poly I:C (a dsRNA mimic) (Li et al. 2017b) [Fitzgerald, 2020 #954]. These "antiviral receptors," upon ligand binding, confer (via TRIF or/and MyD88) activation of interferon regulatory factors 3 and 7 (transcription factors), which in turn facilitates transcription of interferon type I expression.

Taken together, the common interpretation is that TLR activation results in the production of pro-inflammatory cytokines (e.g., tnfa and IL-1ß) and/or in the expression and synthesis of transcription factors involved in protection against viruses, bacteria, and parasites (Sahoo 2020; Kanwal et al. 2014; Rauta et al. 2014; Zhang and Gui 2012; Palti 2011).

Tables 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 2.10, 2.11, 2.12, 2.13, 2.14, 2.15, 2.16, 2.17, 2.18, 2.19, 2.20, 2.21, and 2.22 give an updated overview of *tlrs 1–5*, *7–9*, *12–14*, and *18–28* found in different fish species. This list is continuously growing as genome, and transcriptome sequences from new species are completed and analyzed. This will be done during the "Fish10K" project where the aim is to genome sequence 10,000 fish species during a ten-year period (Fan et al. 2020), and through the ongoing "Fish1K" project (Sun et al. 2016b) and Earth Biogenome Project (Lewin et al. 2018).

2.9.2 Interferon Type I

Interferons (IFNs) are a group of cytokines with important roles in defense against viral pathogens (cf. Chaps. 13 and 14). They are divided into two families, type I and type II, based on structural properties and functions. Both the type I and II IFN systems are essential to antiviral defense in innate and adaptive immunity (Zou and Secombes 2011) (Tables 2.23 and 2.24). In contrast to type I IFNs, which are more important in innate immunity, IFN-γ (type II IFN) is exclusively produced in immune-related cells and is more important later in the immune response. In innate immune responses, IFN-γ is produced by natural killer cells (Jung et al. 2012). During adaptive cell-mediated immune responses, IFN-γ is produced by CD4-positive Th1 cells and CD8-positive cytotoxic T lymphocytes. IFNs induce the expression of a broad array of IFN-stimulated genes (isgs), which encode for proteins with direct antiviral activity, including inhibition of viral transcription, degradation of viral RNA, inhibition of translation, or modification of protein function. Several reviews of the interferon system of teleost fish have been presented over the years (Robertsen 2006; Workenhe et al. 2010; Zou and Secombes 2011; Secombes and Zou 2017). Chaves-Pozo and coworkers investigated the interferon response in the ovary of rainbow trout (*O. mykiss*). They found that the VHS virus strongly upregulated all the *ifn* genes studied, while the IPN virus either had no effect or strongly suppressed *ifn* gene expression (Chaves-Pozo et al. 2010). Valero and coworkers investigated *ifns* in the gonads of gilthead sea bream (*Sparus aurata*) and European sea bass (*D. labrax*). They evaluated the expression after infection with the disease viral nervous necrosis (VNN) in the brain

Table 2.1 Updated list of *tlr1* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr3 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. Tlr1 can associate with tlr2 and tlr6 to expand their ligand-binding specificities. In general, tlr1 may bind bacterial peptidoglycan and triacyl and diacyl peptides. *ND* not determined

TLR no	Species	Tissue expression	References
1	Common carp	Muscle, gills, skin, and others	Gong et al. (2017)
1	Common carp	Peripheral blood leukocytes, mid-kidney, spleen, and others	Fink et al. (2016)
1	Pufferfish	Heart, spleen, anterior kidney, and others	Oshiumi et al. (2003)
1	Pufferfish	Expressed in kidney	Oshiumi et al. (2003)
1	Channel catfish	Anterior kidney	Quiniou et al. (2013)
1	Channel catfish	Spleen, anterior kidney, kidney, gills, and others	Zhang et al. (2013a)
1	Rainbow trout	Spleen, anterior kidney	Palti et al. (2010b)
1	Atlantic salmon	ND	Salazar et al. (2016)
1	Brown trout	ND	Sudhagar et al. (2020)
1	Orange-spotted grouper	Anterior kidney, spleen, gills, skin, brain	Wei et al. (2011)
1	Large yellow croaker	Blood, spleen, heart, liver, kidney	Wang et al. (2013a)
1	Nile tilapia	Kidney, brain, spleen, intestine, muscle + other	Abouelmaatti et al. (2020)
1	Golden pompano	Skin, anterior kidney	Wu et al. (2018)
1	Sea perch	Anterior kidney, intestine, liver, spleen, heart	Li et al. (2018a)
1	Yellow catfish	Midgut, brain, foregut, head kidney, spleen, and other	Zhang et al. (2017b)
1	Blunt snout bream	ND	Lai et al. (2017b)
1	Miiuy croaker	Liver, head kidney, and other	Xu et al. (2016)
1	Grass carp	Spleen, mid-kidney, anterior kidney, and other	He et al. (2016)
1	Rock bream	Spleen, kidney, liver, and others	Elvitigala et al. (2015)
1.1	Zebrafish	Skin, liver, blood, spleen, testis, and others	Jault et al. (2004)
1	Tibetan fish (Przewalski's carp)	ND	Tong et al. (2015)
1	Yellow River scaleless carp	ND	Qi et al. (2017)
1–1 1–2	Spotted sea bass	Details not given	Fan et al. (2019)
1	Maraena whitefish	Anterior kidney, kidney, and others	Altmann et al. (2016)

Table 2.2 Updated list of *tlr2* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. *ND* not determined

TLR no	Species	Tissue expression	References
2–1	Common carp	Gills, spleen, and others	Gong et al. (2017)
2–2	Common carp	Spleen, kidney, and others	Gong et al. (2017)
2a	Common carp	Peripheral blood leukocytes, gut, anterior kidney, and other	Fink et al. (2016)
2b	Common carp	Heart and other	Fink et al. (2016)
2	Japanese flounder	Peripheral blood leukocytes	Hirono et al. (2004)
2	Pufferfish	Expressed in many tissues	Oshiumi et al. (2003)
2	Channel catfish	Anterior kidney, gills, spleen	Quiniou et al. (2013)
2	Channel catfish	Liver, brain, gills, trunk kidney, intestine, and others	Baoprasertkul et al. (2007a)
2	Channel catfish	Anterior kidney, spleen, heart, kidney, brain, and others	Baoprasertkul et al. (2007a)
2	Rainbow trout	Spleen, anterior kidney, thymus, macrophages, B cells, thrombocytes	Brietzke et al. (2016)
2	Orange-spotted grouper	Spleen, gills, anterior kidney	Wei et al. (2011)
2	Large yellow croaker	Blood, spleen, heart, anterior kidney, posterior kidney	Fan et al. (2015)
2b	Large yellow croaker	Intestine, blood, skin, liver	Ao et al. (2016)
2	Indian major carp, rohu	Spleen, intestine, gills, liver, blood	Samanta et al. (2012)
2	Darby's sturgeon	Blood, anterior kidney, brain, heart, muscle	Tang et al. (2020)
2	Golden pompano	Liver, intestine, blood, skin, anterior kidney	Wu et al. (2018)
2	Yellow catfish	Spleen, anterior kidney, foregut, gills, liver, and other	Zhang et al. (2017b)
2	Grass carp	Mid-kidney, anterior kidney, and other	He et al. (2016)
2	Zebrafish	Brain, liver, skin, spleen	Jault et al. 2004)
2	Gibel carp	Brain, gills, anterior kidney, and others	Fan et al. (2018)
2	Turbot	Kidney, liver, spleen, anterior kidney, and others	Zhang et al. (2016)
2	Miiuy croaker	Eye, gills, spleen, intestine, and others	Xu et al. (2013)
2	Indian major carp, mrigal	Gills, liver, kidney, intestine, blood	Basu et al. (2012b)
2	Catfish	Liver, brain, gills, trunk kidney, intestine, stomach, and others	Baoprasertkul et al. (2007a)

Table 2.2 (continued)

TLR no	Species	Tissue expression	References
2	Tibetan fish (Przewalski's carp)	Details not given	Tong et al. (2015)
2	Yellow River scaleless carp	ND	Qi et al. (2017)
2–1 2–2	Spotted sea bass	ND	(Fan et al. (2019)
2	Maraena whitefish	Anterior kidney, kidney, and others	Altmann et al. (2016)
2	Goldfish	ND	Tu et al. (2016)

(Valero et al. 2015). The orange-spotted grouper, *Epinephelus coioides*, is a commercially important fish that is widely farmed in tropical waters, e.g., in Taiwan, Japan, Australia, and also Europe. Chen et al. characterized a type *I ifn* from this fish and determined the expression during nodavirus infection. Groupers infected with nodavirus had elevated levels of *ifn* and administration of recombinant IFN type I, which led to upregulated antiviral activity (Chen et al. 2014). In large yellow croaker (*L. crocea*), a type I group II interferon was identified by Ding and coworkers. The *ifn* was constitutively expressed in all examined tissues, spleen, liver, skin, head kidney, gills, blood, muscle, heart, brain, and intestine. The expression was rapidly upregulated in spleen and head kidney by poly I:C and *Aeromonas hydrophila* (Ding et al. 2019). A type I interferon gene was identified in Japanese eel (*Anguilla japonica*). The *ifn* was expressed constitutively in liver, spleen, intestine, gills, skin, kidney, heart, and muscle. After injection with LPS, poly I:C, and live *A. hydrophila*, expression levels increased in both liver, spleen, and kidney (Feng et al. 2017). A transgenic cell line for the detection of salmon interferons has been established. It is based on a CHSE-214 cell line containing a reporter construct expressing firefly luciferase under the control of a rainbow trout promoter for the IFN-induced *mx1* gene. The *mx* promoter was shown to respond to both salmon IFN type I and trout IFN type II in a dose-dependent manner, while there was no response to recombinant tnfa and ilb (Jorgensen et al. 2007). Three distinct members of type I interferons were identified in the mandarin fish (*Siniperca chuatsi*) by Laghari et al. Fish injected intraperitoneally with poly I:C resulted in an enhanced expression of all three genes in the head kidney. The disease infectious spleen and kidney necrosis virus (ISKNV) caused an increased but delayed response of *ifns* (Laghari et al. 2018). Liu and coworkers studied ifn subgroups of salmonid species like rainbow trout (*O. mykiss*), chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), Atlantis salmon (*Salmo salar*), and Arctic charr (*Salvelinus alpinus*) and compared them with other species. The analysis confirmed that salmonids have a complex (in terms of ifn subgroups present) and (large number of genes) *type I ifn* repertoire relative to other teleost fish (Liu et al. 2020a). Milne et al. studied three

Table 2.3 Updated list of *tlr3* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr3 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. Tlr3 is able to bind double-stranded RNA, which is a molecular signature of certain viruses. ND not determined

TLR no	Species	Tissue expression	References
3	Pufferfish	Digestive tract	Oshiumi et al. (2003)
3	Silvery pomfret	Liver, kidney, intestine	Gao et al. (2020)
3	Common carp	Intestine, liver, kidney, spleen, and others	Yang and Su (2010)
3–1	Common carp	Spleen, skin, blood, and other	Gong et al. (2017)
3–2	Common carp	Gills, skin, blood, and others	
3	Yellow catfish	Liver, brain, swim bladder, and other	Zhang et al. (2017b)
3	Zebrafish	Skin, liver, blood, spleen, and other	Jault et al. (2004)
3	Gibel carp	Liver, gills, anterior kidney, brain, and others	Fan et al. (2018)
3	Sea perch	Spleen, liver, anterior kidney, and others	Wang et al. (2018b)
3	Spiny eel	Spleen, gills, and others	Han et al. (2017)
3	Snow trout	Kidney, liver, spleen, intestine, and others	Belwal et al. (2017)
3	Atlantic salmon	Spleen, anterior kidney, liver, heart, intestine	Vidal et al. (2015)
3	Orange-spotted grouper	Liver, anterior kidney, and others	Lin et al. (2013)
3	Rohu	Spleen, eye, intestine, blood, skin, and others	Samanta et al. (2013)
3	Japanese flounder	Anterior kidney, heart, gills, and others	Hwang et al. (2012)
3	Yellow croaker	Liver, intestine, heart, kidney, gills, and others	Huang et al. (2011)
3	Turbot	Anterior kidney, stomach, intestine, heart, and others	Hu et al. (2015a)
3	Channel catfish	Liver, muscle, spleen, and others	Baoprasertkul et al. (2006)
3	Channel catfish	Expressed in many tissues and organs, minor in blood	Zhang et al. (2013a)
3	Channel catfish	Liver, gills, spleen, stomach, and others	Bilodeau and Waldbieser (2005)
3	Rainbow trout	Liver, intestine, pyloric caeca, posterior and anterior kidneys, and others	Rodriguez et al. (2005)
3	Zebrafish	Gills, spleen, kidney, heart, brain, liver	Phelan et al. (2005)
3	Tibetan fish (Przewalski's carp)	Details not given	Tong et al. (2015)
3	Yellow River scaleless carp	ND	Qi et al. (2017)
3	Spotted sea bass	Details not given	Fan et al. (2019)

(continued)

Table 2.3 (continued)

TLR no	Species	Tissue expression	References
3	Maraena whitefish	Liver, heart, kidney, skin, gills, and others	Altmann et al. (2016)
3	Atlantic cod	Details not given	Solbakken et al. (2016)
3	Grass carp	ND	Liao et al. (2017)
3	Goldfish	ND	Tu et al. (2016)
3	Lamprey	Skin, gills, peripheral blood leukocytes, kidney, and others	Kasamatsu et al. (2010)

distinct type I interferons in meagre (*Argyrosomus regius*), namely *ifnc*, *ifnd*, and *ifnh*. Constitutive expression was analyzed during larval development and in adult tissues (gills, midgut, head kidney, spleen). The spleen had high transcript levels of all three *ifns*. *Ifnd* and *ifnh* were also highly expressed in gills. The expression of each subgroup increased significantly across all four tissues following injection of poly I:C (Milne et al. 2018). In Atlantic salmon, Sun et al. identified an *ifn* multigene cluster encoding three *ifn* subtypes (*ifna*, *ifnb*, and *ifnc*). Each *ifn* subtype was constitutively expressed in head kidney. The three subtypes showed a striking difference in expression properties in response to stimulation with poly I:C. Both *ifna* and *ifnc* transcripts increased, while *ifnb* was only slightly induced by poly I:C (Sun et al. 2009). Type I interferon genes were cloned and characterized in the rock bream (*Oplegnathus fasciatus*). Their expression was upregulated in blood cells and head kidney by LPS, poly I:C, *E. tarda*, *Streptococcus iniae*, and iridovirus, and recombinant ifn I protein induced a rapid and transient expression of the *mx* gene in head kidney cells (Wan et al. 2012). An overview of type I ifns in different fish species with their activities is found in Table 2.23.

2.9.3 Interferon Type II

Interferon-gamma (ifng), the only type II interferon, is a pleiotropic pro-inflammatory and antiviral cytokine. In mammals, it is constitutively produced by NK cells, whereas T lymphocytes produce IFNG after activation or differentiation. IFNG is a key cytokine for innate and adaptive immunity against viral and intracellular bacterial infections and is involved in tumor control. An updated teleost interferon-gamma review has recently been published (Pereiro et al. 2019). Arts and coworkers made recombinant proteins of the carp (*Cyprinus carpio*) IFN-γ sequences of both clusters (*ifng1* and *ifng2*) and tested their effects on expression of pro-inflammatory mediators (Arts et al. 2010). An interferon-responsive stable cell line RTG-3F7 has been developed for rainbow trout by modifying the RTG-2 cell line through transfection with a plasmid construct containing a promoter

Table 2.4 A list of *tlr4* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr4 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. TLR4 binds the lipid moiety of bacterial lipopolysaccharide. *ND* not determined

TLR no	Species	Tissue expression	References
4all	Channel catfish	Gonads, gills	Quiniou et al. (2013)
4mb	Channel catfish	Gills, liver, spleen	
4	Channel catfish	Intestine, gills, and others	Zhang et al. (2013a)
4–1	Common carp	Intestine, gills, brain, and other	Gong et al. (2017)
4–2	Common carp	Intestine, spleen, gills, skin, blood, and others	
4.1	Grass carp	Heart, blood, liver, and others	Pei et al. (2015)
4.2	Grass carp	Skin, intestine, liver, spleen, and others	
4.3	Grass carp	Gills, skin, and others	
4.4	Grass carp	Heart, skin, and others	
4.1	Grass carp	Spleen, intestine, kidney, and others	Huang et al. (2012)
4–2	Grass carp	Spleen, intestine, kidney, and others	
4–3	Grass carp	Spleen, intestine, kidney, and others	
4–4	Grass carp	Spleen, kidney, anterior kidney, gills, and others	
4–1	Yellow catfish	Liver, anterior kidney, gills, brain, trunk kidney, heart, and other	Zhang et al. (2017b)
4.1	Zebrafish	Blood, skin, testis, digestive organ, brain, liver, heart, and other	Jault et al. (2004)
4.2	Zebrafish	Blood	
4	Rohu	Blood, spleen, gills, kidney, and others	Samanta et al. (2017)
4	Blunt snout	Kidney, muscle, heart, liver, and others	Tang et al. (2016)
4	Blunt snout	Blood, skin, heart, spleen, gills, and others	Lai et al. (2016)
4	Tibetan fish (Przewalski's carp)	Details not given	Tong et al. (2015)
4	Yellow River scaleless carp	ND	Qi et al. (2017)

element from the *IFN-γ* responsive gene *TAP2* linked to a luciferase reporter gene and a hygromycin resistance gene. The results indicate that the stable cell line RTG-3F7 is an excellent tool for monitoring the presence of trout ifng in biological samples (Castro et al. 2010). The large yellow croaker (*L. crocea*) is an important mariculture fish species in China, and the bacterium *Vibrio harveyi* and the ciliate protozoan *C. irritans* are the two major pathogens of this species. The nucleotide sequence of *ifng* was obtained, and expression studies were performed. Fish were challenged with *V. harveyi* and *C. irritans*,

Table 2.5 List of *tlr5* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr5 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. Bacterial flagellin is the ligand for TLR5. *ND* not determined, *S* soluble, *m* membrane

TLR no	Species	Tissue expression	References
5 s	Pufferfish	Reproductive organ, heart, gills, brain, skin, eye, liver, spleen	Oshiumi et al. (2003)
5		Digestive tract, reproductive organ, liver,	
5all	Channel catfish	Anterior kidney, liver, gonads, posterior kidney	Quiniou et al. (2013)
5 mb		Anterior kidney, liver, gonads, posterior kidney	
5–1 and 5–2	Channel catfish	Expressed at low levels in many organs and tissues	Zhang et al. (2013a)
5 s		Liver, anterior kidney, kidney, and others	
5	Channel catfish	Ovary, liver, anterior kidney, skin, and others	Baoprasertkul et al. (2007b)
5	Channel catfish	Liver, kidney, and others	Bilodeau and Waldbieser (2005)
5	Golden pompano	Intestine, liver, kidney, blood	Zhu et al. (2020)
5 s		Blood, kidney, spleen, skin, intestine	
5	Common carp	Muscle, spleen, and other	Gong et al. (2017)
5	Yellow catfish	Liver, gills, blood, trunk kidney, and other	Zhang et al. (2017b)
5a	Zebrafish	Low expression in digestive organ, negligible in others	Jault et al. (2004)
5	Spiny eel	Liver, eye, gills, muscle, fins, and others	Han et al. (2017)
5a	Yellow River scaleless carp	ND	Qi et al. (2017)
5 m	Tibetan fish (Przewalski's carp)	Details not given	Tong et al. (2015)
5.1	Ya-fish	Liver, spleen, anterior kidney, and others	Du et al. (2019a)
5.2		Liver, spleen, and others	
5	Spotted sea bass	Details not given	Fan et al. (2019)
5	Silver pomfret	Liver, kidney, spleen, intestine, and others	Gao et al. (2018)
5 s	Miiuy croaker	Liver, kidney, skin, and others	Huo et al. (2018)
5	Dark barbel fish	Anterior kidney, spleen, and others	Qin et al. (2018)
5 s		Anterior kidney, spleen, and others	

Table 2.5 (continued)

TLR no	Species	Tissue expression	References
5	Pacific red snapper	Intestine, liver, anterior kidney, brain, muscle, and others	Reyes-Becerril et al. (2017)
5	Triploid crucian carp	Kidney, liver, anterior kidney, heart, and others	Zhang et al. (2017a)
5 s	Striped catfish	Anterior kidney, kidney, liver, spleen, and others	Jayaramu et al. (2017)
5	Orange-spotted grouper	Skin, anterior kidney, spleen, kidney, and others	Bai et al. (2017)
5 s	Orange-spotted grouper	Liver, spleen, and others	
5	Maraena whitefish	Liver, fins, heart, skin, and others	Altmann et al. (2016)
5 s		Liver and others	
5a	Grass carp	Anterior kidney, kidney, muscle, and others	Jiang et al. (2015)
5b		Anterior kidney, kidney, heart, gills, and others	
5 s	Gilthead sea bream	Liver, peritoneal exudate, blood, spleen, thymus	Munoz et al. (2013)
5	Indian major carp, mrigal	Liver, skin, muscle, and others	Basu et al. (2012a)
5	Japanese flounder	Kidney, liver, heart, gills, and others	Hwang et al. (2010)
5a	Blunt snout bream	Liver, kidney, and others	Zhan et al. (2019)
5b		Spleen, kidney, heart, and others	
5	Atlantic salmon	ND	Salazar et al. (2016)
5 s			
5	Lamprey	Peripheral blood leukocytes, skin, gills, and others	Kasamatsu et al. (2010)

respectively. One day after injection with *V. harvey*, all 10 tissues investigated had a higher expression of *ifng*, while only spleen, muscle, intestine, heart, and skin had higher expression after infection with *C. irritants* (Chen et al. 2015). Jung and coworkers produced a recombinant ifng (rifng) from the olive flounder (*P. olivaceus*). Stimulation of kidney leukocytes in vitro with rinfg induced the gene expression of *il1b*, *signal transducer and activator of transcription 1* (*stat1*), *CXCL-13*-like chemokine (*cxcl13*), and ifng. Intraperitoneal injection of a mixture of rifng and *E. tarda* into olive flounder resulted in a survival rate of 60% compared to 0% in the group treated with *E. tarda* only (Jung et al. 2012).

The extensive use of paraquat (PQ) in agricultural practice throughout the world may compromise the integrity of biological systems in fish. PQ toxicity has been found to be mediated by the production of free radicals, which cause oxidative damage to cells. In a

Table 2.6 Updated list of *tlr7* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr7 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. TLR7 recognizes internalized single-stranded RNA found in viruses. *ND* not determined

TLR no	Species	Tissue expression	References
7	Pufferfish	Kidney, gills, reproductive organ	Oshiumi et al. (2003)
7	Channel catfish	Spleen, anterior kidney, posterior kidney	Quiniou et al. (2013)
7	Channel catfish	Anterior kidney, kidney, brain, spleen, heart, gills, intestine, skin, and others	Zhang et al. (2013a)
7–1	Common carp	Brain, gills, skin, blood, and others	Gong et al. (2017)
7–2		Gills, heart, skin	
7	Common carp	Spleen, liver, heart, kidney, and others	Tanekhy et al. (2010)
7	Yellow catfish	Brain, spleen, swim bladder, anterior kidney, and other	Zhang et al. (2017b)
7	Zebrafish	Heart, skin, blood, brain, and others	Jault et al. (2004)
7	Tibetan fish (Przewalski's carp)	Details not given	Tong et al. (2015)
7	Yellow River scaleless carp	ND	Qi et al. (2017)
7	Spotted sea bass	Details not given	Fan et al. (2019)
7	Maraena whitefish	Anterior kidney and others	Altmann et al. (2016)
7a-c	Atlantic cod	No details given	Solbakken et al. (2016)
7	Zig-zag eel	Gills, muscle, spleen, intestine, and others	Han et al. (2019)
7	Barbel chub	Spleen, brain, heart, and others	Jin et al. (2018)
7	Golden pompano	Spleen, kidney, muscle, gills, skin, and others	Wei et al. (2017)
7	Tongue sole	Kidney, spleen, liver, heart, and others	Li and Sun (2015)
7	Large yellow croaker	Spleen, kidney, muscle, gills, skin, and others	Qian et al. (2013)
7	Grass carp	Spleen, skin, heart, intestine, gills, and others	Yang et al. (2012)
7	Rainbow trout	Spleen, anterior kidney, and others	Palti et al. (2010a)
7	Goldfish	ND	Tu et al. (2016)
7/8a	Lamprey	Peripheral blood leukocytes, skin, and others	Kasamatsu et al. (2010)
7/8b		Peripheral blood leukocytes, heart, and others	

Table 2.7 List of *tlr8* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr8 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. TLR8 may recognize guanosine and uridine-rich single-stranded RNA found in viruses. Tlr8 associates with tlr7. *ND* not determined

TLR no	Species	Tissue expression	References
8	Pufferfish	Reproductive organ	Oshiumi et al. (2003)
8	Channel catfish	Spleen, anterior kidney, posterior kidney	Quiniou et al. (2013)
8a-1	Channel catfish	Anterior kidney, kidney, spleen, and others	Zhang et al. (2013a)
8a-2		Spleen, anterior kidney, kidney, heart, brain, and others	
8-1	Common carp	Gills and other	Gong et al. (2017)
8-2		Gills, intestine, skin, heart, and other	
8-3		Intestine, kidney, spleen, and other	
8	Common carp	Spleen, skin, brain, gonads, and others	Shan et al. (2018b)
8-2	Yellow catfish	Spleen, brain, liver, foregut, and other	Zhang et al. (2017b)
8.1	Zebrafish	Heart, blood, liver, ovaries, digestive organ, and others	Jault et al. (2004)
8.2		Skin, blood, heart, and others	
8	Tibetan fish (Przewalski's carp)	ND	Tong et al. (2015)
8a	Yellow River scaleless carp	ND	Qi et al. (2017)
8b			
8	Spotted sea bass	ND	Fan et al. (2019)
8.1	Maraena whitefish	Anterior kidney, kidney, and others	Altmann et al. (2016)
8.2		Anterior kidney, kidney, heart, and others	
8a-l	Atlantic cod	ND	Solbakken et al. (2016)
8	Zig-zag eel	Spleen, gills, and others	Han et al. (2019)
8	Barbel chub	Spleen, brain, skin, kidney, heart, muscle, and others	Jin et al. (2018)
8	Golden pompano	Spleen, gills, muscle, skin, and others	Wei et al. (2017)
8	Large yellow croaker	Spleen, gills, muscle, skin, and others	(Qian et al. 2013)
8	Grass carp	Swim bladder, spleen, brain, posterior intestine, anterior kidney, skin, and others	Chen et al. (2013)
8a	Grass carp	ND	Liao et al. (2017)
8b			
8a1	Rainbow trout	Spleen, anterior kidney, kidney, and others	Palti et al. (2010a)
8a2		Spleen, anterior kidney, kidney, and others	
8	Turbot	Blood, spleen, anterior kidney, and others	Dong et al. (2016)

Table 2.8 A list of *tlr9* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr9 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. TLR9 binds unmethylated CpG sequences in DNA—often found in prokaryotes. *ND* not determined

TLR no	Species	Tissue expression	References
9	Pufferfish	Kidney, digestive organ, skin, heart	Oshiumi et al. (2003)
9	Channel catfish	Anterior kidney, spleen	Quiniou et al. (2013)
9	Channel catfish	Heart, anterior kidney, kidney, and others	Zhang et al. (2013a)
9	Common carp	Kidney, gills, brain, heart, and others	Gong et al. (2017)
9	Common carp	Kidney, gills, and others	Gong et al. (2017)
9	Yellow catfish	Spleen, anterior kidney, kidney, hindgut, and other	Zhang et al. (2017b)
9	Yellow catfish	Spleen, anterior kidney, gills, intestine, adipose tissue, and others	Zhang et al. (2017b)
9	Zebrafish	Blood, heart, testis, and others	Jault et al. (2004)
9	Tibetan fish (Przewalski's carp)	Details not given	Tong et al. (2015)
9	Yellow River scaleless carp	ND	Qi et al. (2017)
9	Spotted sea bass	Details not given	Fan et al. (2019)
9	Silver pomfret	Spleen, kidney, liver, intestine, gills, and others	Gao et al. (2018)
9	Maraena whitefish	Anterior kidney, gills, and others	Altmann et al. (2016)
9a-e	Atlantic cod	ND	Solbakken et al. (2016)
9	Zig-zag eel	Spleen, muscle, fin, gills, and others	Han et al. (2019)
9	Golden pompano	Spleen, kidney, skin, and others	Wei et al. (2017)
9	Turbot	Brain, anterior kidney, blood, intestine, gills, and others	Dong et al. (2016)
9	Blunt snout bream	Kidney, heart, spleen, gills, liver, and others	Zhan et al. (2019)
9	Grass carp	ND	Liao et al. (2017)
9a	Orange-spotted grouper	Spleen, anterior kidney, liver, kidney	Lee et al. (2015)
9b		Spleen, anterior kidney, liver, kidney	
9	Pacific red snapper	Intestine, leukocytes, liver, skin, anterior kidney	Reyes-Becerril et al. (2015)
9	Rainbow trout	Spleen, anterior kidney, peripheral blood leukocytes, gut	Ortega-Villaizan et al. (2009)
9A		Spleen, skin, kidney, brain, and others	Yao et al. (2008)

(continued)

Table 2.8 (continued)

TLR no	Species	Tissue expression	References
9B	Large yellow croaker	Spleen, skin, liver, and others	
9	Gilthead sea bream	Peripheral blood leukocytes, peritoneal exudate, gills, spleen, anterior kidney, and others	Cuesta et al. (2008)
9	Gilthead sea bream	Gills, spleen, gut, anterior kidney, muscle, and others	Franch et al. (2006)
9	Japanese flounder	Spleen, kidney, intestine, gills, blood, and others	Takano et al. (2007)
9	Goldfish	ND	Tu et al. (2016)

Table 2.9 Tlr12 found in Yellow River scaleless carp (common name). See citation for corresponding Latin names. TLR12 recognizes profilin found in certain parasites. *ND* not determined

TLR no	Species	Tissue expression	References
12	Yellow River scaleless carp	ND	Qi et al. (2017)

study by Ma et al. (2014), the acute toxicity of PQ in common carp (*C. carpio*) was determined. The results suggest that PQ exposure may result in suppression or excessive activation of the immune system that leads to immune dysfunction and reduced immunity (Ma et al. 2014). In the report of Pereiro et al. (2016), an antiviral turbot (*S. maximus*) interferon-gamma gene was characterized, and its expression pattern under basal conditions, after type I ifn administration and viral and bacterial infections, was evaluated (Pereiro et al. 2016). The intramuscular injection of an expression plasmid encoding turbot *ifn* gene was not able to affect the transcription of numerous immune genes directly related to the activity of ifng. It was neither able to reduce the mortality caused by a VHSV nor *A. salmonicida* challenge. Shibasaki and coworkers cloned and characterized two ginbuna crucian carp (*Carassius auratus langsdorfii*)-specific isoforms of *ifng* called *ifng rel1* and *ifng rel2*. Recombinant ifng rel1 and ifng rel2 showed high antiviral activities against the lethal crucian carp hematopoietic necrosis virus (Shibasaki et al. 2014).

The antiviral activity of ifn gamma against IPNV and salmonid alphavirus (SAV) was studied by Sun et al. (2011). The studies were performed in Atlantic salmon TO cells and Chinook salmon embryo cells (CHSE-214). Ifn-γ induced antiviral activity against both IPNV and SAV3 in salmon cells (Sun et al. 2011). The marine flatfish Atlantic halibut (*Hippoglossus hippoglossus*) is of great commercial interest. However, due to poorly developed larva at hatching and a long live-feed stage, aquacultural use of this species is limited. Øvergård and coworkers cloned and characterized the gene encoding the *ifng*. A constitutive expression was found in both lymphoid and non-lymphoid organs with

Table 2.10 Updated list of *tlr13* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr13 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. It has been suggested that tlr12 binds 23S ribosomal RNA (rRNA) found in bacteria. *ND* not determined

TLR no	Species	Tissue expression	References
13	Darby's sturgeon	Anterior kidney, heart, and others	Tang et al. (2020)
13	Orange-spotted grouper	Spleen, anterior kidney, liver, and others	Liang et al. (2018)
13	Tibetan fish (Przewalski's carp)	Details not given	Tong et al. (2015)
13	Yellow River scaleless carp	ND	Qi et al. (2017)
13-1	Spotted sea bass	ND	Fan et al. (2019)
13-2			
13-3			
13	Miiuy croaker	Liver, spleen, anterior kidney, and others	Wang et al. (2016a)
13	Soiny mullet	Anterior kidney, skin, and others	Qi et al. (2020)
13-like genes	Brown trout	ND	Sudhagar et al. (2020)

Table 2.11 A list of *tlr14* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr14 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. The natural ligand for TLR14 is not known. Since it is a member of tlr1 subfamily one can speculate that this receptor may bind similar ligands as TLR1/2/6—which are ligands from bacteria. *ND* not determined

TLR no	Species	Tissue expression	References
14	Golden pompano	Skin, intestine, kidney, blood	Wu et al. (2019)
	Ya-fish (Schizothorax prenanti)	Peripheral blood leukocytes, brain, anterior kidney, spleen, eye	Li et al. (2018c)
14a	Lamprey	Gills	Ishii et al. (2007)
14b	Lamprey	Gills, gut, egg, skin, and others	
14	Spotted sea bass	ND	Fan et al. (2019)
14	Atlantic cod	ND	Solbakken et al. (2016)
14	Japanese flounder	Kidney, spleen, liver, gills, and others	Hwang et al. (2011)
14a	Lamprey	Gills	Kasamatsu et al. (2010)
14b		Gills, peripheral blood leukocytes, intestine, and others	
14c		Peripheral blood leukocytes, heart, gills, brain, eye, and others	
14d		Liver, kidney, intestine, and others	

Table 2.12 List of *tlr18* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr18 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. TLR18 is also a member of the TLR1 subfamily and may bind molecules from bacteria. *ND* not determined

TLR no	Species	Tissue expression	References
18	Channel catfish	Gills, gonads, posterior kidney	Quiniou et al. (2013)
18	Channel catfish	Gills, kidney, brain	Zhang et al. (2013a)
18-1	Common carp	Kidney, intestine, heart, and other	Gong et al. (2017)
18-2		Kidney, intestine, gills, heart, and other	
18	Common carp	Skin, spleen, liver, hindgut, and others	Shan et al. (2018a)
18	Atlantic salmon	Muscle, liver, spleen, gills, and anterior kidney	Lee et al. (2014)
18	Tibetan fish (Przewalski's carp)	Details not given	Tong et al. (2015)
18	Yellow River scaleless carp	ND	Qi et al. (2017)
18	Grass carp	ND	Huang et al. (2012)
18	Grass carp	Spleen, gills, heart, and others	Huang et al. (2015)

Table 2.13 Updated list of *tlr19* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr19 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. Teleost-specific TLR19 is localized in the endosomes and recognizes dsRNA analogs. *ND* not determined

TLR no	Species	Tissue expression	References
19	Channel catfish	Anterior kidney, spleen, posterior kidney	Quiniou et al. (2013)
19	Channel catfish	Heart, anterior kidney, kidney, and others	Zhang et al. (2013a)
19	Common carp	Brain, heart, skin, and other	Gong et al. (2017)
19	Tibetan fish (Przewalski's carp)	Details not given	Tong et al. (2015)
19	Yellow River scaleless carp	ND	Qi et al. (2017)
19	Grass carp	ND	Huang et al. (2012)
19	Atlantic salmon	Spleen, anterior kidney, gills, muscle, liver	Lee et al. (2014)
19	Brown trout	ND	Sudhagar et al. (2020)

Table 2.14 A list of *tlr20* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr20 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. TLR20 is proposed to play a role in the immune response of carp to protozoan parasites. *ND* not determined

TLR no	Species	Tissue expression	References
20	Channel catfish	Anterior kidney, spleen	Quiniou et al. (2013)
20–1	Channel catfish	Anterior kidney, kidney, spleen, heart, gills, and others	Zhang et al. (2013a)
20	Channel catfish	Stomach, anterior kidney, liver, spleen, intestine, gills, and others	Baoprasertkul et al. (2007b)
20a	Atlantic salmon	Spleen, anterior kidney, gills, muscle, liver	Lee et al. (2014)
20b		Spleen, anterior kidney, muscle, gills	
20c		Spleen, anterior kidney, gills, muscle, liver	
20d		Spleen, gills, anterior kidney, liver, muscle	
20	Tibetan fish (Przewalski's carp)	ND	Tong et al. (2015)
20	Yellow River scaleless carp	ND	Qi et al. (2017)
20.2	Grass carp	Spleen, anterior kidney, liver, brain, and others	Huang et al. (2016)
20a	Grass carp	ND	Huang et al. (2012)
20b			
20a	Atlantic salmon	Spleen, anterior kidney, gills, and others	Lee et al. (2014)
20b		Spleen, anterior kidney, muscle, and others	
20c		Spleen, anterior kidney, gills, muscle, liver	
20d		Spleen, gills, anterior kidney, liver, muscle	
20a–d	Zebrafish	ND	Pietretti et al. (2014)
20	Goldfish	ND	Tu et al. (2016)

relatively high expression in the thymus and gills (Overgard et al. 2012). An overview of type II ifng in different fish species is presented in Table 2.24.

2.9.4 Tnfa

Tumor necrosis factor-alpha (tnfa) is a cytokine involved in systemic inflammation, apoptosis, cell proliferation, and regulation of immune cells (Wiens and Glenney 2011). It is produced mainly by activated macrophages as a membrane or secreted form. The main pro-inflammatory effects are mediated through the activation of endothelial cells (Roca et al. 2008). In bony fish, *tnfa* was first discovered in Japanese Flounder (Hirono et al.

Table 2.15 Updated list of *tlr21* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr21 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. It has been suggested, similar to TLR9, that TLR21 may bind unmethylated CpG sequences in DNA. *ND* not determined

TLR no	Species	Tissue expression	References
21	Pufferfish	Gills, heart, reproductive organ	Oshiumi et al. (2003)
21	Channel catfish	Spleen	Quiniou et al. (2013)
21	Channel catfish	Kidney, spleen, heart, gills, anterior kidney, and others	Quiniou et al. (2013)
21	Channel catfish	Stomach, liver, spleen, intestine, kidney, ovary, gills, and others	Baoprasertkul et al. (2007b)
21-1	Common carp	Gills, skin, heart, brain, and other	Baoprasertkul et al. (2007b)
21-2		Spleen, heart, skin, and other	
21	Common carp	Spleen, anterior kidney, gills, and others	Li et al. (2018b)
21	Atlantic salmon	Spleen, gills, anterior kidney, muscle, liver	Lee et al. (2014)
21	Tibetan fish (Przewalski's carp)	Details not given	Tong et al. (2015)
21	Yellow River scaleless carp	ND	Qi et al. (2017)
21	Spotted sea bass	ND	Fan et al. (2019)
21	Maraena whitefish	Gills, heart, kidney, anterior kidney, skin, and others	Altmann et al. (2016)
21	Atlantic cod	ND	Solbakken et al. (2016)
21	Atlantic cod	Kidney, gills, testis, spleen, and others	Sundaram et al. (2012b)
21	Blunt snout bream	Gills, kidney, muscle, liver, and others	Zhan et al. (2019)
21	Grass carp	ND	Huang et al. (2012)
21	Grass carp	Skin, spleen, intestine, anterior kidney, and others	Wang et al. (2013b)
21	Large yellow croaker	Spleen, gills, anterior kidney, intestine, and others	Sun et al. (2016a)
21	Large yellow croaker	Spleen, anterior kidney, stomach, liver, and others	Sun et al. (2018)
21	Olive flounder	Spleen, gills, heart, muscle, and others	Gao et al. (2013)
21	Orange-spotted grouper	Kidney, anterior kidney, heart, spleen, and others	Li et al. (2012)
21	Mudskipper	Gills, reproductive organ, kidney, brain, and others	Qiu et al. (2019)
21	Atlantic salmon	Spleen, gills, anterior kidney, muscle, liver	Lee et al. (2014)

Table 2.15 (continued)

TLR no	Species	Tissue expression	References
21	Zebrafish	ND	Pietretti et al. (2014)
21	Zebrafish	Spleen, gills	Sundaram et al. (2012a)
21	Darby's sturgeon	Gills, intestine, anterior kidney, and others	Qi et al. (2018b)
21	Nile tilapia	Brain, gills, heart, muscle, stomach, intestine, skin, and others	Pang et al. (2017)
21	Yellowtail	Spleen, anterior kidney, intestine, liver, gills	Reyes-Becerril et al. (2016)
21a	Lamprey	Peripheral blood leukocytes, gills, and others	Kasamatsu et al. (2010)
21b	Lamprey	Gills and others	Kasamatsu et al. (2010)
21c	Lamprey	Gills, heart, muscle, kidney, liver, eye, and others	Kasamatsu et al. (2010)

2000) rainbow trout (Laing et al. 2001) and has since been characterized in a number of species. Fish have 14 tumor necrosis family genes. Their genomic existence and location have been investigated in the Japanese pufferfish (fugu) (*Takifugu rubripes*) (Biswas et al. 2015). Fugu was found to possess nine *tnf* superfamily genes including seven newly identified and two that had been previously reported. Poly I:C caused an elevated expression of three fugu *tnf* superfamily 10 genes in head kidney cells. Tnfa is an important factor for bacterial pathogen killing. *A. salmonicida* subsp. *salmonicida* is highly pathogenic for turbot, an economically important cultured flatfish in Europe, China, and Chile. In *A. salmonicida*-infected fish, the number of tnfa immunopositive cells was significantly increased in the kidney and spleen (Coscelli et al. 2016). Immunoreactive cells were also present in the digestive tract, liver, heart, gills, and skin (Ronza et al. 2015).

The striped trumpeter (*Latris lineata* Forster) is a new species in Tasmanian waters. The *tnfa* was cloned, and the expression was analyzed in response to an ectoparasite *Chondracanthus goldsmidi*. A significant upregulation was found in the gills, which are the site of parasite attachment. Head kidney cells showed a significant upregulation of *tnfa*, but spleen cells did not (Covello et al. 2009). The European sea bass (*D. labrax*) is intensely aquacultured in the Mediterranean area. The bacterial pathogen *V. anguillarum* provokes the highest mortality among several pathogens of this species. Available vaccines do not achieve the desired protection. In a recent study, recombinant tnfa was used as adjuvant in a commercial sea bass oral vaccine against *V. anguillarum*. Tnfa significantly enhanced disease resistance and induced recruitment of gut intraepithelial lymphocytes (Galindo-Villegas et al. 2013). In rainbow trout, two *tnfa* genes have been described. Recently, a third *tnfa* (*tnfa3*) that has low identities to known trout molecules was reported.

Table 2.16 List of *tlr22* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr22 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. TLR22 is a typical fish-specific TLR and is suggested to bind double-stranded RNA. *ND* not determined

TLR no	Species	Tissue expression	References
22	Pufferfish	Kidney	Oshiumi et al. (2003)
22	Channel catfish	Anterior kidney, gills, spleen, posterior kidney	Quiniou et al. (2013)
22	Channel catfish	Anterior kidney, kidney, intestine, liver, gills, heart, and others	Zhang et al. (2013a)
22-1	Common carp	Kidney, spleen, gills, heart, and other	Gong et al. (2017)
22-2		Spleen, heart, gills, skin, blood, and other	
22-3		Heart, skin, and others	
22	Common carp	Gills, anterior kidney, brain, hindgut, foregut, and others	Li et al. (2017a)
22	Rohu	Kidney, muscle, spleen, and others	Li et al. (2017a)
22	Rohu	Liver, kidney, gills, heart, eye, and others	Samanta et al. (2014)
22	Catla	Kidney, intestine, brain, and others	Samanta et al. (2014)
22	Yellow catfish	Spleen, anterior kidney, gills, heart	Zhang et al. (2017b)
22	Turbot	Anterior kidney, kidney, spleen, heart, intestine, stomach, and others	Hu et al. (2015b)
22	Rainbow trout	Spleen, anterior kidney, trunk kidney, and others	Rebl et al. (2007)
22L		Spleen, anterior kidney, trunk kidney, gills, and others	
22	Tibetan fish (Przewalski's carp)	ND	Tong et al. (2015)
22	Yellow River scaleless carp	Liver, muscle, and others	Qi et al. (2019)
22a	Yellow River scaleless carp	ND	Qi et al. (2017)
22b			
22	Spotted sea bass	ND	Fan et al. (2019)
22	Triploid crucian carp	Anterior kidney, kidney, muscle, liver, and others	Zhang et al. (2017a)
22a	Maraena whitefish	Anterior kidney, liver, heart, kidney, and others	Altmann et al. (2016)
22b		Anterior kidney, kidney, and others	

(continued)

Table 2.16 (continued)

TLR no	Species	Tissue expression	References
22	Gilthead sea bream	Spleen, thymus, gills, and others	Munoz et al. (2014)
22a	Grass carp	ND	Huang et al. (2012)
22b		ND	
22	Grass carp	Anterior kidney, spleen, kidney, gills, and others	Lv et al. (2012)
22	Grass carp	Gills, anterior kidney, kidney, midgut, and others	Lv et al. (2012)
22a	Mudskipper	Spleen, gills, kidney, intestine, and others	Qiu et al. (2019)
22b		Spleen and others	
22c		Spleen, kidney, and others	
22d		Kidney, brain, spleen, and others	
22	Soiny mullet	Liver, spleen, and others	Qi et al. (2020)
22	Zebrafish	Spleen, gonads, and others	Sundaram et al. (2012a)
22	Darby's sturgeon	Heart, gills, kidney, anterior kidney, and others	Sundaram et al. (2012a)
22	Nile tilapia	Spleen, gills, and others	Pang et al. (2017)
22a	Atlantic cod	Kidney, liver, gills, testis, and others	Sundaram et al. (2012b)
22b		Kidney, liver, gills, and others	
22c		Kidney, gills, testis, liver, spleen, and others	
22d		Gills, kidney, spleen, liver, and others	
22e, f, l		Low in all organs and tissues	
22g		Gills and low in other organs and tissues	
22h		Kidney, gills, liver, spleen, anterior kidney, blood, and others	
22i		Gills, liver, spleen, kidney, and others	
22j		Kidney, gills, liver, spleen, anterior kidney, and others	
22 k		Anterior kidney, kidney, spleen, liver, gills, and others	
22a–l	Atlantic cod	ND	Solbakken et al. (2016)
22-1	Ya-fish	Gills, liver, kidney, and others	Du et al. (2019b)
22-2		Spleen, brain, heart, and others	
22-3		Spleen, kidney, liver, anterior kidney, brain, heart, and others	
22	Atlantic salmon	ND	Salazar et al. (2016)

Table 2.16 (continued)

TLR no	Species	Tissue expression	References
22	Wuchang bream	Similar expression levels in many organs and tissues	Lai et al. (2017a)
22	Orange-spotted grouper	Kidney, anterior kidney, peripheral leukocytes, spleen, heart, and others	Ding et al. (2012)
22	Large yellow croaker	Anterior kidney, heart, spleen, blood, and others	Xiao et al. (2011)
22	Lamprey	Eye, low in other organs	Kasamatsu et al. (2010)

Table 2.17 Updated list of *tlr23* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr23 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. The ligand for TLR23 is unknown, *ND* not determined

TLR no	Species	Tissue expression	References
23	Spotted sea bass	Details not given	Fan et al. (2019)
23	Atlantic cod	ND	Solbakken et al. (2016)
23a	Atlantic cod	Kidney, gills, liver, and others	Sundaram et al. (2012b)
23b		Low in all tissues and organs	
23a	Mudskipper	Kidney, spleen, gills, and others	Qiu et al. (2019)
23b-e		Kidney, spleen, and others	
23f		Kidney and others	
23 g		Spleen, kidney, and others	

Table 2.18 List of *tlr24* found in different fish species (common names). See citation for corresponding Latin name. The main tissue distribution of tlr24 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. The ligand for TLR23 is unknown

TLR no	Species	Tissue expression	Reference
24a	Lamprey	Peripheral blood leukocytes, liver	Kasamatsu et al. (2010)
24b		Peripheral blood leukocytes, heart, and others	

The constitutive expression of *tnfa3* was generally lower than the other two genes in tissues and cell lines. Expression of all three *tnfa* isoforms could be modulated by crude LPS, peptidoglycan, poly I:C and recombinant Ifng in cell lines and primary macrophages, and bacterial and viral infections (Hong et al. 2013). The genomic location of the two *tnfa* genes in zebrafish (*D. rerio*) and medaka (*Oryzias latipes*) was recently determined. Zebrafish *tnfa1* and *tnfa2* were found on chromosomes 19 and 15, and medaka *tnfa1* and *tnfa2* on chromosomes 11 and 16, respectively. There was a constitutive expression of the genes in

Table 2.19 Updated list of *tlr25* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr25 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. The ligand for TLR25 is unknown, *ND* not determined

TLR no	Species	Tissue expression	References
25	Channel catfish	Gills, kidney, anterior kidney	Quiniou et al. (2013)
25	Channel catfish	Gills, anterior kidney, kidney, and others	Lee et al. (2020)
25	Nile tilapia	Spleen, anterior kidney, skin, muscle	Lee et al. (2020)
25-1	Common carp	Muscle, gills, heart, blood, and other	Gong et al. (2017)
25-2		Muscle, heart, skin, blood, and other	
25a–g	Atlantic cod	ND	Solbakken et al. (2016)
25	Grass carp	ND	Huang et al. (2012)
25	Grass carp	ND	Liao et al. (2017)
25	Darby's sturgeon	Kidney, gills, skin, heart, and others	Liao et al. (2017)

Table 2.20 Updated list of *tlr26* found in different fish species (common names). See citations for corresponding Latin names. The main tissue distribution of tlr26 mRNA is also given, where the highest expression is found in the first organ/tissue listed, and so on. *TLR26* is not yet found in any other fish species. The ligand is unknown

TLR no	Species	Tissue expression	References
26	Channel catfish	Anterior kidney, kidney, spleen	Quiniou et al. (2013)
26	Channel catfish	Anterior kidney, kidney, brain, gills, and others	Zhang et al. (2013a)
26	Yellow catfish	Anterior kidney, blood, spleen, heart, and others	Liu et al. (2019)

Table 2.21 *Tlr27* is found in spotted gar, coelacanth, and elephant shark. See citation for corresponding Latin name. *ND* Not determined. Its ligand is unknown

TLR no	Species	Tissue expression	Reference
27	Spotted gar, coelacanth, elephant shark	ND	Wang et al. (2015)

Table 2.22 *Tlr28* is found in miiuy croaker. See citation for corresponding Latin name. Like *tlr27*, the *tlr28* belongs to the *tlr1* subfamily. Its ligand is unknown

TLR no	Species	Tissue expression	Reference
28	Miiuy croaker	Liver, eye, skin, gills, heart	Wang et al. (2016b)

Table 2.23 Innate immunity: interferon type I genes in fish

Fish Species	Subject	References
Rainbow trout	Response in ovary against virus	Chaves-Pozo et al. (2010)
Orange-spotted grouper	Functional analysis in response to virus infection	Chen et al. (2014)
Large yellow croaker	Characterization, function	Ding et al. (2019)
Japanese eel	Identification, expression	Feng et al. (2017)
CHSE-214 cell line	Mx1 promoter–reporter system	Jorgensen et al. (2007)
Mandarin fish	Functional, signaling, and transcriptional differences	Laghari et al. (2018)
Salmonids	Evolution of subgroups	Liu et al. (2020a)
Meagre	Discovery, expression	Milne et al. (2018)
Review		Robertsen (2006)
Review	Evolution of interferons and interferon receptors	Secombes and Zou (2017)
Atlantic salmon	Multigene cluster, identification, expression	Sun et al. (2009)
Gilthead sea bream, European sea bass	Characterization, expression	Valero et al. (2015)
Zebrafish	Interferon-induced proteins (IFITs)	Varela et al. (2014)
Rock bream	Cloning, functional characterization	Wan et al. (2012)
Review		Workenhe et al. (2010)
Review		Zou and Secombes (2011)

Table 2.24 Innate immunity: Presence of interferon type II (*ifng*) genes in fish

Fish Species	Subject	References
Common carp	Functional analysis	Arts et al. (2010)
Cell line (Rainbow trout)		Castro et al. (2010)
Large yellow croaker	Characterization, expression	Chen et al. (2015)
Olive flounder	Response against *Edwardsiella tarda* infection	Jung et al. (2012)
Turbot	Characterization, expression	Pereiro et al. (2016)
Review		Pereiro et al. (2019)
Ginbuna crucian carp	Isoforms	Shibasaki et al. (2014)
Atlantic salmon TO cells, CHSE-214 cells	Antiviral activity	Sun et al. (2011)
Atlantic halibut	Characterization, expression	Overgard et al. (2012)

different tissues. An increased expression of both was induced in head kidney cells by LPS in vitro (Kinoshita et al. 2014). Li and Zhang studied a *tnfa* homologue from the Tongue sole (*Cynoglossus semilaevis*) named *CsTNF1*. Expression of *CsTNF1* was detected in liver, spleen, kidney, blood, gill, brain, muscle, heart, and intestine, and was upregulated by experimental challenge with bacterial and viral pathogens (Li and Zhang 2016).

Meagre (*Argyrosomus regius*) is an emerging aquaculture species found in the Mediterranean area and Black Sea due to its large size, fast growth, low feed conversion ratio, and high processing yield. Two types of *tnfa* were expressed in meagre (type 1 and type 2). *Tnfa1* was more highly expressed in head kidney and gills. Both isoforms increased in expression in head kidney following injection with LPS (Milne et al. 2017). Atlantic bluefin tuna (*Thunnus thynnus*) was introduced into Mediterranean aquaculture in the early nineties, and has become the most valuable finfish aquaculture, representing more than half of the world's total production (Pleic et al. 2014). Tuna aquaculture is a capture-based activity, where wild-caught tuna is cultured in marine cages for a period of time in order to increase their protein and fat content. The full-length cDNA and gene sequences of Bluefin tuna *tnfa1* and *tnfa2* were determined, and expression studies showed that they were constitutively expressed in liver and head kidney at similar levels. Expression of both cytokines was examined in acute and chronic natural infection of the parasites *Pseudocycnus appendiculatus* and *Didymosulcus katsuwonicola* (Pleic et al. 2015). *D. katsuwonicola*-infected gills showed significantly higher expression of *tnfa2*, while *tnfa1* showed no difference in expression with either *Pseudocycnus appendiculatus*- or *Didymosulcus katsuwonicola*-infected gills.

Rainbow trout red blood cells (RBCs) are able to endocytose nanostructured tnfa in vitro despite not being phagocytic cells, and in response to nanostructured tnfa, the expression of different immune genes could be modulated (Puente-Marin et al. 2019a).

Tnfa was cloned in large yellow croaker (*Pseudosciaena crocea*), mainly distributed in coastal regions of East Asia, and is one of the most important cultured marine fish in China. *Vibrio parahemolyticus* challenge demonstrated enhanced expression of *tnfa* in head kidney and blood (Xie et al. 2008). *Tnfa* was also identified in grass carp (*Ctenopharyngodon idella*), and its role in signaling was defined (Zhang et al. 2012). Additionally, *tnfa* is involved in the control of ovulation (Crespo et al. 2010, 2015) (Table 2.25). For more details, please see Chap. 10 ("cytokines") by Dr. C. Secombes.

2.9.5 The Complement System

The mammalian complement system is composed of about 35 plasma and membrane-associated proteins. The main functions of the complement system are opsonization, inflammation, and formation of the cytolytic membrane attack complex. The proteins are mostly produced by liver hepatocytes and secreted to the blood, except for some like factor D and c1q. Several components of the teleost complement contain isoforms like c3, c4, c5, c7, factor B, factor I, and MBL. Most homologs of mammalian complement

Table 2.25 Innate immunity: tnfa genes in different fish species

Species	Subject	References
Japanese pufferfish	Genomic identification, expression	Biswas et al. (2015)
Turbot	*Aeromonas salmonicida* infection, immunohistochemical study	Coscelli et al. (2016)
Three striped trumpeter	Cloning, sequencing, expression in response to ectoparasite *Chondracanthus goldsmidi*	Covello et al. (2009)
Brown trout	Ovulatory mechanism	Crespo et al. (2010)
Brown trout	Ovulation	Crespo et al. (2015)
European sea bass	Oral vaccine adjuvant	Galindo-Villegas et al. (2013)
Rainbow trout	Phylogeny, expression, bioactivity	Hong et al. (2013)
Zebrafish, medaka	Genomic location, expression	Kinoshita et al. (2014)
Tongue sole	Expression, antibacterial and antiviral defense	Li and Zhang (2016)
Review	Regulator in adipose tissue	Liu et al. (2015)
Meagre	Discovery, distribution, expression	Milne et al. (2017)
Atlantic bluefin tuna	Characterization, expression	Pleic et al. (2014)
Atlantic bluefin tuna	Expression, role in acute and chronic parasitic infection	Pleic et al. (2015)
Rainbow trout	Expression of immune genes post-endocytosis of nanostructured tnfa	Puente-Marin et al. (2019a)
Gilthead sea bream, zebrafish	Activator of endothelial cells	Roca et al. (2008)
Turbot	Immunohistochemical distribution	Ronza et al. (2015)
Turbot	Immune response to *Enteromyxum scopthalmi* (Myxozoan)	Ronza et al. (2015)
Review	TNF and TNF superfamilies	Wiens and Glenney (2011)
Large yellow croaker	Characterization, response to bacterial infection	Xie et al. (2008)
Grass carp	Characterization, immune challenge in vitro and in vivo	Zhang et al. (2012)

components are present in teleosts and have been shown to be expressed in a variety of tissues like the kidney, skin, and intestine. Several reviews on the complement system of teleosts are available (Zhang et al. 2013b; Nakao et al. 2011; Uribe et al. 2011; Boshra et al. 2004, 2006). Additional information of the complement system in fish is given in Chap. 9.

2.9.5.1 c3

c3 is the central complement component and has been isolated, purified, and characterized in many teleost species. Recently, four c3 isoforms were purified from the Nile tilapia (*Oreochromis niloticus*) serum and were shown to possess an intrachain thioester bond. All named c3-1, c3-2, c3-3, and c3-4 showed the two-chain polypeptide structure typical of c3 (Abdel-Salam et al. 2014). Forn-Cuni et al. confirmed the presence of three *c3* genes and in addition identified five more *c3* genes in the zebrafish (*D. rerio*) genome (Forn-Cuni et al. 2014). Maternal immunization of female zebrafish with formalin-killed *A. hydrophila* caused a significant increase in c3 and factor b contents in the mother, a corresponding rise in the offspring, and induced a remarkable increase in the hemolytic activities in both the mother and offspring (Wang et al. 2009). The dojo loach (*Misgurnus anguillicaudatus*) is one of the most commercially cultured fish species in Eastern Asian countries including China, Japan, and Korea. Three isoforms of c3 were discovered in *M. anguillicaudatus* named c3-1, c3-2, and c3-3, respectively. The expression of *c3–1* and *c3–3* was mainly detected in liver followed by spleen and gonad. The mRNA levels were upregulated in the gill, skin, liver, and spleen after bath infection with *A. hydrophila* (Xu et al. 2018b). Furthermore, the complete nucleotide sequence of *c3* from two Antarctic teleosts *Trematomus bernacchii* (two isoforms) and *Chionodraco hamatus* (a single isoform) was determined (Melillo et al. 2015).

Rainbow trout c3a and c5a receptors were cloned and functionally characterized. Both anaphylatoxin receptors were expressed at considerable levels by B cells. Treatment by lipopolysaccharide led to a significant upregulation of both receptors, suggesting that B cells play a role in the development of an inflammatory response (Li et al. 2007).

2.9.5.2 Classical Pathway

The classical complement pathway involves c1, c2, and c4. The first complement component (c1) is activated by recognition of antigen-bound immunoglobulins, and proteolytically activates c4 and c2 into c4b and c2a, respectively. Rock bream (*Oplegnathus fasciatus*) is one of the most economically important marine fish species in South Korea, which geographically distribute in the coastal water, especially in coral beds of the Pacific and Indian Ocean. Rock bream complement components c1r and c1s were characterized, and homology analysis showed 73.4% and 58% amino acid identity with orthologs of *Pundamilia nyererei* of Lake Victoria and the Japanese rice fish, *Oryzias latipes*, respectively. c1r was highest expressed in blood and c1s in the liver. The transcription of both components was found to be upregulated in response to pathogenic bacteria *E. tarda* and *S. iniae* and virus (rock bream iridovirus) (Godahewa et al. 2015).

Grass carp (*C. idella*) is susceptible to *A. hydrophila* infections. In the study of Dang et al., grass carps were given intraperitoneal injections of live *A. hydrophila* and 4, 8, 12, 24, 48, and 72 h after RNA sequencing of spleen tissue was performed. Four to 72 h after infection, the complement system, represented by *c2*, *c3*, *c4*, *c5*, *c8a*, *c1q*, and *mbl*, was upregulated with a transitory downregulation at 12 h (Dang et al. 2016).

Tissue of *C. idella* was infected with *A. hydrophila*. Two cDNA sequences of *c4* from the common carp (*C. carpio*) were isolated sharing only 32% identity of amino acid level and having distinct binding specificities (Mutsuro et al. 2005).

2.9.5.3 Alternative Pathway

Factor B and factor D are both components of the alternative pathway of complement. Following activation of the alternative pathway, factor B is cleaved into Ba and Bb fragments. In rainbow trout, factor B is known to act as a c3 convertase, but the function of the Ba fragment is unknown. The expression patterns of tongue sole (*Cynoglossus semilaevis*) factor B and the biological activity of the Ba fragment were studied by Li and Sun (2017). Expression of *factor B* was high in liver, muscle, and heart and low in intestine, blood, and kidney. Bacterial infection (*E. tarda*, *Pseudomonas fluorescens*, and *V. harveyi*) induced an expression in kidney, spleen, and liver in a time-dependent manner. For the first time, it was found that overexpression of *Ba* significantly reduced bacterial dissemination in fish tissues, indicating that Ba possesses antimicrobial activity and may inhibit bacterial infection in fish (Li and Sun 2017).

Rock bream (*Oplegnathus fasciatus*) *complement factor D* (Cfd) was characterized and expression was analyzed. Factor D encodes 277 amino acids for a 30 kDa polypeptide and was most highly expressed in the liver and spleen. Transcription of *factor D* was upregulated in the spleen by lipopolysaccharide, *S. iniae*, rock bream iridovirus, and poly I:C (Godahewa et al. 2016). Rainbow trout liver seems not to be an important transcription site of the genes *c1q*, *factor B* (*cfb*), and *c7–2*. The novel characterized factor D of rainbow trout had 253 amino acids with a molecular weight of 27.2 kDa and shared a sequence identity with its human ortholog of 45% (Kobis et al. 2015).

2.9.5.4 Lectin Pathway

The central components of the lectin pathway are MBL and MASPs. Teleost fish often possess several genes encoding different subtypes. Kania et al. (2010) characterized three homologs of mannan-binding lectin (named *MBL H-1*, *MBL H-2*, *MBL H-3*) (Gene: *mbl and variants*) in the rainbow trout. They were expressed in the spleen, anterior intestine, and liver. MBL H-1 and H-3 were also found in the vascular system. *MBL H-1* had the highest expression level in the anterior intestine followed by gill, thymus, and skin, while the highest expression level of *MBL H-2* and *MBL H-3* occurred in the anterior intestine (Kania et al. 2010).

C. semilaevis mannan-binding lectin (Mbl)-associated protein 34 (MAP34) and Mbl-associated serine protease 1 (MASP1) are key factors involved in complement activation through MAPs' ability to bind to M1 and MBL. Remarkably, in contrast to a negative regulatory role of mammalian MAP, the teleost *C. semilaevis* Map 34 exerts a positive effect on the activation of the lectin pathway (Li et al. 2016).

2.9.5.5 Terminal Pathway

c5, c6, c7, c8, and c9 are the components engaged in building the membrane attack complex. Native c5a of rainbow trout and recombinant infectious hematopoietic necrosis virus glycoprotein (G) fusion protein was constructed to test the adjuvant activity of rainbow trout c5a. At four to sixteen weeks postinjection, the serum Igm antibody levels were higher than those injected with G-protein alone, suggesting that c5a acts as molecular adjuvant in rainbow trout (Wu et al. 2014).

Grass carp (*C. idella*) is an economically important species, and its global production is more than 4.5 million tons per year making it the most highly consumed freshwater fish species in the world. *A. hydrophila* is the causative pathogen of intestinal hemorrhage, which has caused great economic loss in grass carp aquaculture. Fish were intraperitoneally injected with *A. hydrophila*, and the transcriptomic response was tested in the spleen. A total of 1591 genes were upregulated, and 530 were downregulated. *c1*, *c7*, and *c8b* were upregulated indicating activation of the classical pathway (Yang et al. 2016). c7 was isolated and characterized from grass carp. The predicted amino acid sequence of c7 cDNA exhibited 55.4% and 48.3% homology with rainbow trout c7–1 and zebrafish c7, respectively. *c7* gene expression was detected in trunk kidney, liver, head kidney, skin, spleen, heart, and intestine. Significant changes in *c7* transcript expression were detected following *A. hydrophila* infection, especially in head kidney and spleen (Shen et al. 2012).

Full-length *c8a* and *c8b* sequences from a cDNA library of rock bream (*Oplegnathus fasciatus*) and their genomic sequences were obtained. Quantitative real-time PCR analysis showed that both components were expressed in all examined tissues, with highest expression in the liver. Pathogen challenge, including *E. tarda*, *S. iniae*, and rock bream iridovirus, led to upregulation of both (Wickramaarachchi et al. 2013).

Complement component c9 is the last component that is involved in the formation of the membrane attack complex on the surface of target cells. The full-length *c9* cDNA sequence was found in the southern catfish (*Silurus meridionalis*) and showed similarity with other teleost fish. The mRNA expression was highest in the liver and observed also in the spleen, head kidney, stomach, and intestine. Intraperitoneal injection of *A. hydrophila* gave upregulation of *c9* in the liver, spleen, and intestine (Fu et al. 2019).

The large yellow croaker *Larimichthys crocea* is one of the most important marine fish in China and East Asian countries. Complement components c7 and c9 were characterized by Guo et al. (2016). *c7* and c9 were mainly expressed in liver, but low levels were also constitutively expressed in most tissues. Fish challenged with *Vibrio alginolyticus* showed a rapidly upregulated response in the liver and head kidney (Guo et al. 2016). Miiuy croaker, *Miichthys miiuy*, belongs to the family Sciaenidae of the order Perciformes and mainly distributes from the western Japan Sea to the East China Sea. In China, it has been widely cultured since the late 1990s for its good taste and high nutritive and medicinal value. A truncated *c9* cDNA sequence encoding 461 amino acids was cloned and characterized in the miiuy croaker (*M. miiuy*). The c9 of miiuy croaker shows the highest amino acid identity score with fugu c9 (61%) and the lowest with zebrafish c9 (36%). The highest levels of transcripts were detected in liver of both healthy and *V. anguillarum-*

infected fish (Meng et al. 2012). Full-length *c9* sequence was identified from a cDNA library of rock bream (*O. fasciatus*), and its genomic sequence was obtained. Quantitative real-time RT-PCR analysis confirmed that *c9* was constitutively expressed in all the examined tissues, with highest expression occurring in the liver. Pathogen challenge including *E. tarda*, *S. iniae*, lipopolysaccharide endotoxin, and rock bream iridovirus led to upregulation of *c9* in liver but resulted in no change in the peripheral blood cells (Wickramaarachchi et al. 2012). The transcriptional expression of central complement components during the ontogeny of the common sole (*Solea solea*) was studied by Ferraresso et al. (2016). The *c2*, *c3*, and *fb* showed a gradual increase in expression between 4 and 33 days post-hatch (dph). *c4* and *masp1* showed no differences in expression during the development, while c1qb showed a very high level of expression. Terminal components, *c5*, *c6*, *c7*, *c8*, and *c9*, showed an increase in expression until the onset of metamorphosis and a second increase after metamorphosis (Ferraresso et al. 2016).

2.9.5.6 Complement Regulation

Complement activation is controlled by both fluid phase and membrane inhibitors. Factor I regulates complement by proteolytic cleavage of components c3b and c4b. Factor H, the main cofactor of factor I, regulates the alternative pathway by acting in the breakdown of c3b to ic3b. Factor I (*cfi*) and factor H (*cfh*) of rainbow trout were cloned and characterized. The deduced amino acid sequences of factor I and factor H exhibited 42% and 32% identity with human orthologs, respectively (Anastasiou et al. 2011). The deduced amino acid sequence of factor H from large yellow croaker (*Larimichthys crocea*) showed 28% and 34% identity with human and rainbow trout orthologs, respectively. The highest expression levels were found in liver, kidney, and spleen. After injection with *V. alginolyticus*, the expression levels were upregulated in all three tested tissues (Qi et al. 2018a). Black rockfish (*Sebastes schlegelii*) is an important aquaculture species in the Republic of Korea. A *c1 inhibitor gene* from black rockfish was cloned and characterized by Nilojan et al. The *c1 inhibitor* was most highly expressed in the liver followed by the gills (Nilojan et al. 2018) (Table 2.26).

2.9.6   Acute-phase Component

During infection, stimulation with strong danger signals or stress, the fish may respond to produce acute-phase proteins (APPs). Especially, IL-1, IL-6, and tnfa are able to induce acute-phase response, as observed in higher vertebrates. The most common APPs are pentraxins such as serum amyloid A (SAA) and C-reactive protein (CRP). Dissimilar to many mammalian species, the fish show a modest acute-phase response when it comes to concentration of pentraxins in serum. CRP may be able to bind to (opsonize) certain bacteria, fungi, and parasites, activate the complement system, agglutinating particles, and may infer production of cytokines. There are two forms of SAA, one of them being acute-phase SAA. SAA may neutralize pathogen activity, reduce tissue damage, and

Table 2.26 Table of complement components found in the listed fish species

Component	Teleost species	References
c1, c1r, c1s	Rock bream Grass carp	Godahewa et al. (2015) Yang et al. (2016)
c1 inhibitor	Black rockfish	Nilojan et al. (2018)
c3	Nile tilapia Common sole Zebrafish Zebrafish Dojo loach *Trematomus bernacchii*, *Chionodraco hamatus* Grass carp	Abdel-Salam et al. (2014) Ferraresso et al. (2016) Forn-Cuni et al. (2014) Wang et al. (2009) Xu et al. (2018b) Melillo et al. (2015) Dang et al. (2016)
c3a receptor	Rainbow trout	Li et al. (2007)
c4	Common carp	Mutsuro et al. (2005)
c5	Rainbow trout	Wu et al. (2014)
c5a receptor	Rainbow trout	Li et al. (2007)
c7	Large yellow croaker Grass carp	Guo et al. (2016) Shen et al. (2012)
c8	Rock bream	Wickramaarachchi et al. (2013)
c9	Sothern catfish Miiuy croaker Large yellow croaker Rock bream	Fu et al. (2019) Meng et al. (2012) Guo et al. (2016) Wickramaarachchi et al. (2012)
Factor B	Rock bream Tongue sole	Godahewa et al. (2016) Li and Sun (2017)
Factor D	Rainbow trout Rock bream	Kobis et al. (2015) Godahewa et al. (2016)
Factor H	Rainbow trout Large yellow croaker	Anastasiou et al. (2011) Qi et al. (2018a)
Factor I	Rainbow trout	Anastasiou et al. (2011)
MBL	Rainbow trout	Kania et al. (2010)
MAP/MASP	Tongue sole	Li et al. (2016)
Ontogeny	Common sole	Ferraresso et al. (2016)
Bacterial infection	Grass carp	Dang et al. (2016)

restore homeostasis. Transferrin, haptoglobin, ceruloplasmin, alpha-2-macroglobulin, lectins, and complement component c3 are all considered to be AAPs. Most of these have regulatory activities limiting infection and restoring the physiological balance. Several reviews covering this topic are recommended (Roy et al. 2017; Bayne and Gerwick 2001; de Magalhaes et al. 2020; Magnadottir 2014; Nakao et al. 2011).

2.9.7 Chemokines and Their Receptors

The major function of chemokines is to guide the migration of cells. An example is chemokine-guided migration of leukocytes to inflammatory foci. Other functions involve immune surveillance where chemokines direct homing of leukocytes to lymphatic tissues. Some chemokines have function in growth of new blood vessels and wound healing. Chemokines are classified into four main subfamilies (CXC, CC, CX3C, and XC) dependent on the amino acid sequences (first two cysteine amino acid residues). Most of the chemokines bind to specific chemokine receptors on cells. Fish display numerous genes for different chemokines and chemokine receptors, suggested due to gene duplication events. Functional and significance studies of chemokine expression are generally not very well examined in fish. However, exceptions exist. We list the most recent findings within functional chemokine research. It has been shown that grass carp cxcl20b possessed antibacterial activity by attaching to the bacterial membrane (Xiao et al. 2020). In another study, it was shown that common carp Cxcb1 stimulated neutrophil extracellular trap formation—which was suggested to be an antipathogenic event (Pijanowski et al. 2020). Moreover, an ayu (*Plecoglossus altivelis*) CC-like chemokine was found to possess chemotactic activity against monocytes and neutrophils in vivo and in vitro (Yu et al. 2019). Chemotactic activity of Ccl4 has been shown in the golden pompano (*Trachinotus blochii*). This recombinant chemokine had also antimicrobial activity against *E. tarda* and *Escherichia coli* (Sun et al. 2019). Furthermore, a rainbow trout CC chemokine (Ck11) also displayed antimicrobial activity against different gram-positive and gram-negative bacteria by attaching to and disrupting their cell membranes (Munoz-Atienza et al. 2019).

2.9.8 Antibacterial Peptides (AMPs)

AMPs are a diverse class of highly conserved molecules that are produced as a first line of defense in all multicellular organisms, including fish. These small peptides (12–50 amino acids) are essential components of innate immunity capable of antimicrobial activity against a broad range of microbial pathogens (Semple and Dixon 2020; Zhang and Gallo 2016). Functionally, they can be described as either membrane disruptive AMPs, which induce membrane permeabilization, or they can be non-membrane disruptive where they can be internalized in cells and act on intracellular targets (Semple and Dixon 2020). In general, fish AMPs may be categorized into five different classes based on their structure: β-defensins, cathelicidins, hepcidins, histone-derived peptides, and piscidins (Brunner et al. 2020). As for chemokines and TLRs, fish possess numerous genes for antimicrobial peptides. Recently, reviews on the significance of AMPs are published (Brunner et al. 2020; Chaturvedi et al. 2020; Valero et al. 2020; Chen et al. 2020; Shabir et al. 2018).

2.10 Conclusion and Future Research

It is clear that fish are indeed equipped with an arsenal of defense mechanisms to prevent infection. An earlier report has used Rag knockout mutants ($rag1^{-/-}$ zebrafish), which possess no serum Igm, to assess the significance of the innate immune system in comparison with control fish with a $rag1^{+/-}$ genotype. An experimental challenge experiment revealed that the $rag^{-/-}$ zebrafish displayed similar protection as the controls (Tokunaga et al. 2017). This underscores the notion that the innate immune system alone may likely be as effective as a fully immune-equipped fish. However, immunized fish will normally acquire higher disease resistance than naïve fish. The concept of trained innate memory should be addressed as a trained innate immune system likely would add higher protection level during infection. Trained innate immunity involves activation of innate defense factors that in turn confer increased disease resistance to infection by homologous or heterologous pathogens. Trained immunity can be transferred to offspring as training induces heritable epigenetic changes.

The future will bring a vast more knowledge of innate immune factors through fish genomic and transcriptomic studies, and it is likely that many more innate immune factors will be revealed. To find their significance in the innate immune defense, these must be functionally examined.

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Adaptive Immunity

3

Beatriz Abós, Christyn Bailey, and Carolina Tafalla

Abstract

Two fundamental features characterize adaptive immunity: specific antigen recognition and immunological memory development. Thus, B and T cells form the adaptive immune system that detects and fights infection and disease with specificity. B- and T-cell progenitors rearrange their specific receptors (BCR and TCR, respectively) generating a great variability in the antigen recognition domains. The BCR and its secreted form (antibodies) recognize antigens in their native form and induce humoral immune responses. By contrast, the TCRs bind to antigens processed and presented on major histocompatibility complex (MHC) molecules and develop cellular immune responses. Through the development of both B and T memory cells, subsequent encounters with the antigen induce more efficient and faster immune responses, being this the basis for vaccination. Fish constitute the first animal group in which most basic elements of the adaptive immune system are present. Still, given the great diversity of fish, many fundamental differences exist among different species. Likewise, the fish adaptive immune system shows some unique features. These structural and functional differences with their mammalian counterparts anticipate important differences in the way that fish regulate both humoral and cellular adaptive immune responses. In the present chapter, we provide an overview of the basic organization of the adaptive immune system in fish, highlighting its main specific traits and providing some insights as to how these particularities might condition the response to antigens.

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Abbreviations

AID	activation-induced cytidine deaminase
APC	antigen-presenting cell
APRIL	proliferation-inducing ligand
ASC	antibody-secreting cell
BAFF	B-cell-activating factor
BCR	B-cell receptor
Blimp1	B lymphocyte-induced maturation protein 1
CD40L	CD40 ligand
CDR	complementarity-determining region
CH	constant heavy
CHNV	crucian carp necrosis virus
CL	constant light
CSR	class switch recombination
CTLA-4	cytotoxic T lymphocyte-associated protein 4
EBF1	early B-cell factor 1
EPC	epithelioma papulosum cyprini
FasL	Fas ligand
fDC	follicular dendritic cell
FITC-KLH	fluorescein–keyhole limpet haemocyanin
GALT	gut-associated lymphoid tissue
GC	germinal centres
GIALT	gill-associated lymphoid tissue
HK	head kidney
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
ILT	interbranchial lymphoid tissue
IRF4	interferon regulatory factor 4
LN	lymph node
LP	lamina propria
LRR	leucine-rich repeat
MALT	mucosa-associated lymphoid tissue

MBL	mannose-binding lectin
MHC	major histocompatibility complex
MLC	mixed leucocyte culture
MMC	melanomacrophage centre
MZ	marginal zone
NALT	nasopharynx-associated lymphoid tissue
NKEF	natural killer cell enhancement factor
NO	nitric oxide
PALS	periarteriolar lymphoid sheath
PBL	peripheral blood leucocyte
PC	plasma cells
pIgR	polymeric Ig receptor
PKD	proliferative kidney disease
RAG	recombination-activating gene
RSS	recombination signal sequence
RTG2	rainbow trout gonad 2
SALT	skin-associated lymphoid tissue
SHM	somatic hypermutation
Tc	T cytotoxic
TCR	T-cell receptor
TD	thymus-dependent
TdT	terminal deoxynucleotidyl transferase
Tfh	T follicular helper cell
TGF	transforming growth factor
Th	T helper
TI	thymus-independent
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNP-KLH	trinitrophenyl–keyhole limpet haemocyanin
TNP-LPS	trinitrophenyl–lipopolysaccharide
Treg	T-regulatory
V(D)J	variable (diversity) joining
VH	variable heavy
VHSV	viral haemorrhagic septicaemia virus
VL	variable light
VLR	variable lymphocyte receptor
Xbp1	X-box-binding protein 1

3.1   Introduction

The immune system of vertebrates consists of two fundamental components: innate immunity and adaptive immunity. The innate immune system acts as a first line of defence and relies on a set of germline-encoded host receptors that recognize conserved molecular signatures found in microorganisms. These receptors stimulate a broad variety of myeloid and lymphoid cells that can exert rapid microbicidal functions, at the same time initiating the adaptive immune response through the presentation of antigens or through the effect of secreted soluble factors (chemokines and cytokines) that provide co-stimulation to cells of the adaptive immune system (Mogensen 2009).

In contrast, in more complicated or reoccurring scenarios the adaptive immune system has emerged and evolved to provide specific protection against subsequent pathogenic infections. Two fundamental features characterize adaptive immunity: specific antigen recognition and immunological memory development (Gourley et al. 2004). Collectively, B and T cells form the adaptive immune system that detects and fights infection and disease with specificity. Recognition specificity and broad reactivity are enabled via antigen receptors on B and T cells generated by recombination-activating genes (RAG1 and RAG2) (Nishana and Raghavan 2012). B lymphocytes express membrane-bound immunoglobulins (Igs), known as B-cell receptors (BCR), which can recognize specific antigens in their native form. These Igs can be secreted, being then referred to as antibodies. T cells, on the other hand, have their own specific receptor (T-cell receptor, TCR) that recognizes processed antigens presented mostly in the context of major histocompatibility complex (MHC) molecules. T cells provide co-stimulatory signals to promote the activation and differentiation of B cells (helper T cells, Th cells) or mediate cellular immune responses aimed at the destruction of pathogens that are localized inside cells (cytotoxic T cells, Tc cells).

The adaptive immune system is able to recognize millions of many different antigens through the generation of the lymphocyte receptor repertoire. This process can be accomplished with a limited number of available genes owing to the exceptional organization of the genes encoding the BCR and the TCR. These receptor genes contain multiple versions that code for each region of the protein, allowing the lymphocyte to obtain a specific sequence among all the possible combinations and expressing these molecules in the membrane. The repertoire generation occurs randomly and independently from antigen exposition. For this, the BCR and TCR contain variable regions encoded by the variable (V), diversity (D) and joining (J) gene segments. These fragments undergo a process of splicing and somatic recombination as well as the random addition of nucleotides in the junction of VD or DJ segments, increasing the diversity in the potential repertoire of lymphocyte receptors (Nishana and Raghavan 2012). However, this large diversity includes the possibility of generating autoreactive cells that can recognize and attack self-antigens. To avoid this process, these cells undergo a selection process in the thymus and the bone marrow or its equivalent (birthplaces for T and B cells, respectively) that destroys or inactivates lymphocytes with autoreactivity (Strasser et al. 2008).

In mammals, adaptive immunity is the basis of immunological memory as successive encounters with a specific antigen lead to the development of quicker and more vigorous immune responses compared to primary responses. This is achieved via the previous generation of long-lived memory T and B cells during the clonal expansion of antigen-specific lymphocytes in the primary response (Sprent and Surh 2001; McHeyzer-Williams and McHeyzer-Williams 2005). Secondary antibody responses reach higher concentrations and produce Igs with higher affinities for the antigen. The increase in the antibody affinity is called affinity maturation and results from selection events suffered by the BCR throughout the differentiation of B cells (McHeyzer-Williams and McHeyzer-Williams 2005). By contrast, the TCR does not undergo this affinity maturation process, instead memory T cells change their responsive capacity having lower activation thresholds against the antigen (Sprent and Surh 2001).

It has to be taken into account that recent studies have established that the concept of immunological memory is not exclusive to adaptive immunity. Hence, the capacity to respond faster and more efficiently to an antigen re-exposure may also be associated with metabolic and epigenetic reprogramming of innate immune cells (termed trained immunity or innate immune memory), evoked by exogenous or endogenous insults, which lead to an altered response towards a second challenge after the return to a non-activated state (Topfer et al. 2015). In any case, the cognate immunological memory mediated by B and T lymphocytes is the basis for vaccination, the most effective method for preventing infectious diseases. Thus, the artificial induction of immunity against different pathogens constitutes one of the most important applications of immunology to human health. Vaccines pretend to create memory T and B cells that produce specific immune response quicker and of higher magnitude against the antigen to which the individual was immunized (Sallusto et al. 2010). This is achieved modifying the pathogen or its toxins in a way that can induce an immune response without producing disease. The main requirements for an effective vaccine depend on the nature of the microorganism nature. In extracellular infections, antibody responses are essential for the host protection, while activation of CD8 T cells is also required to fight intracellular microorganisms.

Most teleost fish possess an adaptive immune system capable of both humoral and cellular responses, specific antigen recognition and immunological memory development as per their mammalian counterparts. Still, a mosaic of noticeable differences exists among teleost species and with their mammalian counterparts. The goal of the present chapter is to provide an overview of the basic organization of the adaptive immune system in fish, detailing the distinct features that condition fish B- and T-cell responses.

3.2 Appearance of Adaptive Immune Responses in Fish

To date, most studies have suggested that adaptive immunity appeared during the early stages of vertebrate evolution, most likely in the disappeared placoderms (Cooper and Alder 2006). Accordingly, the genes that define adaptive immune systems such as Igs,

TCR, MHC I and MHC II, and RAG1 and RAG2 are present in gnathostomes (jawed vertebrates) including cartilaginous fish such as sharks (the most ancient jawed fish) and teleost fish. While these basic components of adaptive immunity are present, it must be taken into account that the adaptive branch of the immune system continued to evolve in tetrapods, reaching further degrees of specialization and sophistication in mammals.

Agnathans (lampreys and hagfish), on the other hand, are jawless vertebrates that lack these cognate adaptive molecules, as well as the classical primary and secondary lymphoid organs found in gnathostomes. However, agnathans possess cells with lymphocyte morphology and much of the lymphocyte molecular machinery (Mayer et al. 2002; Uinuk-Ool et al. 2002; Rothenberg and Pant 2004). In lampreys, a TCR-like gene containing only one copy of V- and J-like sequences, thus impairing the possibility of rearrangeable events, has been identified (Pancer et al. 2004). Nevertheless, leucine-rich repeat (LRR) proteins were identified in activated lamprey lymphocyte-like cells, and these seem to carry out similar functions to BCR and TCR genes (Pancer et al. 2004). These proteins known as variable lymphocyte receptors (VLRs) contain a high number of variable LRR units placed between less variable amino- and carboxyl-terminal LRR units. Additionally, VLRs contain a stalk-like region of invariant sequence that is bound to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor that permits its secretion. The germline configuration of the VLR gene appears incomplete and contains non-coding sequences that lack typical RNA splicing signals or recombination signal sequences. However, the flanking regions of the VLR gene are formed by a large number of cassettes that encode LRR units, which are randomly incorporated into the VLR gene by a multistep joining process (Alder et al. 2005). This mechanism for receptor variability generation is capable of generating a large VLR repertoire, similar to the repertoire for BCRs and TCRs. Subsequent studies have shown that lampreys contain one B-like and two T-like lymphocyte populations, according to the expression of an exclusive VLR isotype (VLRB in B-like cells and VLRA or C in T-like cells) (Kasamatsu et al. 2010; Hirano et al. 2013). Although agnathans are basal to gnathostomes, VLR features seem to be too different to the BCR/TCR recombination system to be its precursor. Furthermore, LRR proteins are present in a variety of unicellular organisms, plants and animals (especially cephalochordates) exerting different actions, including host defence responses (Pancer and Cooper 2006). Hence, the most accepted theory regarding the appearance of the adaptive immune system in jawed vertebrates established that RAG1 and RAG2 genes were once components of a transposable element (Agrawal et al. 1998).

3.3   Immune Organs Involved in Adaptive Immune Responses

Immune organs can be classified as primary and secondary lymphoid organs. Primary immune organs are sites for lymphocyte generation and maturation and in mammals include the thymus, foetal liver and bone marrow, whereas secondary organs are the sites

where mature lymphocytes interact with antigens and become activated and include the spleen, lymph nodes (LNs) and the mucosa-associated lymphoid tissues (MALTs).

The thymus is where T cells mature, while the foetal liver and bone marrow constitute the B-cell maturation organs in mammals. In the absence of bone marrow, teleost fish primary lymphoid organs include the thymus, the head kidney (HK) and the foetal liver. Recently, a structural analogue to the bursa of Fabricius, a primary immune organ present in birds, has been identified in Atlantic salmon (Løken et al. 2020). However, the expression of RAG1 and the absence of RAG2 transcripts suggest secondary lymphoid organ functions for this structure, in contrast to the primary immune function of its avian analogue.

In most teleosts, the thymus is placed near the gill cavity associated with the pharyngeal epithelium (Zapata 1981). The thymus structure can be divided into the medulla (inner zone) and the cortex (outer zone). The cortex is mostly composed of thymocytes (immature T cells) supported by a network of epithelial cells and it is where TCR rearrangements and positive selection take place. While the medulla contains fewer lymphoid cells and the network of epithelial cells is less organized than in the cortex, this part of the thymus is specialized in the negative selection of autoreactive T cells. In teleost fish, the structure of the thymus can be very variable depending on the species, but generally there is not a clear differentiation between the thymic medulla and cortex as that of mammals (Zapata 1981).

As mentioned before, the haematopoietic function of the mammalian bone marrow is substituted in fish by the HK (Zapata 1979). This organ is formed by two arms that penetrate under the gills and it is composed of a reticuloendothelial stroma with sinusoidal cells and reticular cells, similarly to the mammalian bone marrow. In addition to the lymphopoietic progenitors and mature lymphocytes, the HK contains active and inactive plasma cells (PCs). Similarly to the mammalian bone marrow that constitutes a survival niche for long-term PCs, it seems that plasmablasts and PCs in fish migrate back from secondary lymphoid organs to the HK where they are stored for long time periods (Bromage et al. 2004; Zwollo et al. 2005). Together with its haematopoietic and immune role, the fish HK is an endocrine organ homologous to mammalian adrenal glands. On the other hand, the posterior kidney is the renal organ in teleost fish but seems to also function as a secondary immune site in which many B and T cells are also found (Zapata 1979; Zwollo et al. 2005, 2010).

Once mature, B and T cells migrate into peripheral secondary organs where antigens are recognized and the immune response was triggered. In mammals, during thymus-dependent (TD) immune responses, activated B cells form structures called germinal centres (GCs) in the follicles of secondary lymphoid organs (Victora and Nussenzweig 2012). These GCs are the sites where antigen-specific B cells, T follicular helper cells (Tfh) and follicular dendritic cells (fDCs) interact to activate the differentiation of B cells by TD antigens. Activated B cells proliferate massively with some of them differentiating into PCs and/or memory B cells depending on the signals they receive in the GC. Alternative to this TD response, mammals have additional thymus-independent (TI) pathways to elicit faster antibody responses that are mainly orchestrated by innate B-cell subsets, mainly B1 cells or

marginal zone (MZ) B cells. These TI responses do not require T-cell cooperation, but are activated by BCR stimulation usually in combination with a direct recognition of pathogen motifs by innate receptors also expressed on B cells (Cerutti et al. 2011). Generally, the B-cell differentiation processes that take place outside the GC are designated as extrafollicular immune responses.

Teleost fish secondary immune organs include the spleen, the posterior kidney and the MALTs; however, fish lack lymph nodes and lymphoid follicles and conventional GCs have never been identified in these species. Interestingly, melanomacrophage centres (MMCs), which are aggregates of highly pigmented phagocytes, found mainly in the spleen and the posterior kidney of fish have been proposed as primitive GCs (Steinel and Bolnick 2017). In MMCs, B and T cells are found in association with MMs, which have been shown to retain antigens for long time periods. Despite this, much more evidence should be gathered to unequivocally establish them as primitive GCs (Steinel and Bolnick 2017). Nevertheless, the teleost spleen is the site where mature B cells encounter blood-borne antigens and become activated resulting in differentiated plasmablasts and PCs. In mammals, the spleen can be anatomically divided into white and red pulp. Most of the spleen is composed of red pulp where damaged erythrocytes are destroyed. The white pulp, in turn, constitutes the lymphoid tissue, and it is constituted by different areas: the periarteriolar lymphoid sheaths (PALS); the lymph follicles; and the MZ. In fish, the difference between red and white pulp is less notable than in superior vertebrates and the white pulp appears to be less developed.

Regarding the MALTs, four main mucosal immune tissues have been identified in fish: (1) the gut-associated lymphoid tissue (GALT), (2) the skin-associated lymphoid tissue (SALT), (3) the gill-associated lymphoid tissue (GIALT) and the nasopharynx-associated lymphoid tissue (NALT).

Although the mammalian GALT contains some isolated lymphoid follicles scattered along the lamina propria (LP) and some intraepithelial lymphocytes (IELs), it is mainly formed by Peyer's patches, which are aggregations of lymphoid follicles where B cells, T cells, macrophages and DCs interact and GCs are formed. However, fish do not contain Peyer's patches nor do they organize GCs in the GALT. In fish, B and T cells are dispersed through the mucosa, both within the LP or as IELs. Thus, these B- and T-cell responses might best resemble mammalian extrafollicular responses, but how these cells detect antigens and become activated still remains mostly unknown. This diffuse organization of B and T cells throughout the mucosa has also been described in SALT, GIALT and NALT (Salinas 2015). In the case of the GIALT, an organized lymphoid structure with high numbers of T cells has been detected on the caudal edge of the interbranchial septum in salmonids, designated as interbranchial lymphoid tissue (ILT) (Haugarvoll et al. 2008; Koppang et al. 2010). Yet, the exact role of this tissue in the immune response remains unsolved.

3.4   B cells

Humoral adaptive immune responses are mediated by B cells. B cells have the capacity to recognize soluble antigens and synthesize specific antibodies owing to their membrane antigen receptor or BCR. The BCR complex is composed of an Ig (slightly modified to be tethered to the membrane) and by two invariant chains, CD79α and CD79β (or Igα and β). In this complex, the Ig is the one that recognizes the antigen and the CD79 proteins mediate the signal transduction to the cellular nucleus.

In addition to producing antibodies, B cells in both mammals and fish are also involved in antigen presentation. The antigen presentation requires a first interaction of the BCR with the native antigen and its subsequent internalization and processing to expose the antigenic fragments in membrane MHC class II molecules (Rodriguez-Pinto 2005). The presentation of this specific antigen through the BCR occurs with very high efficiency and is associated with B-cell activation, resulting in the activation of cognate T cells. In mammals, however, the presentation of non-specific antigens by B cells is dissociated from B-cell activation generally leading to the inactivation of T cells (Rodriguez-Pinto 2005).

In contrast to conventional mammalian B cells, teleost B cells have a strong phagocytic capacity (Li et al. 2006). This additional capacity to internalize particulate antigens is thought to increase the potential of teleost B cells as antigen-presenting cells (APCs) (Zhu et al. 2014), especially in what concerns particulate antigens. Furthermore, this phagocytic capacity is also related to a microbicidal role, demonstrated in rainbow trout (*Oncorhynchus mykiss*) (Zhang et al. 2017). Hence, it seems that teleost B cells also play an important role in innate responses and early clearance of pathogens, similarly to mammalian B1 cells. In mammals, B1 cells arise early during the ontogeny and are considered components of the innate immune system. They are found primarily in the peritoneal cavity, mucosal surfaces, and spleen where they account for 2% of the spleen lymphocyte population. These cells are responsible for an early recognition of pathogens and production of natural IgMs that help clear pathogens until a specific conventional B-cell response is mounted (Martin and Kearney 2001). This first wave of antibodies is produced by B1 cells independently of T cells outside GCs (extrafollicularly) (Martin et al. 2001). In this context, recent studies have reported many functional and phenotypical resemblances between teleost B cells and mammalian B1 cells, in addition to the strong phagocytic capacity also identified in mammalian B1 cells (Gao et al. 2012; Parra et al. 2012a). These include the transcription of a broad range of innate receptors (Abos et al. 2013); a low IgD and high IgM surface expression; extended survival in cell culture; lack of proliferation upon BCR engagement (Abos et al. 2018a); and expression of B1-specific markers, such as CD9 and CD5 (Castro et al. 2015; Abos et al. 2018a). Collectively, these lines of evidence point to the hypothesis that mammalian B1 lineage arose from ectotherm IgM$^+$ B cells and that B2 cells emerged later in evolution as a more efficient population specialized in adaptive immune responses (Sunyer 2012; Zhang et al. 2017). These shared

characteristics between fish B cells and mammalian B1 cells will surely condition the way in which fish B-cell responses are orchestrated.

3.4.1 B-cell Development

In mammals, B-cell generation starts in the foetal liver from multipotential stem cells. In early stages of the development, the liver is replaced by the bone marrow that becomes the main B-cell production site (Kantor and Herzenberg 1993). From the bone marrow, B cells are generated during all the lifespan decreasing progressively over time. Early precursor cells in the bone marrow have not started to rearrange their Ig genes yet, having them in the germline configuration. B-cell differentiation requires the presence of stromal cells in the bone marrow (adipocytes, fibroblasts, reticulocytes, endothelial cells) that provide direct contacts and soluble factors to induce B-cell maturation. This maturation process produces important changes in the phenotype of B cells with the expression of specific proteins in each stage (summarized in Fig. 3.1). The Ig gene rearrangement is accomplished during late pro-B- and pre-B-cell differentiation phases, and it is possible to see IgM heavy chains in the membrane of pre-B cells, conforming the pre-BCR. Pre-B cells become immature B cells that express membrane IgM molecules, but it is only when B cells mature that they exhibit IgM and IgD molecules at the same time in the membrane. Throughout the maturation process, immature B cells that interact with self-antigens abundant in the bone marrow are eliminated or inactivated in a negative selection process to avoid autoreactive immune responses (Kantor and Herzenberg 1993). In teleosts, given the lack of bone marrow, this B-cell development occurs in the HK. Accordingly, the rainbow trout HK has been shown to express genes encoding RAG-1/2, terminal deoxynucleotidyl transferase (TdT) (enzyme that adds N-nucleotides to the V, D and J exons) and Ikaros (a transcription factor that regulates lymphocyte differentiation/proliferation) and to contain B cells in multiple maturation stages (Zwollo et al. 2005, 2010). Despite this, to date, a full phenotypical characterization of the developmental stages of B cells has not yet been undertaken in fish.

3.4.2 Immunoglobulins and B-cell Subsets

Igs are composed typically of two identical heavy chains (IgH) and two identical light chains (IgL) bound by disulphide bonds. Each chain contains one N-terminal variable domain (VH and VL) and one or more C-terminal constant domains (CH and CL). The variable regions from both, the heavy and the light chains, are assembled via somatic gene rearrangement from an array of multiple V, D (not present in light chains) and J segments during B-cell development. The variable domains contain three hyper-variable regions named complementarity-determining regions (CDR) that are crucial for antigen specificity. The constant region on the other hand mediates the effector functions of the different Igs.
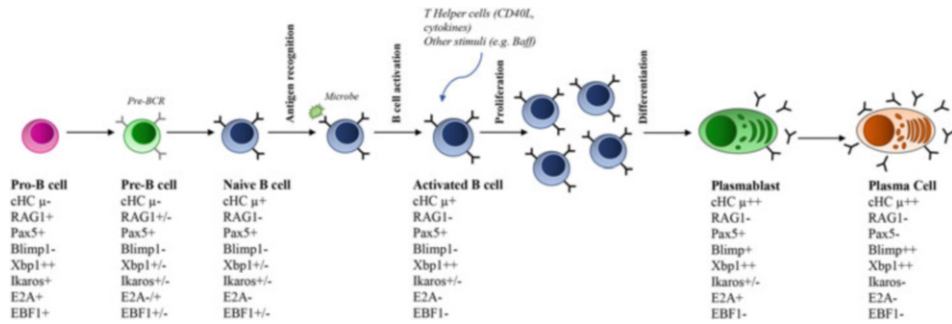
Fig. 3.1 Stages of B-cell development and differentiation. The levels of expression of the cytoplasmic Ig heavy chain μ, the recombinase RAG1 and the transcription factors Pax5, Blimp1, Xbp1, Ikaros, E2A and EBF1 are indicated in each stage. Adapted from Zwollo (2011)

Three types of Igs have been identified in teleost fish: IgM, IgD and IgZ/T since no homologues to mammalian IgA, IgG or IgE are present. As studied in zebrafish (*Danio rerio*) and rainbow trout, the genes in the IgH chains show a particular organization, with upstream VH-DJCμCδ, elements for the H chains of IgM (μ) and IgD (δ) and an exclusive VH-DJCτ for the H chain of IgT (τ) (Danilova et al. 2005; Hansen et al. 2005). Thus, alternative splicing between the recombined VDJ region and Cμ (IgM) or Cδ (IgD) occurs to generate IgM or IgD, rendering IgM and IgD of the same specificity expressed on a single naïve B cell. On the contrary, fish B cells express either IgM or IgT as V segments rearrange either to DJCτ or to DJCμ (Zhang et al. 2011). Therefore, IgT cells seem to constitute an independent B-cell linage in which IgM and IgD are not expressed (Zhang et al. 2010).

IgM was the first Ig identified in fish and is the most abundant Ig in plasma (Flajnik 2002). It can be found as a transmembrane surface protein or secreted as an antibody. Surface IgM is one domain shorter than the secreted form, lacking the Cμ4 domain due to an alternative splicing (Ross et al. 1998). IgM forms tetramers in both serum and mucus, although some studies have reported monomers of IgM in species such as margate (Clem 1975), sheepshead (*Archosargus probatocephalus*) (Lobb and Clem 1981) or rainbow trout (Elcombe et al. 1985). As in higher vertebrates, the teleost IgM isotype achieves multiple effector functions in the humoral immune responses. Fish IgM can activate the complement (Cooper 1985; Boshra et al. 2004), induce agglutination, bind mannose-binding lectin (MBL) motifs (Arnold et al. 2006) and mediate cellular cytotoxicity (Ohlander and Perlmann 1982; Stafford et al. 2006). Furthermore, teleost IgM has been shown to increase the phagocytic capacity of macrophages through antigen-specific opsonization (Soto et al. 2011a, b).

IgD has been described in many vertebrate classes, including teleost (Ohta and Flajnik 2006), but its precise role remains an enigma. Interestingly, orthology between IgD and IgW (also known as IgX, IgNARC or IgR), an Ig-like molecule present in cartilaginous fish, lungfish and coelacanths, suggested that IgD may be as ancient as IgM (Ohta and

Flajnik 2006; Saha et al. 2014). This implies that IgD may play an important but still elusive role in adaptive immunity (Gutzeit et al. 2018). Teleost IgD is characterized by a Cμ1 domain followed by a different number of Cδ domains that can range from 2 to 16, depending on the species. Thus, the fish IgH δ locus is composed of a rearranged VDJ spliced to Cμ1, a variable number of C domains and a transmembrane tail (Bengten et al. 2006; Stenvik and Jorgensen 2000; Srisapoome et al. 2004; Xiao et al. 2010). Both membrane and secreted IgD have been found in different species of teleost fish including channel catfish (*Ictalurus punctatus*), rainbow trout (*O. mykiss*), Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*) and grass carp (*Ctenopharyngodon idella*) (Bengten et al. 2006; Ramirez-Gomez et al. 2012).

IgT is an exclusive Ig of bony fish discovered in 2005 in rainbow trout (IgT) and zebrafish (where it was designated as IgZ) (Danilova et al. 2005; Hansen et al. 2005). The majority of teleost IgT contains 4 C domains, although this can differ in some species (Salinas et al. 2011). IgT has been reported to be specialized in mucosal responses, since it was found that the ratio of IgT:IgM was much higher in gut mucus than in the serum of rainbow trout (Zhang et al. 2010). Similarly, the ratio of IgT$^+$ B cells to IgM$^+$ B cells was also higher in mucosal tissues than in systemic compartments (Zhang et al. 2010). Furthermore, in response to some parasitic infections, IgT production was increased in the mucosal surfaces such as the gut (Zhang et al. 2010), gills (Xu et al. 2016) or skin (Xu et al. 2013), while the changes in IgM were limited to systemic compartments. IgT is a monomer in rainbow trout serum and a tetramer in the mucus (Zhang et al. 2010), where it has the capacity to pass through the mucosal epithelium owing to the polymeric Ig receptor (pIgR). Despite these results, it should be noted that systemic IgT responses have also been reported (Castro et al. 2013, 2014b; Abos et al. 2018b), along with mucosal IgM and IgD responses; therefore, the precise role of IgT in the adaptive immune response of fish remains largely unexplored.

In addition to IgT$^+$ B cells, which constitute an independent B-cell linage from IgM- and IgD-bearing cells (Zhang et al. 2010), different B-cell subsets can be distinguished in fish according to the pattern of surface IgM and IgD expression. As in mammals, mature IgM$^+$IgD$^+$ B cells constitute the main B-cell subset in lymphoid tissues and peripheral blood (Tafalla et al. 2017; Simon et al. 2019). Upon activation, these cells lose surface IgD to become IgM$^+$IgD$^-$ B cells with a plasmablast profile (Tafalla et al. 2017; Granja and Tafalla 2019). Moreover, through some still unknown mechanisms, certain cells lose surface IgM and become IgD$^+$IgM$^-$ B cells. These cells have been identified in catfish blood (Edholm et al. 2010) and in rainbow trout gills (Castro et al. 2014a) and gut (Perdiguero et al. 2019). In rainbow trout, these cells were shown to secrete IgD, which was found bound to intestinal bacteria (Perdiguero et al. 2019), as previously shown for IgT and IgM (Zhang et al. 2010). Interestingly, the intestinal bacteria were found to regulate IgD transcription in the gut (Perdiguero et al. 2019). This suggests a two-way interaction between IgD and the intestinal microbiota that points to an important role of IgD in intestinal homeostasis, maybe complementing the functions of IgM and IgT in this mucosa. In humans, a small subset of mammalian B cells has also been shown to undergo an

unconventional form of class switch recombination (CSR) from IgM to IgD and thereafter differentiate into IgD$^+$IgM$^-$ plasmablasts specifically secreting IgD (Arpin et al. 1998; Koelsch et al. 2007; Chen et al. 2009; Rouaud et al. 2014; Choi et al. 2017). Although also detected in the general circulation, these IgD class-switched cells mostly inhabit the organized lymphoid tissue from nasopharyngeal cavities, including human tonsils (Arpin et al. 1998; Koelsch et al. 2007; Chen et al. 2009), but have never been identified in the human gut. Secreted IgD in humans has been shown to bind to basophils through a calcium-mobilizing receptor, inducing the release of antimicrobial peptides and pro-inflammatory cytokines (IL-1, IL-4) (Arpin et al. 1998; Chen et al. 2009; Koelsch et al. 2007). Whether secreted IgD plays a similar regulatory role in fish is still unknown.

3.4.3 B-cell Differentiation

In mammals, naïve mature B lymphocytes developed in the bone marrow recirculate throughout secondary lymphoid organs where they can encounter specific antigens. Antigen recognition together with the adequate co-stimulatory signals (provided by Th cells in the case of TD antigens or by innate signals in the case of TI antigens) activates B cells inducing their proliferation and differentiation. B cells have the capacity to endocytose the complex Ig/antigen, allowing the antigen to be processed and presented by their MHC II molecules to Th cells. For this, both B and T cells must be specific for the same antigen but not necessarily the same epitope. When Th lymphocytes recognize the specific peptides, they proliferate and differentiate to further activate antigen-specific B lymphocytes (Lanzavecchia 1985). In this interaction, the recognition of CD40 ligand (CD40L) expressed on the surface of Th cells by CD40, expressed on the surface of B cells, is essential for the activation of B cells. In addition, the secretion of cytokines by Th cells further conditions B-cell activation. For example, interleukin 4 (IL-4) secreted by Th2 cells induces B-cell clonal expansion in synergy with CD40L. In rainbow trout, the effect of cytokines commonly produced by T cells, namely IL-2, IL-4/13B (homologous to both IL-4 and IL-13), IL-10 and IL-21, on splenic IgM$^+$ B cells alone or in combination with CD40L was recently studied (Abos et al. 2020). The authors reported that the cytokines had significant effects on IgM$^+$ B-cell survival, proliferation and IgM secretion either individually or in cooperation with CD40L. Additionally, factors secreted by innate cells such as DCs or macrophages are known to condition B-cell activation both within the GCs and in extrafollicular responses. Among these cytokines, additional members of the tumour necrosis factor (TNF) family such as BAFF (B-cell-activating factor) (Tafalla et al. 2017) or APRIL (a proliferation-inducing ligand) (Soleto et al. 2018b) have been shown to play an important role in B-cell survival and differentiation also in fish.

Within GCs, mammalian B cells undergo profound changes in the structure of their Igs by means of two different processes: CSR and somatic hypermutation (SHM). Through CSR, the constant region of IgM is replaced by that of other Igs with higher affinity and different effector functions. This process is mediated by the activation-induced cytidine

deaminase (AID) that replaces the constant region of IgM by that of IgG, IgA or IgE (Nera et al. 2015). Concerning IgD, it is generally accepted that the δ gene is co-expressed with μ by differential transcription and alternative splicing, both sharing the same antigen-binding variable region (Enders et al. 2014). However, as mentioned above, a possible unconventional form of CSR has been suggested in the case of IgD-secreting plasmablasts (Rouaud et al. 2014). Throughout the differentiation process that takes place within the GC, the Igs undergo affinity maturation as a consequence of SHM. In this context, point mutations are introduced in the Ig variable regions through the action of AID. Subsequently, B cells with high-affinity receptors are rescued in a positive selection, while those with less affinity than the B cell they derive from will be eliminated. These positively selected B cells will then differentiate to long-lived PCs or to memory B cells according to the signals they receive in the GC. Memory B-cell differentiation relies on CD40/CD40L new contacts with Tfh cells and memory B cells are characterized by a slow division capacity and non-Ig secretion. Meanwhile, plasmablast/PC differentiation appears to depend on the CD23-expressing fDC and the co-receptors CD19/CD21/CD81 in the B cell. Plasmablasts have an increased capacity to secrete Igs, but they are still dividing and share many of the characteristics of activated B cells that allow them to interact with T cells. After several days, plasmablasts stop their proliferation and either die or differentiate to PCs. Hence, PCs have a greater capacity to secrete Igs but lose their dividing capacity. The terminal differentiation of B cells is controlled in mammals by a complex regulatory network in which the transcription factor Blimp1 (B lymphocyte-induced maturation protein 1) acts as a key regulator. Blimp1 acts as a transcriptional repressor of relevant genes involved in the functionality of undifferentiated B cells, along with other molecules such as X-box-binding protein 1 (Xbp1) and interferon regulatory factor 4 (IRF4), also known to be critical to acquire a plasma cell phenotype (Calame 2001; Shapiro-Shelef and Calame 2005). Blimp1 also represses the promoters of genes involved in functions of mature naïve B cells such as Pax5 (Lin et al. 2002), Bach2 (Ochiai et al. 2006) or Bcl6 (Shaffer et al. 2002). Eventually, PCs migrate to the bone marrow where they receive signals from the stromal cells that allow them to survive for a long time maintaining high-affinity antibodies in the serum (Slifka et al. 1995).

Affinity maturation in fish and other ectotherms has been shown to be much less efficient than in mammals (Wilson and Warr 1992; Warr 1995) even though the expression of AID has been reported in many fish species, with this teleost AID maintaining the capacity to induce point mutations and even mediating class switch in murine B cells (Barreto et al. 2005; Quinlan et al. 2017). For instance, rainbow trout immunized with a TD antigen such as TNP-KLH along with an adjuvant developed IgM antibodies that after a few months reached a 100-fold increase in antigen affinity when compared to antibodies produced the first 5 weeks post-immunization (Ye et al. 2011). However, this increase in antigen affinity is much lower than that reported in mammals. Nevertheless, the presence of plasmablasts and PCs has been demonstrated in some fish species, especially rainbow trout, where studies characterizing these two cell subsets were performed (Bromage et al. 2004; Zwollo et al. 2005; Zwollo 2011).

Despite all the innate-like features of fish B cells and the lack of organized structures where B and T cells can meet, a recent study performed in rainbow trout demonstrated that fish B cells respond to CD40L in a similar way to mammalian B2 cells, upregulating IgM$^+$ B-cell survival and proliferation and inducing PC differentiation (Granja et al. 2019). It should be taken into consideration, however, that some innate cells such as DCs also have the capacity to produce CD40L, as demonstrated in both mammals (Tough 2009) and fish (Granja et al. 2019); thus, it may be possible that under some circumstances innate cells can provide B cells with these 'TD' stimulatory signals.

In the absence of additional adjuvants, rainbow trout B cells were much more responsive to TI antigens (TNP-LPS) than to TD antigens (TNP-KLH) (Granja et al. 2019). TI antigens, opposed to TD antigens, provide B cells with co-stimulatory signals through innate receptors in addition to the BCR (Cerutti et al. 2011). Therefore, these results suggest the importance of innate activation to induce the differentiation of fish B cells. In this sense, fish B cells have been shown to be equipped with a wide range of innate receptors such as Toll-like receptors (TLRs) (Abos et al. 2013) and to be highly responsive to TLR ligands such as LPS (Abos et al. 2016) and CpGs (Simon et al. 2019) that on their own have the capacity to induce the proliferation and antibody secretion of rainbow trout IgM$^+$ B cells. Along this line, a recent study by Soleto and collaborators revealed that TLR signalling is essential for the activation of IgM$^+$ B cells by the bacterial pathogen *Aeromonas salmonicida* (Soleto et al. 2020).

Regarding the characterization of the B-cell differentiation process in fish, already in 2011, Zwollo and collaborators established different phenotypes associated with the expression of different transcription factors such as EBF (early B-cell factor 1), Pax5 and Xbp-1 (X-box-binding protein 1), being able to discriminate between early B-cell progenitors, late developing B cells, resting B cells, activated B cells, plasmablasts and PCs (Zwollo 2011). Additionally, Blimp1 transcription was shown to increase upon activation of B cells with IL-6 (Abos et al. 2016). Interestingly, other stimuli able to activate B-cell proliferation and IgM secretion did not imply an upregulation of Blimp1 transcription. This is the case for BAFF (Tafalla et al. 2017), APRIL (Soleto et al. 2018b) or LPS (Abos et al. 2016). Finally, a recent work provided insights on how the Blimp1 gene family evolved in fish, demonstrating that four homologues of Blimp1 are present in salmonids, all of them with a yet not well-defined role in B-cell activation (Perdiguero et al. 2020). Therefore, it might be possible that different Blimp1 genes are involved in the terminal differentiation of different B-cell subsets or that they are involved in different steps. Alternatively, B cells could be activated to a plasmablast state in response to some stimuli that might not require the expression of any of these Blimp1 homologues.

3.4.4 Antibody Responses

The production of specific IgM antibodies has been demonstrated against viruses (Lorenzen and LaPatra 1999), bacteria (Ellis 1999) and parasites (Alvarez-Pellitero 2008;

Sitja-Bobadilla et al. 2016). In the case of viral agents, the capacity of IgM to neutralize viral replication has also been revealed in some conditions. For example, neutralizing serum IgMs produced against fish rhabdoviruses in rainbow trout have been broadly characterized (Winton et al. 1988; Lorenzen and LaPatra 1999). Neutralizing IgM antibodies with the capacity to limit pathogen progression have also been reported against several bacteria and parasites such as *Aeromonas salmonicida* (Hirst and Ellis 1999), *Francisella asiatica* (Soto et al. 2011b), *Ceratomyxa shasta* (Zhang et al. 2010) or *Cryptobia salmositica* (Ardelli and Woo 1997). In some cases, the capacity of these specific antibodies to provide protection against the pathogen has been demonstrated in passive immunization experiments (LaPatra et al. 2010; Hershberger et al. 2011; Soto et al. 2011b). Still, the precise contribution of pathogen-specific serum IgMs to pathogen clearance in natural infections in fish is still not clear, as in many cases specific IgM levels peak weeks after the mortality terminates (Bernard et al. 1983; Olesen and Verstergaard Jørgensen 1986).

Most studies aimed at investigating antibody production in response to different pathogens are focused on IgM and very few on other isotypes such as IgD or IgT. Although IgD is known to be secreted in many teleost fish species (Ramirez-Gomez et al. 2012), the production of antigen-specific IgD has never been demonstrated in fish. However, a few studies have revealed changes in total IgD levels, suggesting a role of this Ig isotype in the response to pathogens. In rainbow trout, for example, immunization with an attenuated *Flavobacterium psychrophilum* significantly increased the levels of secreted IgD in gills and gut (Makesh et al. 2015). The levels of transcription of IgD and the presence of IgD$^+$ B cells also increased in the skin of rainbow trout infected with *F. psychrophilum* (Munoz-Atienza et al. 2019). Finally, an upregulation of IgD transcription in response to viral, bacterial or parasitic antigens has been reported in freshwater carp (*Catla catla*) (Banerjee et al. 2017) and rohu (*Labeo rohita*) (Basu et al. 2016).

As with IgD, only a few studies have demonstrated the production of antigen-specific IgT. In rainbow trout infected with the intestinal parasite *Ceratomyxa shasta,* specific IgMs to the parasite were found in serum, whereas specific IgT was exclusively found in the gut mucus (Zhang et al. 2010). Other studies have further pointed to the *preferential production* of IgT in mucosal secretions when compared to systemic compartments, but analysing total Ig levels and not antigen-specific Igs (Piazzon et al. 2016; Buonocore et al. 2017; Tongsri et al. 2020). On the other hand, several studies have demonstrated significant IgT responses outside the mucosal compartments that point to the presence of low but significant IgT production in systemic compartments in response to some stimuli/pathogens. For example, IgT responses were evident in the spleen after intramuscular injection with inactivated VHSV (viral haemorrhagic septicaemia virus) (Castro et al. 2013), in the muscle of fish vaccinated with a DNA vaccine against VHSV (Castro et al. 2014b) or in the kidney of rainbow trout affected by proliferative kidney disease (PKD) (Abos et al. 2018b).

Interestingly, some teleost species such as the Atlantic cod lack MHC II genes, CD4 and the invariant chain (Ii) that facilitates peptide loading onto MHC II (Landsverk et al. 2009).

This loss is accompanied by an elevated number of MHC I genes (Persson et al. 1999), but it is unclear if this expansion is related to the loss of the MHC II pathway (Malmstrom et al. 2013). As a consequence, the production of specific antibodies seems impaired in this species, although there is some controversy. Some studies have reported a poor antibody response of cod (Pilstrom et al. 2005), whereas other studies obtained a significant specific IgM response in this species to different bacteria such as *Vibrio anguillarum, Aeromonas salmonicida* and *Francisella* sp. (Schroder et al. 2009) or to antigens of the anisakis nematode *Contracaecum osculatum* (Mohamed et al. 2020).

3.4.5 B-cell Memory Responses

Very few studies have explored whether immune memory is established in teleost fish. One of the first studies that described B-cell memory was performed in rainbow trout. In that study, when fish were immunized with TNP-KLH along with an adjuvant, a more potent response was found in the secondary immune response in comparison with the primary response (Arkoosh and Kaattari 1991). Nevertheless, most reports sustain that affinity maturation of serum IgM after immunization is weak (Cain et al. 2002; Kaattari et al. 2002; Ye et al. 2011). For example, Kaattari et al. showed a two- to threefold increase in affinity in IgMs obtained from rainbow trout immunized with FITC-KLH from week 4 to week 12 post-immunization (Kaattari et al. 2002). As mentioned before, other studies reported increases in affinity of up to 100-fold (Ye et al. 2011), although this increase is still much lower than that observed in mammals.

Primary and secondary antibody responses in teleost show a similar pattern to mammalian responses regarding the distribution of antibody-secreting cells (ASCs) (Ma et al. 2013). After the intraperitoneal immunization of rainbow trout with either TNP-LPS or TNP-KLH, the spleen was the tissue that contained the highest number of ASCs during the primary response, while the HK was the predominant organ during the secondary immune response, showing the strongest PC activity (Ma et al. 2013). Interestingly, the numbers of ASCs reached in response to TNP-LPS during the secondary response were higher than those obtained in response to the TD antigen TNP-KLH (Ma et al. 2013). Similarly, when rainbow trout were anally immunized with TNP-LPS or TNP-KLH, the spleen was the site where most TNP-specific ASCs were found at early time points, while it was the kidney where these cells were mostly found at later time points (Martin-Martin et al. 2020). In this case, in the absence of additional adjuvants, the response to TNP-LPS was much higher than that observed in response to TNP-KLH.

3.5   Adaptive Cellular Responses: T Cells

Cellular adaptive immune responses are mediated by T lymphocytes. Unlike B cells that can directly recognize antigens, T cells generally react to antigens that have been processed by APCs and are exposed on the cell surface in the context of MHC I or MHC II molecules. This recognition is performed throughout their antigen receptor or TCR that always remains membrane-bound. Attending to the nature of their TCR, T cells can be classified into αβ T cells (formed by an α and a β chain) and γδ T cells (formed by a γ and a δ chain). In mammals, αβ T cells are the most abundant T cells in peripheral blood and lymphoid organs, whereas γδ T cells are less common and predominantly found in mucosal tissues (Bonneville et al. 2010). Interestingly, most γδ T cells are activated by antigens in an MHC-independent manner.

From a functional point of view, T cells can be divided into two general populations: cytotoxic T cells (Tc) that express the CD8 co-receptor that interacts with MHC class I and helper T (Th) cells that express CD4 molecules that interact with MHC class II. Both co-receptors stabilize the interaction between the TCR and the MHC molecules. CD8$^+$ T cells are cytotoxic T cells responsible for directly destroying target cells, whereas CD4$^+$ T cells are T helper cells that contribute to the activation of other immune cells. Among these effects, the activation of B cells by CD4$^+$ T cells after the recognition of specific antigens in the context of MHC II on the surface of the B cell delivers the needed activating signals for B cells to mount efficient TD responses as already explained.

3.5.1   T-cell Development

T cells develop in the thymus from common lymphoid progenitors coming from the bone marrow or foetal liver. Thymic stromal cells that include epithelial cells, DCs and macrophages interact with the thymocytes through their MHC I and MHC II molecules, accessory molecules and cytokines in order to orchestrate the maturation of T cells (Takahama 2006). The most immature thymocytes lack TCR, CD4 and CD8 molecules, but then quickly divide and express a pre-TCR that precedes the definitive αβ receptor. This pre-TCR contains a rearranged TCRβ chain together with an invariant protein called the pre-Tα. Later, the thymocyte starts the rearrangement of its TCRα chain that will replace the pre-Tα, interrupting any further recombination process to guarantee allelic exclusion (ensuring that only one specific TCR is expressed in each T lymphocyte) (von Boehmer et al. 1998). At this stage, thymocytes often express CD4 and CD8 simultaneously and suffer a process of positive selection in the thymic cortex by which only the thymocytes that recognize self-MHC molecules will survive (Fowlkes and Schweighoffer 1995). Later, autoreactive thymocytes are negatively selected in the thymic medulla by APCs. The result of this process is a population of mature naïve αβ T cells that will abandon the thymus and recirculate between the blood and lymphoid organs searching for a specific antigen through its TCR (Takahama 2006).

Cartilaginous and bony fish are the most primitive vertebrates with a histologically distinguishable thymus (Chilmonczyk 1983). All evidence suggests that the primary T-cell differentiation and maturation occur in the thymus similarly as in mammals. Interestingly, experiments performed in adult fish from different teleost fish species have confirmed a high presence of T cells in mucosal surfaces such as gills (Koppang et al. 2010) or intestine (Rombout et al. 1998; Romano et al. 2007). This together with the identification of RAG1 expression in these tissues (Huttenhuis et al. 2006; Picchietti et al. 2011) suggests the possibility of an extra-thymic development of T cells in some specific mucosal surfaces.

3.5.2   T-cell Receptors (TCR) and Co-receptors

Similarly to the BCR, the TCR undergoes RAG-mediated V(D)J rearrangement to create a wide range of repertoires. The structure of the TCR is well conserved in most vertebrates, containing a heterodimer of type I transmembrane glycoproteins (α and β chains or γ and δ chains) with extracellular V and C Ig domains and a short cytoplasmic tail. The constant region is proximal to the cell membrane, while the variable region binds to the peptide/ MHC complex. The TCR is attached to the signalling CD3 complex and recognizes co-stimulatory and co-inhibitory molecules to regulate the activation/inhibition state of the T cell (Castro et al. 2011).

The TCR genes are TRA, TRB, TRG and TRD encoding for the TCR-α, β, γ, and δ chains, respectively. The TCR can be formed by a heterodimer of α and β chains (αβ-TCR) or a heterodimer of γ and δ chains (γδ-TCR). TRA and TRG undergo VJ recombination, whereas TRB and TRD go through VDJ recombination. All these TCR genes are well conserved in evolution and have been described in many fish species (Castro et al. 2011). Similarly to mammals, the TCR gene segments in fish are organized in a translocon arrangement (Criscitiello et al. 2010; Castro et al. 2011). The variable domain of the α and β chains has 3 CDRs from which CDR3 is the main responsible for recognizing the processed antigen. The constant domain of the TCR consists of short connecting sequences in which a cysteine residue forms disulphide bonds, which link the two chains. Interestingly, a new TCR chain (TCRμ) was discovered in 2007 in marsupials and monotremes with high similarity to a TCRδ isoform in sharks, probably arising from an ancient receptor system (Parra et al. 2012b).

CD4⁺ T cells recognize peptides presented via MHC II molecules. Once activated, they release cytokines that can activate and regulate the immune responses. The CD4 molecule is a monomer with four extracellular Ig-like domains and a cytoplasmic tail that contains a CxC motif. This motif binds to the tyrosine kinase Lck, activating the intracellular signalling (Gratton et al. 2000). Even though fish CD4 protein sequences do not show a high similarity with those of higher vertebrates, the gene organization and the crucial residues are well conserved. In teleost, CD4 has been identified in several species, including fugu, rainbow trout, channel catfish, sea bass (*Dicentrarchus labrax*), Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic salmon [reviewed in Ashfaq et al.

(2019)]. Two different types of CD4 molecules have been described in bony fish: CD4-1 containing four Ig domains, as in tetrapods, and CD4-2, which contains either two or three Ig domains, depending on the species (Laing et al. 2006). CD4-2 might be the primitive CD4 molecule from which CD4-1, with its 4 Ig domains, would have emerged following duplication of the two Ig domains (Laing et al. 2006). Interestingly, CD4-2 was expressed in a Treg-like subset with a CD25-like$^+$Foxp3-like$^+$ phenotype from a pufferfish model, suggesting different functional roles between the two CD4 populations (Wen et al. 2011). Finally, salmonids contain two CD4-2 molecules: CD4-2a, also known as CD4-related; and CD4-2b (Moore et al. 2009).

CD8$^+$ T cells recognize peptides presented via MHC-I molecules and release the cytotoxins perforin, granzymes and granulysin. As a result of the perforin action, granzymes enter the cytoplasm of the target cell and activate apoptosis. The CD8 molecules may appear as homodimers composed of two α chains (CD8αα) or heterodimers formed from one α chain and one β chain (CD8αβ) (Cole and Gao 2004). In mammals, mature Tc cells containing αβ-TCRs mostly contain CD8αβ molecules. In contrast, most γδ T cells do not express CD8 (Kabelitz et al. 2000). Interestingly, a minor population of intestinal epithelial T cells (both αβ and γδ T cells) express CD8αα (Jarry et al. 1990). In teleost fish, both CD8 chains have been well characterized in different species and the structure of the CD8 genes seems to be conserved throughout evolution. Interestingly, CD4 and CD8 molecules are expressed not only on T cells but also in other cell types as reported in mammalian DC subsets (Vremec et al. 2000). For example, Takizawa et al. identified a CD4$^+$ monocyte/macrophage population in rainbow trout (Takizawa et al. 2016), while CD8α is also expressed in a DC-like population described in different rainbow trout mucosal surfaces (Granja et al. 2015; Soleto et al. 2018a, 2019).

T-cell activation requires the CD3 protein complex associated with the TCR to generate the activation signal within the cell. CD3 is composed of four distinct chains: a CD3γ chain, a CD3δ chain, two CD3ε chains and two CD3ζ chains. The extracellular domains of CD3 ε, γ and δ contain an Ig-like domain, and the cytoplasmic tail contains a single ITAM (immunoreceptor tyrosine activation motif). CD3ζ chains, in turn, possess three ITAMs in their cytoplasmic domains. CD3 in teleost fish seems to have a conserved structure with that of mammalian CD3 (Shang et al. 2008; Overgard et al. 2009).

Finally, additional co-stimulatory signals are required for the complete activation of naïve T cells. This further stimulation is given by the T-cell receptor CD28, when it binds to their ligands: B7.1 (CD80) and B7.2 (CD86) expressed on the APCs. On the other hand, CTLA-4 (cytotoxic T lymphocyte-associated protein 4) is expressed after T-cell activation and transmits inhibitory signals to T cells. This protein also binds to CD80 and CD86 molecules but with higher affinity than CD28. Both CD28 and CLTA-4, as well as orthologues of CD80 and CD86 ligands, have been found in different teleost species (Bernard et al. 2006; Hansen et al. 2009; Sugamata et al. 2009; Zhang et al. 2009, 2018). The binding sites for CD80 and CD86 are evolutionarily conserved in teleost CD28 and CTLA4 molecules (Bernard et al. 2006). Similarly to mammals, rainbow trout increases

CTLA-4 expression after a viral infection, while CD28 remains constitutively expressed, suggesting similar roles to their mammalian orthologs (Bernard et al. 2006).

3.5.3 T-cell Populations

In comparison with conventional αβ T cells, γδ T cells can directly recognize unconventional antigens such as phosphorylated microbial metabolites and lipid antigens (Hayday 2000). The recognition of this kind of unprocessed antigens seems to be similar to that of pattern recognition receptors. CD4 or CD8 expression is absent in the majority of γδ T cells in agreement with the non-MHC I and MHC II restriction. In mammals, low percentages of γδ T cells are found in the blood (1–10% of all T cells), while higher proportions have been identified in the intestine, lungs, the reproductive tract or the skin, suggesting an essential role of γδ T cells in mucosal immunity (Sheridan et al. 2013). This evidence points to γδ T cells as primitive lymphocytes that seem to be implicated in both innate and adaptive immune responses (Hayday 2000). Regarding teleost fish, γδ T cells have been described in zebrafish, accounting for 8–20% of total lymphocytes in different tissues. These cells were shown to phagocytose soluble and particulate antigens and to initiate antigen-specific CD4$^+$ T-cell proliferation and B-cell activation. They have also been shown to be essential for the production of antigen-specific IgZ secretion in the intestinal mucus (Wan et al. 2016).

Regarding Tc cells, two mechanisms by which these cells kill target cells have been described in mammals: the secretory and the non-secretory pathway. The secretory pathway secretes perforin and granzymes that induce cell apoptosis, whereas the non-secretory pathway uses target cell death receptors, such as Fas, that induce caspase-dependent apoptosis when FasL expressed on activated Tc cells attaches to it (Elmore 2007). In relation to the secretory pathway, perforin-like molecules have been described in different fish species such as Japanese flounder (*Paralichthys olivaceus*) (Hwang et al. 2004), rainbow trout (Athanasopoulou et al. 2009) or zebrafish (Varela et al. 2016). Studies performed in ginbuna crucian carp (*Carassius langsdorfii*) revealed that the mode of action of fish perforin was similar to its mammalian homologue (Toda et al. 2011a). Similarly, fish granzyme has also been found to have a similar primary structure than that of mammals (Praveen et al. 2006; Matsuura et al. 2014). On the other hand, the FasL protein involved in the non-secretory pathway has been identified in gilthead sea bream (*Sparus aurata*), channel catfish and Nile tilapia (*Oreochromis niloticus*) (Cuesta et al. 2003; Long et al. 2004; Ma et al. 2014). Moreover, in Japanese flounder, addition of recombinant FasL in a flounder cell line induced apoptosis, showing a similar mechanism to that of mammals (Kurobe et al. 2007).

As stated above, Th cells play an important role in cooperating with B cells, while they also regulate the immune responses of B cells and other cells through the secretion of different cytokines. After activation, naïve Th cells can differentiate into specific subpopulations characterized by specific cytokine secretion patterns and therefore with
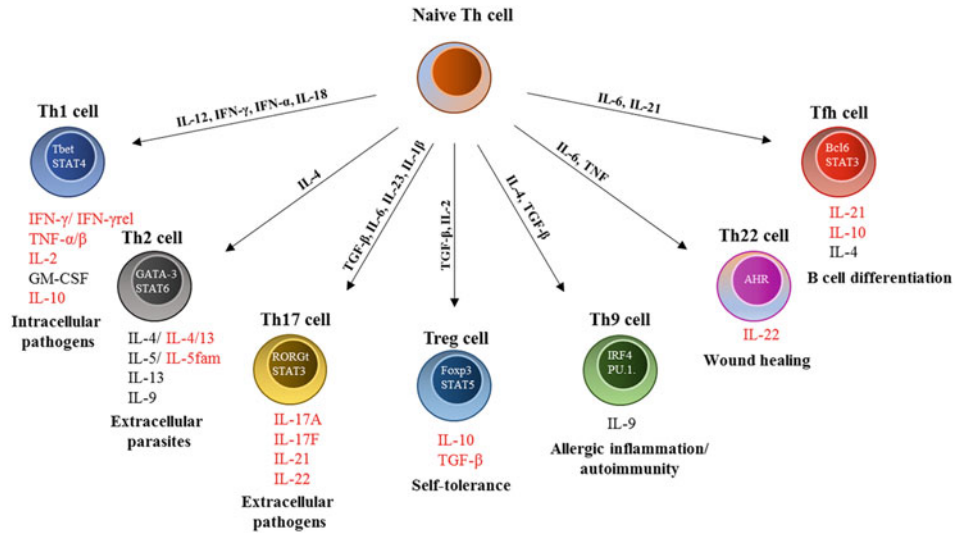
Fig. 3.2 Differentiation of naïve T helper (Th) cells to distinct Th subsets in response to a variety of cytokines. These Th cell subsets express specific transcription factors (indicated inside the cells), secrete specific cytokines (indicated below the cells) and are involved in the immune response against different types of pathogens or immune disorders. Those cytokines identified in teleost fish are highlighted in red

differentiated actions in the immune responses. These Th subsets mainly include Th1, Th2, Th17 and inducible T-regulatory (Treg) cells, although additional subsets are sometimes described in mammals such as Th9, Th22 or Tfh (Geginat et al. 2013). Figure 3.2 shows the main Th cell subsets generated from naïve T cells.

Th1 cells are known to promote cell-mediated immune responses against intracellular viral and bacterial pathogens. The main cytokines produced by Th1 cells upon activation include IFN-γ, IL-2, IL-10 and TNF-α/β. These cytokines induce macrophage activation, nitric oxide (NO) production and the proliferation of Tc cells. The differentiation process to Th1 response is promoted by IL-12, IL-18, IFN-α and IFN-γ, and controlled by the transcription factors T-bet, STAT1 and STAT4. All these molecules have been identified in fish. Two forms of IFN-γ (IFN-γ and IFN-γ-related) have been identified in some species of teleost fish, including Atlantic salmon, rainbow trout and ginbuna crucian carp, while only one form has been found in fugu [reviewed in Pereiro et al. (2019)].

Th2 cells play an important role in the immune response against extracellular pathogens. These cells mainly secrete IL-4, IL-5, IL-9, IL-13 and IL-17E/IL-25. IL-4 regulates clonal expansion of Th2 cells, and together with IL-13, promotes B-cell antibody secretion and alternative macrophage activation. IL-5 stimulates eosinophil activation and survival, and IL-9 promotes mast cell activation. In salmonids, genes with shared homology with IL-4 and IL-13 designated as IL-4/13 have been identified in variable number depending on the fish species (Ohtani et al. 2008; Wang and Secombes 2015; Yang et al. 2016). In rainbow

trout, recombinant IL-4/13A was shown to modulate the expression of several Th2 genes (Wang et al. 2016). Furthermore, GATA3 and STAT6, the transcription factors responsible for Th2 differentiation, have been identified in several fish genomes [reviewed in Nakanishi et al. (2015)].

Th17 cells are involved in immune responses against specific fungi and extracellular bacteria. These cells secrete IL-17A, IL-17F, IL-21 and IL-22 that stimulate chemokine secretion by resident cells leading to the recruitment of neutrophils and macrophages to the sites of inflammation. In teleost, the IL-17 cytokine family has various forms (A-F) of structurally related cytokines to the orthologous proteins in mammals (Kono et al. 2011). The ROR family of transcription factors that control Th17 differentiation have also been identified in fish (Nakanishi et al. 2015).

The main function of Treg cells is to maintain the immune homeostasis, through mechanisms that include the suppression of immune responses and the control of self *versus* non-self recognition. Treg cells are characterized by the expression of the transcription factor FoxP3 and by the secretion of IL-10 and TGF-β. Most of the teleost species studied possess one copy of the IL-10 gene, while two copies have been found in rainbow trout and European common carp (Harun et al. 2011; Piazzon et al. 2017). As per the other Th subsets, the presence of true Treg subsets in fish requires additional investigations to generate a greater understanding and characterization of their response.

On the other hand, Th22 and Th9 subsets have been more recently described in humans and mice. Th9 cells are involved in allergic asthma and autoimmunity and depend on the transcription factor PU.1 and IRF4 (Chen et al. 2019). In turn, Th22 cells express the transcription factor AHR that drives to IL-22 secretion (Jia and Wu 2014). Finally, fTh cells have been sometimes identified as a distinctive Th subtype as they are regulated through an exclusive array of transcription factors. These cells are specialized in inducing B-cell proliferation and differentiation to ASCs through the expression of CD40, ICOSL and the secretion of IL-21, IL-10 or IL-4 (Chtanova et al. 2004).

3.5.4   T-cell Response and Immunity

3.5.4.1 Helper Activity of CD4^{+} T Cells

The helper function of CD4^{+} T cells in teleost fish has been assessed in several studies using mixed leucocyte culture (MLC) assays. In these MLC assays, Th responder cells proliferate in response to allogeneic MHC II-expressing APCs. This proliferative capacity has been demonstrated in different fish species after in vitro incubation with allogeneic leucocytes (Meloni et al. 2006). In channel catfish, for example, Ig^{−} lymphocytes were found to be the responder cells, collaborating with B cells and macrophages in the MLC (Miller et al. 1985). CD4^{+} and CD8^{+} T cells both proliferated after the allogeneic stimulation with distinct types of APCs; however, it was after the pre-sensitization of the fish with ovalbumin that an antigen-specific proliferation of CD4^{+} T cells was observed (Toda et al. 2011b).

When naive ginbuna crucian carp were transplanted with crucian carp necrosis virus (CHNV)-sensitized donor cells, a significantly increased antibody response was reported relative to that observed in fish that received non-sensitized donor cells, or cells in which CD4$^+$ cells had been depleted. This same pattern was observed when cell-mediated cytotoxicity was measured (Somamoto et al. 2014). In rainbow trout, two independent studies have addressed the characterization of CD4$^+$ Th cells. In 2016, Maisey et al. combined antibodies specific for CD3 and CD4-1 to functionally characterize Th cells. They demonstrated that upon stimulation with an antigen, this population proliferated and secreted IFN-γ1, IL-4/13A, IL-15, IL-17D, IL-10 and TGF-β1, suggesting a differentiation to different Th subsets (Maisey et al. 2016). That same year, Takizawa et al. identified two CD4$^+$ Th populations combining monoclonal antibodies against CD4-1 and CD4-2. They found a predominant population, which co-expressed surface CD4-1 and CD4-2 along with a minor subset expressing only CD4-2 (Takizawa et al. 2016). Although both subsets produced equivalent levels of Th1, Th17 and regulatory T-cell cytokines upon bacterial infection, cells expressing only CD4-2 were less proliferative and displayed a more restricted TCRβ repertoire. These data led the authors to hypothesize that these cells represented a functionally distinct population (Takizawa et al. 2016). Similar studies have been undertaken in zebrafish, where an antibody against CD4-1 was combined with an anti-ZAP70 (a T-cell-specific transcription factor) to identify Th cells that increased the expression of cytokines and master transcription factors relevant to Th1/Th2-type responses in response to antigenic stimulation (Yoon et al. 2015).

3.5.4.2 Cytotoxic Activity of CD8$^+$ T Cells

Somamoto and collaborators described Tc virus-specific activity for the first time in fish, generating CHNV-specific ginbuna crucian carp leucocytes after infection with CHNV. This effect was virus-specific and MHC-restricted as in mammals (Somamoto et al. 2004). In addition, the production of virus-specific cytotoxic T cells in MLC has been shown in ginbuna crucian carp. This response seems to be antigen-specific since the effector cells lysed CHNV-infected syngeneic cells but not CHNV-infected allogeneic cells or non-infected syngeneic cells (Somamoto et al. 2009).

Moreover, when PBLs from viral haemorrhagic septicaemia virus (VHSV)-infected rainbow trout were cultured with MHC I-matched VHSV-infected cells, these were efficiently lysed (Utke et al. 2007). This activity went along with an increased expression of CD8 and the natural killer cell enhancement factor (NKEF)-like gene in the leucocyte cultures, suggesting an important role of NK and Tc cells in VHSV protection. Further investigations in rainbow trout responses against VHSV showed that PBLs from fish immunized with a DNA vaccine coding for the VHSV G protein killed both RTG2 (VHSV-infected MHC I-matched target) and EPC (VHSV-infected xenogeneic target) cell lines. Interestingly, the PBLs obtained from fish immunized with a DNA vaccine coding for the VHSV N protein only lysed VHSV-infected RTG-2 cells and not EPC cells. This phenomenon suggests that the G protein has the capacity to activate both adaptive and

NK cell-mediated responses, whereas the N protein would not activate innate cells (Utke et al. 2008).

Regarding bacterial infections, specific Tc cells were shown to have an important role in protection against the intracellular bacteria *Edwarsiella tarda* in ginbuna crucian carp (Yamasaki et al. 2013). Moreover, adoptive transfer of sensitized lymphocytes against *E. tarda* to isogenic naïve ginbuna carp showed that both CD4$^+$ and CD8$^+$ T cells conferred protection against infection. Sensitized CD8α$^+$ cells increased the transcription of IFN-γ and perforin, highlighting the crucial role of cell-mediated cytotoxicity and IFN-γ in this response (Yamasaki et al. 2014). In addition to their specific cytotoxic activity, mammalian Tc cells were shown to have the capacity of directly killing extracellular pathogens through what seems to be an MHC-independent process. In teleost fish, CD8α$^+$, CD4$^+$ T cells and surface IgM$^+$ cells were shown to all have the capacity to directly destroy bacteria. In both sensitized and non-sensitized ginbuna carp, CD8α$^+$, CD4$^+$ T cells and sIgM$^+$ cells showed antibacterial activity against extracellular and cell-associated bacteria, with lower killing activity in non-immunized fish (Nayak and Nakanishi 2013).

3.6   Conclusion

Fish and mammals appear to share many components of the adaptive immune system. Still, pronounced differences exist concerning the sophistication of this arm of the immune system, in which mammals have possibly reached a greater degree of evolution than that of fish. Collectively, the knowledge reviewed within this chapter has deepened our understanding of the two key processes of adaptive immunity: specific antigen recognition and immunological memory development. These processes depend on both the humoral and cell-mediated arms of the adaptive immune system via the production of effector cells and memory cells.

Although vaccines are available for some fish diseases, often the efficacy is not ideal, while for other diseases no vaccine is available. As adaptive immunity is the immunological baseline for vaccination, a greater elucidation of the features and mechanisms of the adaptive immune system are needed to refine the design of these preventive tools. In this context, recent biotechnology advancements in omics have improved the ability to precisely analyse the molecules involved in the immune response. In particular, transcriptomics are becoming more widely applied in fish immunology as they afford a robust, cost-effective method of quantifying many genes within species. However, reliance of a singular approach cannot suffice for providing a complete understanding of an immune response and this is something that requires complementary approaches in terms of the development of new tools and assays, such as specific antibodies against the different lymphocyte subpopulations. Still, there is great potential to expand our knowledge of the adaptive immune response and to improve our universal understanding of the evolution of the immune system.

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Cellular Immune Responses

4

Uwe Fischer and Fumio Takizawa

Abstract

Generally speaking, cellular immune responses are comprised of innate and adaptive cell-based immune mechanisms in which all leukocyte subpopulations are involved. This includes effector functions such as phagocytosis, NETosis, and cell-mediated cytotoxicity. The main players are macrophages/monocytes, dendritic cells, granulocytes, NK cells, and cytotoxic T cells. These effector functions can only be executed and controlled through receptor/ligand interactions and by humoral factors produced by leukocytes or somatic cells. Thus, it is hard to draw a static line between cellular and humoral components of the immune systems since one system cannot exist independently from the other. Similarly, adaptive responses cannot be efficiently induced without innate triggers.

This chapter describes cellular immune mechanisms in teleost fish and relates them to mammalian immunology.

Keywords

Cellular immunity · Phagocytosis · NETosis · APC · Cytokines · Receptors · Cell-mediated cytotoxicity · CTL · NK cell

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Definitions

somatic cell: bodies own cell

tissue: aggregate of cells having the same structure and function

antigen: from anti, Greek = against; gennan, Greek = produce; in the original sense: substances that the body recognizes as foreign via antibodies; in a broader sense: substances against which an immune response is mounted

Abbreviations

aa   amino acid
ADCC   antibody-dependent cell-mediated cytotoxicity
APC   antigen-presenting cell
CD   cluster of differentiation
CMC   cell-mediated cytotoxicity
CTL   cytotoxic T cell
DC   dendritic cell
HSC   hematopoietic stem cell
IFN   interferon
IL   interleukin
MHC   major histocompatibility complex
NET   neutrophil extracellular trap
NK   natural killer
PBL   peripheral blood leukocytes
rb   reviewed by
TCR   T-cell receptor
Th   T helper

4.1   Introduction

One of the most important requirements for the survival of multicellular organisms is to maintain its integrity and to prevent the invasion by other organisms or foreign (to the body) material. The first challenge consists of distinguishing somatic cells from nonself cells to avoid autoaggression. Secondly, the organism has to make a decision whether to tolerate, or to reject foreign cells, pathogens (parasites, bacteria, fungi, and viruses) or altered somatic cells. In mammals, immune response elements are historically divided into cellular and humoral (Greek: juice or sap = liquid) and innate and adaptive. However, these components cannot exist or act independently, thus defining them is difficult. While some definitions for cellular immune responses include only adaptive cellular elements, others

are more global, also incorporating innate immune cells. In this book chapter, cellular immune responses are defined as the reactions of leukocytes where the innate players are granulocytes, macrophages (monocytes), thrombocytes, dendritic cells as well as natural killer (NK) cells and where the adaptive cell-mediated responses are executed by cytotoxic T lymphocytes (CTLs). Fundamental functions of myeloid leukocytes are extracellular trapping and phagocytosis followed by the destruction of pathogens and altered material. Another important function associated with phagocytes is the processing of incorporated antigenic material in a way so that their components can efficiently be presented to, and recognized by cells of the adaptive immune system. While extracellular pathogens can be directly attacked by immune cells, intracellular pathogens, such as viruses and some bacteria, can only indirectly be recognized and their replication hindered, e.g., through cell-mediated cytotoxicity (CMC). To mount an efficient cellular immune response, leukocytes communicate among each other and with nonlymphoid cells through cytokines/cytokine receptors and through receptor/ligand interactions. These interactions are of interest when shaping vaccines that address cellular adaptive immune response mechanisms.

The aim of this chapter is to provide the latest information on cellular defence mechanisms of fishes, and put this knowledge into a comparative context of what we know from tetrapods.

4.2 Cell-Mediated Immune Functions

This subchapter refers to cells that directly act against pathogens, allogeneic/xenogeneic or altered and infected somatic cells.

4.2.1 Phagocytosis

After a pathogen or foreign material has passed epithelial barriers, the immediate cellular immune response is initiated by cells of the innate immune system. One main innate response mechanism is phagocytosis. Phagocytosis was first described by Metchnikoff in 1883 in frogs as the first cell-mediated immune mechanism ever discovered.

Phagocytosis is a variation of endocytosis, where endocytosis is characterized by one common characteristic: after contact with extracellular material, the cell membrane invaginates around the material, forming a pocket and finally an intracellular vesicle or a vacuole. Phagocytosis is defined as the internalization of particulate material (including bacteria and other "foreign" cells or their debris) by professional phagocytes, usually leading to the destruction of the ingested material.

Several cell types are able to execute phagocytosis in vertebrates. Professional phagocytes are monocytes (blood "macrophages"), macrophages (called so in tissues; for more information on macrophages see Chap. 6), dendritic cells (DC), neutrophils, and mast cells. Nonprofessional phagocytes such as epithelial cells, endothelial cells, fibroblasts, and

mesenchymal cells can also engulf foreign material. Some organs harbor certain specialized professional phagocytes in mammals as well as in teleosts: osteoclasts in bone tissue (rb Witten and Huysseune 2009), microglial cells in nervous tissue (rb Cuoghi and Mola 2007), and Langerhans cells in the skin (He et al. 2018). Despite the evidence for the existence of specialized macrophages, the so called Kupffer cells, in the fish liver (Moller et al. 2014), most scientist avoid the use of this term in fish and instead refer to "liver resident macrophages" (rb Shwartz et al. 2019).

Although phagocytes belong to the innate part of the cellular immune response, this distinction is somehow insufficient since B cells, as representatives of the adaptive immune system, are also able to phagocytose as shown in fish, amphibians (Li et al. 2006), and in mice (Parra et al. 2012; Martínez-Riaño et al. 2018).

Mammalian neutrophils and macrophages are myeloid cells which originate from the multipotential hematopoietic stem cell (HSC) in the bone marrow. Their final maturation occurs in the blood and tissues. However, bony fishes do not have bone marrow and the origin of myeloid cells is suggested to be the kidney (rb Katzenback et al. 2012; Kobayashi et al. 2016).

Mature mammalian neutrophils and macrophages are attracted to the site of pathogen growth or inflammation by chemotaxis. Several chemokine orthologs have been described in teleosts that are able to attract fish myeloid cells (rb Alejo and Tafalla 2011) in vitro and in vivo (Torraca et al. 2017). Goldfish neutrophils exhibit chemotaxis toward mycobacteria and efficiently internalize them (Hodgkinson et al. 2015). A critical role in the recruitment of mammalian neutrophils is performed by the chemokine CXCL8 (also known as IL-8), and several sequences similar to mammalian CXCL8 have been found in teleost fish (rb Havixbeck and Barreda 2015).

Uptake of particulate material into mammalian phagocytes, e.g., of bacteria (rb Kaufmann and Dorhoi 2016), small parasites (Ueno and Wilson 2012), and apoptotic cells (rb Li 2012) is receptor-mediated. Receptors are multifold. Bacterial uptake and killing by macrophages and neutrophils can be mediated through pattern recognition receptors (PRRs), or through opsonization by elements of the complement cascade via complement receptors (rb Lukácsi et al. 2020). This process can also be more antigen-specific, when antibody-opsonized bacteria are bound to the surface of macrophages (rb Hamdan et al. 2020) and neutrophils (rb Rosales 2017) via Fc receptors (FcR). Fish neutrophils have also been described as scavengers of dead cells. In goldfish, neutrophils internalize dead or dying macrophages during bacterial infection (Havixbeck et al. 2017).

Thrombocytes are nucleated cells in nonmammalian vertebrates, and along with their main function of blood clotting they also show phagocytic activity. Putative phagocytic activity of chicken thrombocytes was first described in 1968 (Carlson et al. 1968). However, true phagocytosis was often not clearly distinguished from uptake into the canalicular system, which forms an open network of interconnected channels of the outer cell membrane to broaden the cell surface of thrombocytes. Thus, phagocytosis by thrombocytes in lower vertebrates was questioned for a long time (e.g., Meseguer et al. 2002). The first serious evidence of phagocytosis by thrombocytes in fish was demonstrated in common and ginbuna crucian carp, in goldfish and in Japanese flounder

(Nagasawa et al. 2014; rb Esteban et al. 2015). Since goldfish and ginbuna crucian carp thrombocytes express the major histocompatibility complex-encoded thrombocyte marker G6F (Ohashi et al. 2010) and since thrombocytes from several fish species express MHC class II they are also regarded as important in antigen presentation of processed phagocytosed antigens (rb Stosik et al. 2019).

4.2.2 Extracellular Trapping

Another strategy of neutrophils (and less importantly in macrophages) to neutralize bacteria is the formation of extracellular traps (ETs). ETs are extracellular matrices formed by DNA and granular proteins that immobilize and kill bacteria (rb Kaufmann and Dorhoi 2016).

The formation of ETs is a phylogenetically ancient mechanism and not unique to the vertebrate kingdom, including fish. It can also be found in invertebrates and even plants. The formation of neutrophil ETs (NETs) is called NETosis. In teleost fish, NETosis can be induced by stimuli mimicking fungal, viral, and bacterial pathogens, and like in mammals, degradation of entrapped pathogens is executed by myeloperoxidase (MPO), nitric oxide (NO), histones, chymotrypsin-like elastases, and nucleases (rb Neumann et al. 2020). However, there are species-specific differences in the inducibility of NETosis in fish. While Phorbol 12-myristate 13-acetate (PMA) is a potent inducer of ET release in common carp (Pijanowski et al. 2013) and tongue sole (Zhao et al. 2017), PMA triggers only a weak ET release in fathead minnow, zebrafish (Palić et al. 2007), and rainbow trout (Van et al. 2020).

4.2.3 Cell-Mediated Cytotoxicity (CMC)

4.2.3.1 Myeloid Cells

Since neutrophils belong to the innate immune system, their recognition mechanisms are not antigen-specific. In addition to phagocytosis, mammalian neutrophils can destroy bacteria by degranulation and release into the extracellular environment of antimicrobial substances such as cathepsin G, defensins, lysozyme, MPO, NE, and proteinase (Gullberg et al. 1997) as well as of antimicrobial peptides. They also produce and release reactive oxygen (ROI) and nitrogen intermediates (RNI) which further contribute to the destruction of extracellular bacteria, foreign cells, and accidently, also somatic cells (rb Kumar and Dikshit 2019; rb Korhonen et al. 2005). Neutrophils are long known to play a decisive role in pus formation. Pus usually consists of bacteria, neutrophils, and cellular debris from bacteria as well as debris of damaged somatic cells. Damage of somatic cells in phlegmons due to injury and wound infection arises not only from the cytotoxic activity of bacteria but also of neutrophils. To protect the surrounding tissues, a capsule is usually built around the pus to form an abscess. Although abscesses are common findings in ornamental fish

practice, scientific articles describing their cellular and molecular mechanisms are rare in fish. A zebrafish larval model for mammalian abscess formation has been described by Prajsnar et al. (2012). While bacteria (*S. aureus*) were shown to survive within phagocytes in infection foci, no capsule formation was described around abscess-like structures, probably due to the early stage of fish development in this study.

The destructive role of mammalian neutrophils is also critical in many other acute inflammatory diseases, e.g., in sepsis (e.g., rb Shen et al. 2017) or organ damage (e.g., rb Jaeschke and Hasegawa 2006). Sepsis is defined as a systemic inflammatory response syndrome (SIRS) in response to an infectious process (rb Soong and Soni 2012). It is largely initiated by bacterial endotoxins and although pattern recognition is different between mammals and teleost fishes, e.g., due to different TLR signaling, a sepsis model has been described in zebrafish (Philip et al. 2017).

Another adverse effect of mammalian neutrophils affects the outcome of transplantation. Neutrophils are recruited to the graft leading to the risk of allograft rejection (e.g., rb Oliveira et al. 2018). Similarly, in carp (Kurata et al. 1995) and rainbow trout (Sasaki et al. 2002), neutrophils kill xenogeneic cells in a nonspecific manner.

Graft-versus-host reaction is characterized by an immune response of grafted cells against the recipient. Although the main effector cells in fish have been described as CD8+ lymphocytes (Shibasaki et al. 2010), the involvement of neutrophils in this process cannot be ruled out (Nakanishi and Ototake 1999). A kind of nonspecific autoaggression has also been described for neutrophils in ginbuna where neutrophils have killed isogeneic erythrocytes in vitro requiring cell to cell interaction (Fischer et al. 1998). However, since the neutrophil effector to erythrocyte target cell ratios in these in vitro studies were much higher (up to 40:1) than those usually found in blood under physiological conditions (approx. 1:1000; Fänge 1992), the probability of neutrophil effectors to encounter an erythrocyte was much higher than under physiological conditions. Further, some senescent or damaged erythrocyte targets might have activated neutrophils which in turn killed additional intact erythrocytes.

4.2.3.2 Natural Killer Cells

Mammalian NK cells are large, granular, and cytotoxic lymphocytes with the ability to recognize and kill tumor cells, stressed cells, and cells infected with viruses and other intracellular pathogens. NK cells do not express antigen-specific receptors, such as the B-cell or T-cell receptors, with highly variable regions. However, they possess members of various families of activating and inhibitory receptors to monitor an aberrant expression of cellular stress markers, MHC class I, and MHC class I-related molecules. Thus, NK cells play important roles in the clearance of altered cells, especially in the early innate response to viral infections, before adaptive immune responses by cytotoxic T cells take part. NK cells can also sense the missing expression of self MHC class I on foreign allogeneic cells without prior sensitization (rb Kärre 2002).

Origin and Distribution of NK Cells

The majority of our knowledge on NK cells originates from studies on mice and humans. NK cells are widely distributed in most murine organs, and represent only a minor population of total lymphocytes in most tissues (e.g., ~2% and ~ 10% in murine spleen and lung, respectively) (rb Grégoire et al. 2007). In humans, NK cells (CD3−/CD56+) are relatively abundant, comprising 5%–20% of lymphocytes in blood, bone marrow, spleen, and lung with low frequencies in the lymph nodes, tonsil, and gut (rb Freud et al. 2017; rb Dogra et al. 2020).

In adult mammals, the bone marrow is the major site of early phase NK cell development (rb Yu et al. 2013). Common lymphoid progenitor cells (CLPs) committed from HSCs are the earliest lymphoid progenitor cells and can generate T cells, B cells or innate lymphoid cells (ILCs) including NK cells. Stromal cells in bone marrow produce certain cytokines, Fms-like tyrosine kinase 3 ligand (FLT3L), and stem cell factor (SCF), crucial for NK cell differentiation by inducing the common IL-2 receptor subunit β (CD122) shared by IL-2 and IL-15 signaling pathways (rb Leonard et al. 2019). The acquisition of CD122 directs CLPs to become immature NK cells (Carotta et al. 2011). Committed immature CD122+ NK cells in humans and mice further develop through distinct stages, which differ in their cell surface receptor repertoires, such as the activation receptors, natural killer group 2D (NKG2D; alias CD314), natural cytotoxicity receptor 1 (NCR1; alias CD335, NKp46, Ly94), and NK1.1 (NKR-P1C, CD161) (rb Goh and Huntington 2017). Precursor and immature NK cells migrate into secondary lymphoid organs, and these sites are considered as reservoirs for NK cell maintenance (rb Huntington et al. 2007; rb Abel et al. 2018). In humans, the appearance of CD56 (NCAM, neural cell adhesion molecule) further indicates a final transition of immature to mature NK cells (rb Freud et al. 2017; rb Di Vito et al. 2019). Most immature NK cells develop into a minor CD56-bright population and then convert into a major CD56-dim population with the acquisition of the antibody binding-Fc receptor CD16. Human CD56-bright NK cells exhibit reduced lytic capacity while being potent producers of inflammatory cytokines. These subsets reside primarily in lymph nodes and in the intestine. In contrast, the CD16 expressing CD56-dim NK cells represent the major NK cell population in peripheral blood, bone marrow, spleen, and lung, and are potent cytolytic effector cells, rapidly secreting cytotoxic mediators (granzymes and perforin) following receptor-mediated activation (rb Freud et al. 2017).

In mice, mature NK cells are defined by the acquisition of C-type lectin-like Ly49 receptors (rb Goh and Huntington 2017). Since murine NK cells show no expression of CD56, mature NK cells are further classified into three functionally distinct subsets by their expression of CD27 and CD11b (Hayakawa and Smyth 2006; Chiossone et al. 2009). In detail, mature CD11b-/CD27+ NK cells, representing the first maturation stage, are abundantly present in the bone marrow and lymph nodes. Intermediate CD11b+/CD27+ NK cell stages are equally distributed in the lymphoid organs, liver, and lung while most mature CD11b+/CD27- NK cells are found in blood, spleen, lung, and liver. This maturation step is associated with the functional development of mature NK cells (Walzer et al. 2007; Chiossone et al. 2009).

Mammalian NK cells use activating and inhibitory receptors to recognize both healthy and altered cells, such as infected, stressed, and tumor cells. Within one individual, NK cell activation is largely regulated by MHC class I molecules which are expressed by most nucleated cells marking these cells as "self" (rb Höglund and Brodin 2010). When the NK cell inhibitory receptors recognize self MHC class I molecules on another cell, the NK cell's cytotoxicity function is inactivated to prevent killing of nonaltered self cells. To acquire the capacity to recognize "non-self" target cells with "wrong" MHC class I expression (in the case of grafted cells) and self target cells with down-regulated or altered MHC class I expression (e.g., during virus infection or in tumor cells), mammalian NK cells must be educated to detect host MHC class I molecules using their inhibitory receptors. Only NK cells that have engaged their inhibitory receptors with self MHC class I molecules during development are functionally mature and competent (rb Sun and Lanier 2011; rb Shifrin et al. 2014). This process of NK cell development is termed NK cell licensing, NK cell tuning or classical NK cell education. NK cell licensing brings forth two types of self-tolerant NK cells. The licensed cells effectively patrol for missing MHC class I targets. In contrast, unlicensed NK cells lacking the expression of inhibitory MHC class I-specific receptors diminish the capacity to respond to MHC class I-deficient targets (rb Anfossi et al. 2006). Thus, the unlicensed NK cells have a low potential to attack normal cells and are not autoreactive. In addition to classical MHC class I molecules, nonclassical MHC class I, or even other than MHC class I molecules are involved in NK cell education (rb He and Tian 2017).

In teleost fish, two types of homologous cells to mammalian NK cells have been described: nonspecific cytotoxic cells (NCCs) and NK-like cells (rb Fischer et al. 2013). NCCs, which have been well characterized in catfish, are small and agranular lymphocytes that spontaneously kill a variety of xenogeneic targets and can be recognized by a monoclonal antibody, 5C6, specific to the NCC receptor protein 1 (NCCRP1) (rb Shen et al. 2002). NCCRP-1+ cells in catfish are present at concentrations of ~30%, ~40–50%, and ~ 2% in pronephros, spleen, and blood, respectively (Evans et al. 1988). Given NCCRP-1 is a marker of NCCs in teleost fish, expression analysis of NCCRP-1 transcripts in carp and grouper suggests its ubiquitous distribution in the fish body (Sakata et al. 2005; Huang et al. 2014). In contrast to fish NCCs, NK cells have not been isolated in fish yet, as no specific markers or reliable antibodies are available, although cell lines with NK cell activity have been isolated from catfish (discussed below). However, large granular lymphocytes similar to mammalian NK cells were identified in rag1−/− mutant zebrafish (Muire et al. 2017). Moreover, single cell transcriptomes in several fish species clearly identified NK-like cell populations (Moore et al. 2016; Carmona et al. 2017; Guslund et al. 2020). These data are valuable not only for analysing the function of fish NK cells, but also to isolate them in the future using suitable reporter genes (e.g., GFP) and/or antibodies targeting NK cell-specific molecules.

Induction and Regulation of NK Cell-Mediated Cytotoxicity

Mammalian NK cells play a crucial role in immune responses against viral infections and tumors. However, the killing activity of NK cells must also be tightly regulated to assure tolerance to healthy self tissues. NK cells monitor the surface of each host cell using germ-line-encoded receptors. Some cell surface markers are commonly associated with healthy cells, and others are expressed mainly by damaged or infected cells. NK cells have receptors for each of these types of molecules: one type, called activating receptors, recognizes the "unhealthy" markers, and another type, called inhibitory receptors, recognizes the "healthy" markers (rb Pegram et al. 2011). Whether NK cells kill a target cell or not is determined by the overall balance of signaling by these inhibitory and activating receptors. If more activating receptors are stimulated than inhibitory receptors, the NK cell decides to attack the target cells which are infected or badly damaged, and releases cytoplasmic granules which destroy the target cells (rb Vivier et al. 2008).

In mammals, genes encoding NK cell receptors (NKRs) are clustered in two main gene complexes: the natural killer complex (NKC) encoding C-type lectin-like molecules, and the leukocyte receptor complex (LRC) encoding the immunoglobulin-like receptors (rb Carrillo-Bustamante et al. 2016). These NKR gene clusters show species-specific expansion resulting in divergent activating and inhibitory receptors. The main NK cell receptors for MHC class I in humans are the killer cell immunoglobulin-like receptors (KIRs), which are located in the LRC. Mice lack KIR genes and instead predominantly harbor Ly49 receptors encoded in the NKC to regulate their NK-cell activity. These Ly49 receptors show high polymorphism between different strains of mice. In contrast, humans lack functional Ly49 genes (rb Kelley et al. 2005; rb Rahim et al. 2014).

Human KIRs have either two or three extracellular immunoglobulin-like domains and contain either long cytoplasmic tails with immunoreceptor tyrosine-based inhibition motifs (ITIM) or short cytoplasmic tails comprising a charged residue in their transmembrane regions that associate with signaling adaptor molecules with immunoreceptor tyrosine-based activating motifs (ITAM), such as DAP12, or FcγR (rb Purdy and Campbell 2009; rb Pegram et al. 2011). Most KIRs are inhibitory receptors with cytoplasmic tails containing ITIMs. The ligands of human KIRs are classical MHC class I molecules, such as HLA-A, B, and C molecules. The ligand binding to either activating or inhibitory KIRs induces the phosphorylation of tyrosine residues in ITAMs or ITIMs, respectively. This activation process through activating receptors leads to the release of cytotoxic granules. In contrast, the phosphorylation of ITIMs in inhibitory receptors recruits phosphatases and neutralizing activating signals (rb Long 2008). As regular and healthy cells express MHC class I, the recognition of KIRs on NK cells inhibits its killing process (Fig. 4.1).

In mice, instead of KIR receptors, a family of NKC-encoded lectin-like receptors called Ly49 is used for recognition of polymorphic classical MHC class I and related proteins. Similar to KIRs, inhibitory Ly49 receptors have an ITIM in their cytoplasmic tail although activating Ly49 receptors use the DAP-12 molecule for signaling. An important feature of the NK-cell population is that not all NK cells in an individual are identical and NK cells express only a subset of the receptors in its potential repertoire. The decision which KIRs
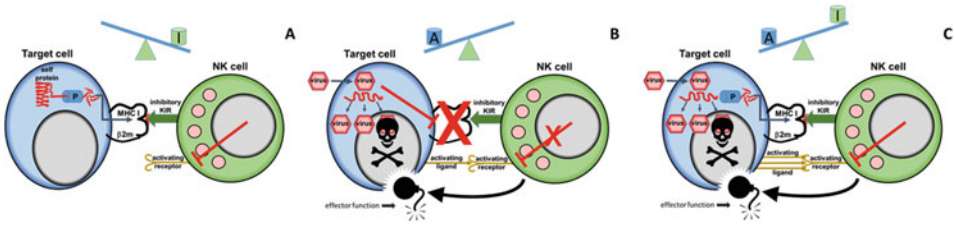
Fig. 4.1 Human NK cells possess inhibitory and activating receptors. In a normal situation, KIRs recognize self-peptide-loaded self MHC class I molecules on the surface of somatic cells which program NK cells into an inert state (**a**). Some viruses down-regulate MHC class I expression and induce the expression of activating ligands in infected cells. While the inhibitory function of KIRs is missing, activating receptors are engaged and drive NK cells into an activated stage (**b**). Even if MHC class I expression is not affected or up-regulated during certain virus infections, the balance might still be shifted toward NK cell activation through interaction between activating ligands expressed on infected cells and activating receptors displayed on NK cells (**c**). The inert state of NK cells can be shifted into an activated state in any situation where the signals from activating receptors are stronger than those from inhibitory receptors

and Ly49s are expressed on each NK cell seems to be random and is regulated by the methylation of KIR gene loci (Santourlidis et al. 2002; Liu et al. 2009).

Mammalian NK cells also express C-type lectin-like receptors, CD94 and NKG2 as heterodimeric receptors with activating or inhibitory effects. NKG2 consists of several members (A-F, H) (rb Pegram et al. 2011, rb Wilk and Blish 2018). NKG2A, 2C, and 2E are displayed as covalently linked heterodimers with CD94. NKG2B and NKG2H are alternative splice isoforms of the NKG2A and NKG2E genes, respectively, and are also expressed as heterodimers with CD94. These heterodimers interact with nonclassical (also nonpolymorphic) MHC class I-like molecules, including HLA-E in humans and Qa1 in mice. NKG2A and 2B contain two ITIMs and are important in educating NK cell tolerance to self cells and contribute to the inhibition of NK cell-mediated immunity to infections and tumors. Whilst CD94/NKG2C and CD94/NKG2E can associate with DAP12 and work as activating receptors, NKG2D makes homodimers but not heterodimers with CD94, and functions as an activating receptor. It binds several MHC class I-like molecules that are induced by various types of cellular stress (rb Waldhauer and Steinle 2008).

Natural cytotoxicity receptors (NCRs) are an additional group of mammalian activating receptors. This group belongs to the Ig-superfamily and includes NKp46 (NCR1/CD335), NKp44 (NCR2/CD336), and NKp30 (NCR3/CD337) (rb Barrow et al. 2019). Among NCRs, NKp46 is conserved in both humans and in mice; however, NKp30 and NKp44 in mice are pseudogenes. Thus, NKp46 is the most selective marker of NK cells in mammalian species. In humans, activated and resting NK cells express NKp46 and NKp30, which can associate with the transmembrane regions of the ITAM-containing CD3ζ-homodimers (Khakoo et al. 2004). Moreover, NKp46 can also engage with FcεRIγ-CD3ζ heterodimers, both of which possess ITAM motifs. In contrast, NKp44 is expressed on some NK cells upon IL-2 stimulation and can directly associate with DAP12. Some of the ligands

recognized by the NCRs are virus-derived molecules, such as influenza virus hemagglutinin (HA), that activates effector functions of NK cells against infected cells. Intracellularly localized proteins from tumors and stressed cells are also possible ligands for NCRs. Nuclear proteins (e.g., BAG6, MLL5, and PCNA) can be transported to the cell surface on stressed and tumor cells via exosomes. The cytosolic protein proliferating cell nuclear antigen (PCNA) released by mammalian tumor cells can inhibit the function of NKp44 (Rosental et al. 2011). NKp30 can bind to B7-H6 which is mainly expressed on cancer cells, triggering NK cell activation (Brandt et al. 2009). Interestingly, NKp30 can interact with BAG6 secreted by tumor cells in an exosomal or soluble form, which in turn can activate or inhibit NKp30-mediated NK cytolytic functions, respectively (Reiners et al. 2013). The NKp30 gene is the most ancient among the NKR family and is also found in cartilaginous fish (Flajnik et al. 2012). Sharks also possess B7H6 genes, suggesting that B7H6-NKp30 interaction arose early in vertebrate evolution. However, animals including bony fish which have lost NKp30 genes have also lost B7H6 genes.

Although NK-like cells have been identified in teleost fish, there are no orthologous genes of mammalian NKRs (e.g., KIR and Ly49 families). However, it is believed that multiple multigene families of immunoglobulin domain-containing innate immune receptors (IIIRs) in teleost fish represent the counterparts of mammalian innate immune receptor families such as the KIRs, leukocyte immunoglobulin-like receptors (LILRs), Fc receptors, triggering receptors expressed on myeloid cells (TREMs), and CD300s (rb Wcisel and Yoder 2016). These IIIR families in bony fish include the novel immune-type receptors (NITRs); diverse immunoglobulin domain containing proteins (DICPs); polymeric immunoglobulin receptor-like proteins (PIGRLs); novel immunoglobulin-like transcripts (NILTs); and leukocyte immune-type receptors (LITRs).

Novel immunoglobulin-like transcripts (NILTs) which show structural similarities with mammalian TREM and NKp44 receptors were identified in carp, trout, Atlantic salmon, and zebrafish, but not in fugu genomes (rb Wcisel and Yoder 2016). NILTs basically encode one or two Ig domains and possess ITIM or ITAM motifs. The number of NILTs have diverged greatly in cyprinids, whereas only a few (<10 genes) have been found in salmonids. The function and ligands of NITRs are still unknown.

IIIRs may function as NKRs in fish, and one of the most investigated IIIRs in teleost fish is the novel immune-type receptor (NITR) family (rb Yoder and Litman 2011). Multiple genes of the NITR family including activating or inhibitory receptors are present in teleost fish and show similar structures to mammalian KIRs. The majority of NITRs have a cytoplasmic ITIM while some NITRs possess a positively charged residue within their transmembrane domain for possible association with adaptor proteins containing ITAMs. In fact, activating and inhibitory NITRs can activate and inhibit the ERK/MAPK pathways in human cell lines, respectively. Moreover, NITR9 representing the only activating NITR in zebrafish can interact with Dap12 resulting in activating signaling (Wei et al. 2007). In addition, cross-linking of a recombinant zebrafish NITR9 on a human NK cell line resulted in increased target cell killing. Importantly, recent single-cell transcriptome analyses of

lymphocytes in zebrafish demonstrate that certain NITRs may be markers for NK cells (Carmona et al. 2017; Tang et al. 2017).

LITRs are teleost's unique innate immune receptors containing two to six immunoglobulin domains. They are well described in catfish and likely have a role in target cell recognition and signal transduction. The structure of LITR Ig domains is similar to that of mammalian FcRs. Catfish LITRs consist of multiple genes and include inhibitory and activating forms. The mAb (CC41) was established to target LITRs, and members of the LITR family were identified as markers for catfish NK cells, alloantigen-specific CTLs, and antiviral cytotoxic cells (Shen et al. 2004; Taylor et al. 2016).

Another NK-like cell subset in teleost fish, NCCs, expresses NCCRP-1. This receptor is a type III membrane protein with functional domains for antigen binding, signaling, and transcriptional activation (Jaso-Friedmann et al. 2001). Cross-linking of NCCRP-1 induces receptor tyrosine and serine phosphorylation (Evans et al. 1999). It binds to the natural killer target antigen (NKTag) of tumor antigens and protozoan parasites resulting in target cell killing (Jaso-Friedmann et al. 1997). NCCRP-1 genes have also been identified in a number of other animal species including axolotl, mouse, and man. However, mammalian NCCRP-1, a member of the lectin-type FBXO gene family, is not specific to immune tissues and is expressed only in the cytosol (Kallio et al. 2011).

NK cells isolated from uninfected mammals can spontaneously kill targets, but this activity is enhanced when NK cells are exposed to interferons or certain cytokines produced by dendritic cells and macrophages during infection by various types of pathogens. Cytokines IL-2, IL-12, IL-15, IL-18, and type I interferons positively control NK cell function, either independently or in cooperation (rb Wu et al. 2017). As CD122 (IL-2Rb) is critical for NK cell development from CLPs, IL-2 and IL-15 are the most extensively studied cytokines for NK cell activation in humans and mice (rb Meazza et al. 2011). IL-2, which was originally found to be a T-cell growth factor (its roles in T-cell function are discussed later), has been shown to induce proliferation and cytokine production of NK cells as well as to activate cytotoxic effector mechanisms in NK cells. Interleukin-15, discovered by its "IL-2-like" stimulatory role, also has important roles in NK cell development and function. IL-2 and IL-15 share not only properties regarding NK cell function, but also with respect to receptor binding. Both can bind to CD122 (IL-2Rβ) and CD132 (IL-2Rγ). The binding of IL-2 and IL-15 to respective receptors is due to their corresponding α chains, IL-2Rα (CD25) and IL-15Rα (CD215), respectively (rb Leonard et al. 2019). Moreover, IL-2 secreted as a free cytokine can bind directly to the IL-2Rαβγ complex with high-affinity or to dimeric IL-2Rβγ with intermediate affinity while IL-15 can function as a cell surface-bound form on IL-15Rα-expressing professional antigen-presenting cells. IL-15Rα binds to IL-15 with high affinity, which effectively activates NK cells at relatively low concentrations when compared to IL-2 (rb Waldmann 2006). JAK-STAT5 signaling through IL-2 or IL-15 regulates NK cell activation, and the IL-15-STAT5 signaling pathway is especially critical for NK cell development and homeostasis (rb Gotthardt and Sexl 2017). Interestingly, and different from mammals, teleost fish have no IL-2Rα gene, meaning that both fish IL-2 and IL-15 require IL-15Rα

for signaling (Dijkstra et al. 2014). In teleost fish, IL-2 and IL-15 genes have been identified, and its biological function analyzed in numerous fish species, including rainbow trout (Wang et al. 2018a, b; Yamaguchi et al. 2020). Trout IL-15/IL-15Rα heterodimers induce the up-regulation of transcription of IFNγ and perforin, and the phosphorylation of STAT5 in trout CD4-CD8α-IgM-splenocytes, a subpopulation of lymphocytes presumably containing NK cells (Yamaguchi et al. 2020). Grass carp IL-15 also induces type 1 immune responses (as concluded from IFNγ and T-bet expression) and NK cell activation (as concluded from perforin and eomesodermin aka EOMES-a expression) (Wang et al. 2020). Thus, teleost IL-15 is also suggested to be a critical factor in NK cell activation.

Mammalian IL-12 was originally named natural killer cell stimulatory factor (NKSF) based on its activities to induce a large amount of IFNγ. Like IL-18, it is mainly produced by professional antigen-presenting cells, such as DCs, monocytes, and macrophages. The cooperative effect of these cytokines, IL-12 and IL-18, is required for proper and efficient IFNγ production by NK cells, and this is crucial in controlling viral infections before cytotoxic T cells become activated (rb Wu et al. 2017).

Viral infections trigger the expression of type I IFNs (IFNα/β). They are one of the most potent regulators of NK proliferation and cytotoxicity. IFNα induces DCs to produce IL-12, IL-15, and IL-18, thereby activating the IFNγ production by NK cells (rb Paolini et al. 2015). The production of IFNγ by NK cells in the early immune response enhances the capacity of macrophages in pathogen killing, activates DCs, and promotes the differentiation of CD4 T cells into the Th1 subset, which further leads to the production of IFNγ.

In addition to cytokines, multiple transcription factors coordinate the development and effector functions of different lymphocyte subsets. In mammals, ID2 (Inhibitor of DNA binding 2) is a critical regulator of all ILCs including NK cells (Boos et al. 2007). Its expression continues throughout early NK cell development and during NK cell maturation while repressing the development of CLPs into T cells (Zook et al. 2018; rb Brillantes and Beaulieu 2019). The transcription factors NFIL3 (Nuclear factor, interleukin 3 regulated), ETS1 (ETS proto-oncogene 1), and TOX (Thymocyte selection-associated high mobility group box) are expressed in early stages of NK cell development and control Id2 transcription. NFIL3 (alias E4BP4) can bind to the EOMES promoter during early stages of NK cell development (Male et al. 2014), while TOX1 is involved in later stages of NK cell maturation (Aliahmad et al. 2010).

The mammalian T-box transcription factors EOMES and T-bet are both required for late NK cell development and effector function (Gordon et al. 2012). The balance of their expression is well regulated during NK cell development (rb Brillantes and Beaulieu 2019). For example, increased T-bet expression in NK progenitors in bone marrow can suppress EOMES expression and stabilize the transition from immature to mature NK cells. EOMES is thought to promote NK cell development and maturation following T-bet in NK cell development. Importantly, EOMES can induce CD122 expression in both NK cells and CD8+ CTLs, resulting in an enhancement of IL-15 responsiveness. Finally, T-bet in association with Zeb2 controls terminal maturation of NK cells. However, tissue-residing NK cells show distinct expression patterns of EOMES and T-bet in various tissues,

suggesting unique developmental pathways (rb Simonetta et al. 2016). T-bet and EOMES genes have been identified in a few fish species. In rainbow trout, T-bet and EOMES are expressed in both CD8+ T cells and IgM-/CD8- lymphocytes (possibly including NK cells) (Takizawa et al. 2011, 2014). Overexpression of Atlantic salmon EOMES induces the expression of IFNγ and granzyme A in salmon splenocytes (Kumari et al. 2013). These results indicate that teleost T-bet and EOMES may also play a critical role in cell-mediated cytotoxicity.

Effector Functions of NK Cells

In mammals, both NK cells and CTLs contain lytic granules in their cytoplasm including perforins, granzymes, and NK-lysin (alias granulysin) to eliminate pathogens and altered cells. Perforin can polymerize to form transmembrane pores in the membrane of target cells, and granzymes belonging to the family of serine proteases mediate apoptosis of target cells. This can effectively induce target-cell apoptosis of virus-infected cells to inhibit viral spread. NK-lysin is an antimicrobial peptide, destroying cholesterol-poor microbial membranes, and NK-lysin and granzyme can cooperatively kill intracellular bacteria and parasites after disruption of host cell membranes by perforin (Walch et al. 2014; rb Dotiwala et al. 2016). In addition to lytic granules, mammalian NK cells also utilize death receptor-mediated apoptosis with Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) to kill viral-infected cells and altered cells (rb Prager and Watzl 2019).

Although a variety of leukocytes are involved in nonspecific cell cytotoxicity in teleost fish, two types of NK cell homologs with nonspecific cytotoxicity have been well described in channel catfish thus far: NCCs and NK-like cells. Homologous genes to cytolytic granules, FasL and TRAIL have been cloned in many fish species, and their functions are likely conserved between mammals and teleost fish (Yamaguchi et al. 2019a; Tafalla and Granja 2018). However, the association of these molecules to teleost NK cells is unknown in several fish species. Catfish NCCs possess granzyme-like serine proteases (Praveen et al. 2004) and express transcripts of perforin and granulysin genes (Praveen et al. 2006), indicating that teleost NCCs use cytotoxic mechanisms similar to mammalian NK cells. Tilapia and catfish NCCs do not express the membrane-bound type of FasL, but constitutively release soluble FasL proteins (rb Evans et al. 2001; Bishop et al. 2002). NCC activity has been found in various fish species, such as rainbow trout (Hayden and Laux 1985; Greenlee et al. 1991), carp (Bielek 1988; Meseguer et al. 1994), damselfish (McKinney and Schmale 1997), and tilapia (Faisal et al. 1989). In channel catfish, a subpopulation of peripheral blood leukocytes (PBL) was found to exhibit NK cell activity against allogeneic cells (Yoshida et al. 1995; Stuge et al. 1995) and virus-infected cells (Hogan et al. 1996). Later, several NK-like cell lines were established from catfish PBL that were shown to be negative for TCR, as well as neutrophil and monocyte marker genes, but also negative for mAb 5C6, a specific marker for catfish NCCs, indicating the existence of distinct populations of NK-like cells in catfish (Stuge et al. 2000, Shen et al. 2004). Moreover, these NK-like cells contain cytoplasmic granules and kill allogeneic target cells by induction of apoptosis via perforin/granzyme mechanism (Hogan et al. 1999;

Shen et al. 2004). Current research from single cell transcriptomes identified additional candidates of marker and effector molecules in teleost NK-like cells (Moore et al. 2016; Carmona et al. 2017; Guslund et al. 2020). However, further progress is needed to understand the mechanisms by which fish NK cells recognize altered target cells and are activated.

Antibody-dependent cell-mediated cytotoxicity (ADCC) is another mechanism by which mammalian NK cells can kill their target cells. ADCC is based on the interaction of cytotoxic Fc receptor (FcR)-expressing cells with the Fc part of antibodies followed by direct recognition of the respective antigens on target cells by FcR-bound antibodies (rb Gómez Román et al. 2014) (Fig. 4.2). Alternatively, surface antigen-expressing cells are already opsonized by antibodies, and NK cells bind to opsonized cells by means of their FcR. In mammals, where ADCC can be critical in viral infection and cancer clearance, IgG antibodies represent the major ADCC-mediating Ig isotype. Mammalian NK cells generally express CD16 (FcγRIII) and upon recognition of IgG on target cells, NK cells are activated and kill opsonized target cells by cytotoxic granule release and Fas signaling. NK cell-mediated ADCC is also used as effector mechanism for immunotherapy against tumor cells using antitumor antibodies (rb Wang et al. 2015). In channel catfish, NK-like cells armed with IgM were shown to kill target cells through ADCC (Shen et al. 2003, 2004). However, orthologous genes of FcμR and FcαμR (CD351) in fish, amphibians, birds, and reptiles have not been identified yet (rb Akula et al. 2014; Kortum et al. 2014). Strangely, although catfish possess an FcμR homolog that can bind to catfish IgM, this molecule is only expressed as soluble form (Stafford et al. 2006). Therefore, it remains to be explored which receptor is utilized in teleost fish to induce ADCC.

4.2.3.3 Cytotoxic T Lymphocytes (CTLs)

Origin of CTLs

Mammalian cytotoxic T cells have their origin in the thymus, and T-cell precursors are recruited from HSCs in the bone marrow. While traversing the thymus cortex and medulla, progenitor T lymphocytes develop from immature CD4/CD8 double-negative (DN) into CD4/CD8 double-positive (DP) and finally either CD8 or CD4 single-positive (SP) T cells, depending on their preference to either bind to MHC class I or II molecules, respectively. During mammalian T-cell maturation, two important processes occur: T-cell receptor (TCR) rearrangement and selection. TCR rearrangement of the variable (V), diversity (D), and joining (J) gene segments occurs during the DN and DP stages yielding TCRs with great diversity. The TCR finally defines the antigen specificity of T cells enabling CD8 SP and CD4 SP T cells to recognize antigenic peptides presented by MHC class I or II. In a strict selection process, during which only a small fraction ($<5\%$) of developing T lymphocytes survive, both weak interaction of the TCR with self-peptide–MHC ligands as well as excessive signaling result in T-cell apoptosis (negative selection). Negative selection ensures that mature T cells are not committed to react against the body's own cells. Only T cells showing an intermediate level of TCR signaling are allowed to further
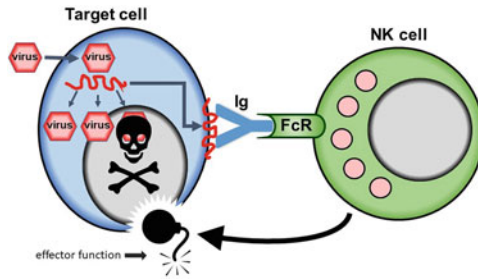
Fig. 4.2 NK cells can kill target cells by Antibody-Dependent Cell-mediated Cytotoxicity (ADCC). During certain virus infections, viral proteins are incorporated into the membrane of infected cells. Antibodies can bridge the contact between Fc receptors (FcR) expressed on the surface of NK cells and the Fc part of antibodies opsonizing virus-infected cells

mature (positive selection) (rb Germain 2002). Mature naïve SP T lymphocytes leave the thymus and recirculate through secondary lymphoid tissues such as lymph nodes, Payer's patches, and spleen. In these tissues, CD8+ cytotoxic T lymphocytes (CTL) and CD4+ T helper (Th) cells are activated through binding of their TCRs to antigenic peptides displayed by MHC class I or MHC class II molecules on the surface of antigen-presenting cells (APC), respectively. Activated T cells undergo additional development and differentiate into effector and memory cells (rb Boes and Durham 2017). However, there are also examples where DP T cells can be found outside the thymus, e.g., in pigs (Saalmüller et al. 1987).

The T-cell development in teleost fishes is similar to that in mammals although there are still many gaps in our knowledge (rb Bajoghli et al. 2019). In the zebrafish embryo, HSCs originating from dorsal aorta migrate to the caudal hematopoietic tissue (CHT) and finally home to the thymus and the kidney. Two days post fertilization (dpf), the zebrafish thymus region is colonized by HSC and T lymphopoiesis is initiated following the expression of RAG1 and later on by the formation of cortex and medulla. Based on the consecutive expression of RAG1, Ikaros, TCRα, TCRβ, TCRγ, and TCRδ and other T-cell markers in the zebrafish thymus, it may be concluded that CD8α is first transcribed at 6 dpf (rb Ma et al. 2013). In rainbow trout, CD8α is expressed as early as between 7 and 10 dpf, while first TCR expression can be seen between 14 and 40 dpf (Fischer et al. 2005; Heinecke et al. 2014, respectively). In sea bass, CD8 transcription was first recorded 51 days after hatch (Picchietti et al. 2009). In ginbuna crucian carp and rainbow trout, thymocytes traverse a stage where they are CD4/CD8 DP cells while such cells cannot be found outside the thymus. Thus, DP cells reach a SP state prior to leaving the thymus (Toda et al. 2011; Takizawa et al. 2011, 2016).

Although CD8+ cells can be found in all trout and ginbuna organs, their distribution is unequal both when comparing organs, and within the same organ (Table 4.1).

In contrast to humans where the percentage of CD8α+ cells among PBL is between 16 and 38% (rb Virella 2007), the corresponding share in teleost fish is remarkably low

Table 4.1 Percentages of CD8α+ (ginbuna crucian carp and rainbow trout) and CD8β+ (Japanese flounder) lymphocytes in different organs of adult fish

Lymphocyte source	Rainbow trout (%)	Ginbuna crucian carp (%)	Japanese flounder %	References
Thymus	70	35	nd	
Intestine	54	nd	nd	rb Nakanishi et al. (2015) for trout and ginbuna
Gill	25	nd	nd	Xing et al. (2017) for flounder
Spleen	2	3.5	2.8	
Pronephros	4	7	3.2	
PBL	0.3	1.7	2.1	Takizawa et al. (2011) for trout Toda et al. (2011) for ginbuna Xing et al. (2017) for flounder

(Table 4.1). While trout CD8α+ lymphocytes are equally distributed in most tissues, they are found in higher concentrations in the internal (basal) zones of trout thymus (Takizawa et al. 2011), and in the cortical zones of sea bass thymic lobes (Picchietti et al. 2009). Although teleost fishes lack lymph nodes, Payer's patches, and distinct T-cell and B-cell zones with germinal centers, CD8α+ lymphocytes form follicle-like structures in the trout spleen (Takizawa et al. 2011). According to investigations by Leal et al. (2016), abundance of CD8α+ T lymphocytes among skin lymphocytes is higher in its anterior parts (12%) than in posterior sections (6%).

Induction and Regulation of CTL-Mediated Cytotoxicity

Before mammalian CTLs (like NK cells) use their effector molecules perforin, granzyme, and FasL for target cell killing, they need to be activated and proliferated. In order to create a systematic approach of CTL (and Th cell) induction, two main activation signals were initially suggested. According to this model, signaling starts with the binding of TCR complexes on T cells to the peptide-MHC complex on APCs (signal 1). Generally speaking, MHC class I is expressed by all nucleated cells (in fish this includes erythrocytes and thrombocytes!) and presents peptides derived from intracellularly produced proteins, while MHC class II expression is restricted to professional APCs and is important for displaying peptides from endocytosed/phagocytosed antigens. While CD8 serves as a coreceptor of TCR to stabilize contact of CTLs to MHC class I, CD4 supports binding of Th cells to MHC class II expressing APCs (rb Murphy and Weaver 2017). Signal 2 is characterized by costimulatory signaling between additional receptors on T cells and their corresponding ligands on APCs. To more comprehensively explain additional activation of
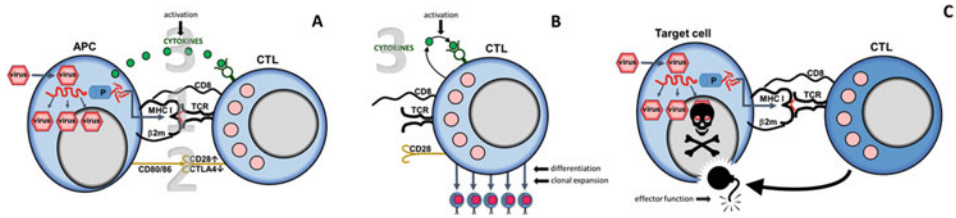
Fig. 4.3 Three signals are needed for CTL activation in mammals, differentiation, clonal expansion, and finally effector function: (1) Presentation by APCs of antigenic peptides through MHC class I to TCR of CTLs; (2) Accessory receptor/ligand interactions; (3) Paracrine cytokines produced by APCs (**a**). After this, autocrine cytokine signaling leads to further CTL activation resulting in CTL differentiation, clonal expansion (**b**), and effector function (CMC) (**c**)

T cells by cytokines, Curtsinger et al. (1999) introduced signal 3 (rb Mescher et al. 2006). This signal theory does not fully explain the complex activation system for T cells, but it reflects its most important steps that appear to be conserved throughout vertebrates (Fig. 4.3).

Before mammalian CD8+ CTLs utilize their TCR complexes to recognize peptide–MHC class I complexes on target cells, proteins intracellularly synthesized by viruses or by intracellular bacteria are proteolytically cleaved to peptides of around 10 amino acids (aa) in the proteasome. Peptides are then translocated into the endoplasmic reticulum (ER) by transporters associated with antigen processing (TAP). In the ER, the MHC class I heavy chain folds into three domains (α1, α2, and α3) and associates with a fourth domain, the β2-microglobulin (β2M). The α1 and α2 domains of MHC class I form the peptide-binding groove where antigenic peptides of 8–10 aa in length are loaded. Peptide-loaded MHC class I molecules are finally transported to the cell surface of APCs and presented to the corresponding specific TCR complex on CD8+ lymphocytes (rb Hewitt 2003). However, peptide specificity does not imply that a certain TCR can only bind to peptides with identical aa sequence. TCRs can also cross-react with other peptides of different avidities depending on the presence of anchor residues in the respective peptides (rb Singh et al. 2017).

Orthologous genes for MHC class I and peptide-loading pathway molecules, e.g., tapasin, proteasome subunit beta molecules of the immunoproteasome, and TAP (TAP1 and TAP2) have been described in a number of fish species (rb Yamaguchi and Dijkstra 2019). While signature motifs and domains of these molecules are conserved among teleost fishes, MHC class I signaling cascades remain largely unexplored. Orthologous genes of MHC class I, the TCR complex (consisting of the TCR chains and the CD3 subunits), and the TCR coreceptor CD8 are known in teleost fish, while in grass carp, CD8$\alpha\alpha$ homodimers were reported to bind specifically to the pMHC-I complex (Wang et al. 2018a, b). Additionally, genes associated with TCR complex signaling namely the zeta-chain-associated protein kinase (ZAP)70 and lymphocyte-specific protein tyrosine kinase (LCK) have been reported in teleosts. However, the Lck-binding motif of the teleost CD8

molecules is different from tetrapods. While in tetrapods, a bicysteine Lck-binding motif (CxC) is only present in the cytoplasmic tail of CD8α chains, both teleost CD8α and CD8β chains possess the possible Lck-binding motif CxH suggesting that both CD8 chains can be involved in Lck signaling. However, since Lck binding to CxH motif is zinc-dependent in CD8α but not CD8β, CD8α seems to be the main TCR coreceptor to recruit Lck molecules in teleost CTLs.

CD8 is the main CTL marker, however does not reveal the antigen specificity of CTLs. MHC class I multimer technology utilizes labeled and multimerized recombinant MHC class I proteins that are loaded with peptides of a known aa sequence and that are stabilized with recombinant β2m, for staining of antigen-specific CTLs expressing a certain (peptide-) specific TCR mimicking signal 1 during CTL activation. Such multimers have been used in grass carp to identify antigen-specific CTLs against grass carp hemorrhagic virus (GCHV) and in rainbow trout against infectious hematopoietic necrosis virus (IHNV) (rb Yamaguchi et al. 2019a).

There are several studies on the regulation of CD8 expression in virus-infected teleost fish. In rainbow trout, the expression levels remained unchanged or down-regulated after IPNV infection, while in Atlantic halibut infected with nodavirus and in Atlantic salmon inoculated with infectious salmon anemia virus (ISAV), CD8 expression was decreased. The reasons for such down-regulations are unknown. In mammals, herpesviruses, pox viruses, and the human immunodeficiency virus suppress MHC class I pathway components to prevent antigen presentation to CD8+ lymphocytes thereby suppressing signal 1 of CTL activation. Other reasons for CD8 suppression could be simply a cytolytic infection of CD8+ cells or an attrition of CTL responses by increased type I interferon expression (rb Yamaguchi et al. 2019a).

Monoclonal and polyclonal antibodies against CTL markers, particularly against CD8 are valuable tools to study CTL responses. They are available for ginbuna, rainbow trout (rb Nakanishi et al. 2015), and for olive flounder (Xing et al. 2018). Five days after infection with viral hemorrhagic septicemia virus (VHSV), the number of CD8α+ cells in the spleen was found to be decreased while a concurrent increase occurred in the liver (Castro et al. 2014). VHSV infection also increases the number of CD8+ cells in the adipose tissue (Pignatelli et al. 2014). CD8α+ cells separated from trout spleen showed a skewed TCR repertoire 3 weeks after a secondary VHSV infection suggesting clonal expansion of CD8+ CTLs upon viral infection. Several papers describe the attraction of CD8+ cells to the site of infection. In trout, interaction of IHNV with olfactory sensory neurons in the nasal cavity triggered the recruitment of CD8+ T cells to the olfactory mucosa (Sepahi et al. 2019) and an influx of CD8+ cells was also seen during ichthyophthiriasis in infection foci of rainbow trout gills (Olsen et al. 2011), while in another parasite infection (proliferative kidney disease) of the same species, no major changes among CD8+ cells were recorded (Bailey et al. 2020). During red mouth disease (caused by *Yersinia ruckeri*), a transient early decrease in densities of CD8α+ cell was seen shortly after infection of vaccinated trout followed by an increase at 30 days after infection (Deshmukh et al. 2013).

Costimulatory signal 2 interactions between B7 family molecules (B7s and B7-homologous family members expressed on APCs) and CD28 family molecules (expressed on T cells) are essential for the balance between T-cell activation and T-cell tolerance following TCR-mediated signal 1 transduction in mammals. Within the CD28 family, there are two positive costimulatory regulators: CD28 and inducible costimulatory signal (ICOS), and three inhibitors: cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death-1 (PD-1), and B- and T-lymphocyte attenuator (BTLA) (rb Riley and June 2005). Mammals possess two B7 molecules: B7-1 (CD80) and B7-2 (CD86). In teleost fish, a single CD80/86 gene is believed to be the ancestor from which CD80 and CD86 arose by gene duplication during evolution. Trout CD80/86 is expressed on B cells and up-regulates the expression of IL-2 in PBL (Zhang et al. 2009). Since teleost B cells have been associated with phagocytosis and zebrafish B cells were shown to express CD80/86, they are suggested to play a role as professional APCs (Zhu et al. 2014). Additional B7 family members such as H1/DC, B7-H3, and B7-H4, have been identified in teleost fishes too. Although signaling motifs are apparently missing in the cytoplasmic region of teleost CTLA-4, both CD28 and CTLA-4 are structurally highly conserved among vertebrates suggesting similar functions (rb Yamaguchi et al. 2019a, b).

To fully activate naïve mammalian CD8+ CTLs, a third signal provided by cytokines is required. Interferon (IFN)γ is a signature cytokine for this activation. Additional highly relevant cytokines that can activate mammalian CTLs are IL-2, IL-15, and IL-21. IFNγ is expressed by CD8+ CTLs, by NK cells and Th1 cells and is thereby a key inducer of type 1 immunity. Upstream, this type 1 response IFNγ production is stimulated by IL-12, a cytokine produced by DCs. During intracellular infections, mammalian DCs not only provide this signal 3, but also the aforementioned signals 1 and 2 to CTLs (Henry et al. 2008). IFNγ homologs have been described in many fish species. Rainbow trout and zebrafish express two IFNγ genes (IFNγ1-1 and IFNγ1-2). While in zebrafish, IFNγ1–1 expressing cells do not transcribe effector molecules of cytotoxic cells, IFNγ1–2 is coexpressed with granzymes and granulysins. In trout, IFNγ is expressed by CD8α+ lymphocytes and triggers the expression of IL-15, suggesting its role in Th1 responses. However, direct effects of piscine IFNγ homologs on CTL activity still need to be shown. Channel catfish, common carp, ginbuna, goldfish, grass carp, Indian major carp, and zebrafish, additionally express IFNγ-related proteins (IFNγrel). However, the functions of IFNγrel still need to be explored (rb Yamaguchi et al. 2019a).

Another important cytokine stimulating the clonal expansion of mammalian T cells and the growth of antigen-stimulated T cells is IL-2 (rb Nelson 2004). IL-2 plays a crucial role in the differentiation of naïve CD8+ T cells into CTL effector cells expressing perforin, granzyme B, and IFNγ, but also into CTL memory cells (Pipkin et al. 2010). Additionally, IL-2 is involved in the formation of immune tolerance (rb Malek and Bayer 2004). IL-15, another member of the IL-2 cytokine family, stimulates CTLs and has been shown to be even more important than IL-2 in the development and maintenance of CTL memory (rb Sim and Radvanyi 2014). Mammalian IL-2 binds to the IL-2Rαβγ with high affinity or to the IL-2Rβγ with intermediate affinity, while IL-15 is predominantly expressed in

association with IL-15Rα either on the surface of APCs or released as a soluble heterodimer with IL-15Rα. IL-15Rα significantly stabilizes IL-15 which then binds to IL-2Rβγ, e.g., on CD8+ lymphocytes (rb Waldmann 2006). However, teleost fishes do not possess IL-2Rα implying that both cytokines must only rely on IL-15Rα (Dijkstra et al. 2014). Trout and yellow croaker IL-2 trigger gene expression of IFNγ. Trout IL-15 induces IFNγ gene expression in splenocytes (rb Yamaguchi et al. 2019a) and recently, Yamaguchi et al. (2019b) have shown that trout IL-15/IL-15Rα heterodimers possess a higher bioactivity with respect to the activation of CD8+ lymphocytes than IL-15 alone.

Another cytokine that promotes CTL function by induction of perforin, granzyme B, and IFNγ in mammals is IL-21. However, IL-21 rather works in concert with other cytokines such as IL-7 and IL-15 to expand and activate CD8+ T cells (rb Leonard et al. 2019). In rainbow trout, IL-21 triggers gene expression of IFNγ, IL-10, IL-21, IL-22, CD8, and IgM; and it maintains transcription of CD8 at a later stage of in vitro stimulation (rb Wang et al. 2011a, b). Synergistic effects of IL-21 with additional cytokines are still unknown in teleost fishes.

Besides mammalian cytokines that activate CTLs, there are also cytokines that suppress their function. One of them, IL-10, can reduce the production of IFNγ as well as T-cell proliferation and effector activity. By inhibiting signal 1 through decreased antigen presentation by professional APCs, IL-10 can have an additional negative feedback on T-cell responses. However, IL-10 has both immunosuppressive and stimulatory effects in mammals. Although IL-10 triggers the proliferation and effector function of CD8+ T cells against tumors, it could also inhibit CTL activity against tumor cells by decreasing antigen presentation (rb Ouyang and O'Garra 2019). IL-10 activity has been described in several fish species. It down-regulates IFNγ gene expression in goldfish and promotes the survival and proliferation of T cells isolated from immunized common carp. From stimulation experiments in common carp, it was concluded that IL-10 promotes the development of CD8+ memory T cells, while down-regulating CD4+ memory T cells. In infected amberjack, IL-10 was suggested to shift the Th1/Th2 balance toward Th2 (rb Yamaguchi et al. 2019a).

Mammalian cytokines transduce signals through their corresponding receptors resulting in transcription factor induction and finally in the expression of certain effector molecules. The T-box transcription factors EOMES and T-bet are signature transcription factors promoting Th1 and consequently CTL (and NK cell) activation, trafficking and regulation in mammals. They are engaged by IL-2, IL-12, IL-21, IFNγ, type 1 interferons, and even by IL-4. T-bet and EOMES are usually expressed in different ratios. While mammalian effector T cells are rather T-bet[high] and EOMES[low], this situation is inversed in memory cells (rb Pritchard et al. 2019 and Dejean et al. 2019). Homologous genes to T-bet and EOMES have been identified in many fish species. In virus-infected grass carp, in bacterial-infected Atlantic salmon, and in vaccinated ginbuna, T-bet and IFNγ expressions were found to be up-regulated. T-bet (along with perforin and granzyme) expression can be induced in allo-grafted ginbuna. In rainbow trout, high expressions of EOMES and T-bet were recorded in CD8α+ cells (rb Yamaguchi et al. 2019a).

Apart from these three signals, chemokines also contribute to CTL recruitment and differentiation in mammals. Several chemokines and their corresponding receptors are involved in CTL priming, effector function, and memory formation in mammals (rb Griffith et al. 2014). In the teleost orange-spotted grouper, recombinant CCL4 was found to induce chemotactic activity in PBL and to up-regulate TNF-α1, TNF-α2, IFNγ, Mx, T-bet, and both CD8α and CD8β expressions, suggesting the induction of a Th1-skewed response. Recombinant CK12a, a CCL19-like chemokine was found to trigger the expression of CD8α, granulysin, and IFNγ while increasing the number of CD8α+ cells at the site of administration in rainbow trout (rb Bird and Tafalla 2015; Sepahi et al. 2017).

Effector Functions of CTLs

CTL-mediated cytotoxicity is MHC class I restricted in mammals, meaning that the MHC class I of the APC must fit into the TCR of a CD8+ CTLs. However, MHC molecules were not discovered during investigations on immune responses against pathogens, but in transplantation medicine. Dausset, Snell, and Benacerraf were awarded the Nobel Prize in physiology in 1980 for the discovery of the so-called histocompatibility ("tissue matched") antigens during early 1970s. Subsequently, Doherty and Zinkernagel found the MHC class I restriction of CTL-mediated cytotoxicity against virus infection in mice (Nobel Prize in 1996).

The first studies on cell-mediated cytotoxicity in teleosts also used allograft rejection models. However, the initial lack of assay systems requiring MHC class I matched effector and target cells have delayed investigations on antiviral CTL-mediated cytotoxicity. A respective assay system was introduced in clonal ginbuna crucian carp by establishing a syngeneic cell line from the respective fish clone. In clonal rainbow trout, a syngeneic MHC class I matched target cell line was identified after sequencing of well-established trout cell lines. Another approach was used in channel catfish and orange-spotted grouper where autologous target cells isolated from the same individual effector cell donor were applied. The latter system offers the possibility to use CMC assays even if clonal fish are not available. Infected MHC class I mismatched target cells are usually not killed by CTLs. Such killing indicates the contribution of NK cells to cell-mediated cytotoxicity (see subchapter on NK cell responses). Phenomena related to CTL-mediated cytotoxicity in fish are comprehensively reviewed in a recent publication by Yamaguchi et al. (2019a).

Strong suggestion for MHC class I restricted CMC has been shown in ginbuna against two rhabdoviruses (carp hematopoietic necrosis virus—CHNV; eel virus from America—EVA) and against a birnavirus (infectious pancreas necrosis virus—IPNV), in rainbow trout against two rhabdoviruses (VHSV and IHNV) as well as in orange-spotted grouper against nervous necrosis virus (NNV)-infected fish. DNA vaccines are suggested to be potent triggers of CTL responses since they mimic a viral infection in terms of intracellular antigen processing and presentation through MHC class I (rb Wang et al. 2011a, b). In trout, CMC against VHSV was shown after intramuscular administration of DNA encoding the G or the N protein of VHSV (rb Yamaguchi et al. 2019a).

Further characterization of effector cells in MHC class I restricted CMC has brought additional evidence that teleost CTLs are among the effector cells contributing to antiviral and allo-specific cytotoxicity. In ginbuna and rainbow trout, such effector cells express mRNA encoding TCR and CD8. Moreover, CD8+ lymphocytes that were separated from CHNV-infected ginbuna crucian donors were able to kill CHNV-infected MHC class I matched target cells and to protect syngeneic recipients against CHNV infection. By using a system of allo-specific CMC in ginbuna, it was shown that CD8α+ effector cells kill their target cells by utilizing perforin and granzyme pathways (rb Yamaguchi et al. 2019a). Recently, Taylor et al. (2020) succeeded to clone four different antiviral cytotoxic cell lines expressing rearranged TCR genes, perforin, granzyme, and IFNγ. Surprisingly, none of these T-cell lines expressed CD8, while three of them expressed CD4-like molecules.

Mammalian CTLs (and NK cells) use several effector molecules to drive target cells into apoptosis. They store in their cytolytic granules perforin, granulysin (or its ortholog NK-lysin), and serine protease granzymes that are released into the cytotoxic synapse formed by signal 1 related and adhesion molecules. Perforin polymerizes to form trans-membrane pores in the target cell membrane resulting in the delivery of granulysin and granzymes into target cells. Another granula-independent mechanism is based on interaction between FasL on activated CTLs and Fas on the target cell membrane. Both mechanisms lead to activation of caspases and finally to target cell apoptosis (rb Voskoboinik et al. 2015).

Genes encoding CTL effector molecules have been sequenced in several fish species and a few studies also investigated their activities such as antimicrobial activity, proteolytic activity, caspase, and apoptosis induction. CD8+ cells have been shown to express perforin and granulysin (rb Yamaguchi et al. 2019a). However, deep insights into mechanisms of CTL effector functions in fish are still missing.

There is little information on CTL memory in fish. Effector cells isolated from ginbuna reinfected with IPNV or CHNV were more effective in killing syngeneic cells infected with the respective homologous viruses than against syngeneic cells infected with a heterologous virus (EVA). The use of syngeneic (clonal) fish offers the possibility to transfer immune cells from one individual to another without risking rejection of donor cells by the recipient. In clonal ginbuna, adoptive transfer of CD4− cells (containing CD8α-expressing cells) from CHNV-infected donors to naïve syngeneic recipients induced an antiviral immune response (Somamoto et al. 2014). Using adoptive transfer experiments, CD8+ cells have also been proven to be important in intracellular bacterial infections in fish such as with *Edwardsiella tarda*. Transfer of CD8+ cells from ginbuna donors sensitized by *E. tarda* to isogenic naïve recipients resulted in up-regulation of IFNγ and perforin and conferred protection against *E. tarda* challenge (Yamasaki et al. 2014). Adoptive transfer of leukocytes from clonal rainbow trout donors that were previously immunized against the bacterium *Yersinia ruckeri* to syngeneic recipients resulted in protection of the latter. However, expression of CD8 versus IgM and IgT in organs as well as IgM levels in sera of recipients suggest a B- rather than a T-cell memory response (Yamaguchi et al. 2019b).

4.3 Vaccination Targeting CTL-Mediated Immune Responses

Vaccination is based on B-cell and/or T-cell (including Th and CTL) memory. Due to intracellular processing of antigens during intracellular infections, CTL responses seem to be highly important in antiviral responses in vertebrates. Nevertheless, in several vaccines against human viral diseases, antibodies alone were found to be sufficiently protective. An antibody-based protective response has also been suggested against IHNV in Atlantic salmon, as high antibody levels corresponded with the reduction of postchallenge mortality. However, most licensed vaccines are inactivated and thus antigens are rather taken up by APCs resulting in an MHC class II presentation pathway. Peptides presented by MHC class II, however, are recognized by Th cells but not by CTLs. Part of the peptides produced after uptake may also be cross-presented through MHC class I. Although the existence of cells with a phenotype of cross-presenting DCs in rainbow trout suggests cross-presentation in teleost fish, this phenomenon remains to be demonstrated (rb Yamaguchi et al. 2019a).

DNA vaccines seem to be best suited to mimic a viral infection and to trigger CTL responses through MHC class I pathways. Indeed, a CTL-like response could be shown after DNA vaccination against VHSV in an MHC class I matched system of vaccinated rainbow trout effector cell donors and virus-infected target cells. Moreover, effector cells isolated from the injection site of DNA vaccinated clonal trout donors migrated to the injection sites of the homologous DNA vaccine after transfer to vaccinated fish of the same (isogenic) trout clone suggesting antigen-specific effector cell homing (rb Yamaguchi et al. 2019a).

Our current knowledge on MHC class I restricted cell-mediated cytotoxicity in teleost fish gives rise to the hope of using peptide vaccines. For this, antigenic peptides conforming to the groove of MHC class I need to be initially analyzed or predicted. The correctness of the corresponding synthesized peptides needs to be confirmed by analyzing their capacity to stabilize recombinant MHC class I and β2m heterodimers. This has been demonstrated in grass carp for peptides derived from GCHV and in rainbow trout for IHNV peptides. However, high polymorphism among MHC class I alleles makes the use of MHC class I peptide-based vaccines difficult in an outbred population with high genetic variability (rb Yamaguchi et al. 2019a). Moreover, peptide presentation profiles are even more variable due to the fact that diploid bony fish possess two β2m loci which may lead to structural changes in the peptide-binding groove of the MHC class I α1 and α2 domains (Li et al. 2020).

Very important components of vaccine formulations are adjuvants. In mammals, several adjuvants are known that can support CTL responses. Although adjuvants are widely used in commercial fish vaccination, their capacity to enhance T-cell responses remains unknown. To overcome the side effects of oil adjuvants and to design vaccines that preferentially trigger CTL memory, molecular adjuvants are the focus of current research. Both IL-2 and IL-15 are known to stimulate CTL responses, and IL-15 contributes to T-cell

memory in mammals. Recently, Yamaguchi et al. (2019b) have shown that recombinant IL-15/IL-15Ra heterodimers trigger type 1 immune responses in rainbow trout.

4.4 Conclusion/Outlook

Cell-mediated immune functions are an integral part of a concerted immune response. While nonvertebrates rely on pattern recognition only and have evolved without the need of a true adaptive immune system, vertebrates have developed cells that specifically recognize and counteract infectious agents. As in their mammalian counterparts, evolutionary ancient cells of the innate immune system such as macrophages and granulocytes act in concert with TCR bearing Th cells and CTLs of the adaptive immune system in teleost fish. Many of the cells involved in cell-mediated immune responses have been described in fish and the increasing availability of tools to identify them have been developed during the last decades. While evolutionary ancient cell-mediated functions such as phagocytosis and NETosis could clearly be shown in fish, the phenomenon of cell-mediated cytotoxicity requires further research efforts. Currently available tools in fish immunology still do not always allow to clearly distinguish between NK cell and CTL responses, even where MHC class I matched effector and target cell systems are available. Another aspect that should attract our attention is the early stage of fishes in evolution where immune cells might not have reached a stage of specialization as in mammals thus exhibiting several functions. Even in mammalian immunology, former clear textbook borders between innate and adaptive immune responses are not a dogma anymore (cf. innate memory, NKT cells, etc.). Another challenge for future studies is to better understand how cell-mediated immune response mechanisms are embedded in the whole process of disease protection and resistance. Finally, basic and applied research in fish immunology will help to design vaccines that are able to selectively target those immune mechanisms that are responsible for disease protection.

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Lymphocytes of Teleosts

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Abstract

Increasing evidences suggest that there is a great degree of similarity in the morphological and functional organization of immune defences across jawed vertebrates, and lymphocytes are among the most conserved elements. Some peculiarities make lymphocytes unique cells, they contain enzymes (RAGs) able to recombine somatically genome sequences, genome sequences prone to recombination (Ig, TcR), and MHC machinery able to recognize extracellular and intracellular antigens, thus representing an extraordinary and efficient evolutionary machinery to fight nonself. The evolutionary success of lymphocytes is evident in the similarities between cell populations and physiological functions conserved among vertebrates. Teleost fish represent the base of the evolutionary pathway that shaped lymphocyte functions, and this review summarizes the available knowledge on fish lymphocyte populations and subpopulations, and their main physiological features.

Keywords

Teleost fish · T cells · B cells · Lymphocytes · Comparative immunity · Evolutionary immunology

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Abbreviations

APC	Antigen-presenting cells
BcR	B-cell receptor
CD	Cluster differentiation antigen
CDR	Cell-determining region
CTL	Cytotoxic T lymphocytes
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
GALT	Gut-associated lymphoid tissue
HC	Heavy chain (immunoglobulin)
IEL	Intraepithelial leukocytes
Ig	Immunoglobulin
IHC	Immunohistochemistry
ILT	Interbranchial lymphoid tissue
ISH	*In situ* hybridization
LC	Light chain (immunoglobulin)
LITRs	Leukocyte immune type receptors
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MHC	Major histocompatibility complex
NITRs	Novel immune type receptors
NK	Natural killer cells
pAb	Polyclonal antibody
PAMPs	Pathogen-associated Molecular Patterns
qPCR	Quantitative real-time PCR
RAG	Recombination-activating genes
TcR	T-cell receptor

5.1   Introduction

Lymphocytes are cells with distinguishing peculiar features, among which the most important is the recombination of germline-encoded immunoglobulin-type genes, the only gene recombination process occurring outside germinal tissues. Somatic recombination of immunoglobulin genes is an acquisition of jawed vertebrates, very likely deriving from the domestication of pre-RAG genes in invertebrate chordates (Zhang et al. 2019). Additional evolutionary steps that originated jawed vertebrates included whole genome duplication events and functional RAGs (Dehal and Boore 2005), whose activity met the intrinsic plasticity of immunoglobulin domain-containing genes, already employed to recognize nonself throughout all animal phyla (Miccoli et al. 2021). Other features of lymphocytes are the presence on the plasma membrane of BcRs, TcRs, MHC class II, and

lymphocyte-specific coreceptor molecules. These features, in concert with those of other cell populations and molecules, give to jawed vertebrates the extraordinary capability of a long-lasting immune memory for infectious pathogens. Teleost fish represent one of the most extant ancient clade of jawed vertebrates and hence represent the anatomical and physiological basis through which the evolution shaped following groups. For this feature, and considering the high degree of similarities in the anatomy and physiology of jawed vertebrates, teleost fish are widely employed as animal model in biomedical research, with the zebrafish as the most used. In addition, farmed fish are a fundamental food source for humans and the control of their health and immune status in farming conditions is of great interest for optimal industrial production in an environment-friendly fashion.

The knowledge on the physiology of fish lymphocytes is increasing, and available data strongly suggest that the lymphocyte subpopulations are present in teleosts as they are known in mammals, together with an increasing knowledge on evolutionary relationships between the immune system in vertebrate classess, in particular with mammals. Recent data also suggest that starting from teleosts, a complex of "immune layers" accumulated toward mammals during evolution, with superimposing layers endowed with new gene products or gene isoforms from which additional specialization emerged. Despite the absence of bone marrow and lymph nodes, it is now accepted that B cells of teleosts represent the ancestors from which amphibian, reptilian, avian, and mammalian B cells evolved and differentiated. In this respect, there are clear morphological and physiological similarities between mammalian B-1 B cells and fish IgM-bearing B cells (Scapigliati et al. 2018).

This review summarizes available knowledge on the main in vivo and in vitro cellular features of teleost fish lymphocytes, with the aim of supplying a better comprehensive view on vertebrate cellular immunology, on the lymphocyte responses against pathogens, and on the possible use of the knowledge for animal and human health.

5.2 Early Data and Identification of Fish Lymphocytes

The first reports describing immune activities clearly attributable to lymphocytes date back about 80 years ago employing the trout as target species. These studies reported on antibody production after immunization with bacteria (Cushing 1942; Duff 1942), development of antibacterial protective humoral immunity following vaccination (Snieszko and Friddle 1949; Krantz et al. 1964), and antibody responses against viral antigens (Sigel and Clem 1965). The presence of mucosal immunity in fish was first supposed in 1960s with the discovery of antibodies in the mucus of plaice (Fletcher and Grant 1969).

Following the pioneering studies, fish immunology became important for either comparative immunology and application of studies to fish farming, and from the 1970s investigations on the immunobiology of lymphocytes bloomed. The presence of lymphocytes in fish was indirectly supposed by morphological observations, and by adapting to fish the experimental knowledge developed for mammals. With this approach, proliferative responses of leukocytes to mitogens were reported (Etlinger et al. 1976;

Sizemore et al. 1984), together with the presence of T-like and B-like cells (Cuchens and Clem 1977). It is interesting to note that despite a clear evidence of a thymus presence in teleosts, the tissue was initially not directly related to an involvement in T-cell immunity (Parkhouse 1975). Soon later, the presence of lymphoid tissues in fish was confirmed, and early data on ontogenetic development of lymphoid cells became available (Ellis 1977). Following, the presence of B- and T- lymphocytes with features similar to those of mammals was supposed by the expression of genes coding for lymphocyte master genes, and lymphocyte-driven biological activities directly observed by employing specific markers for B cells and T cells subpopulations.

5.3   B Cells

Three gene families encoding immunoglobulin heavy chains (HC) are known in fish, namely IgM, IgZ/IgT, and IgD, that associate with three immunoglobulin light chains (LC), $-\kappa$ and $-\lambda$, $-\sigma$, to form the mature antibody molecules. The three Ig HC germline forms are functionally rearranged in corresponding B lymphocyte populations that undergo antigen-driven somatic recombination and clonal proliferation, although poor knowledge is available on the clonal structure of IgM, IgD, and IgT B-cell isotypes. As for other vertebrates, resting fish B lymphocytes are cells of ca. 5–8 μm in diameter with a high ratio of nucleus/cytoplasm, upon antigen stimulation undergo dramatic cytoplasmic changes becoming antibody-secreting plasma cells. The IgM-, IgD-, and IgZ/T- B cells display peculiar tissue distribution in healthy fish, but can migrate between anatomical sites upon antigen priming and perform tissue-related peculiar physiological activities. Without the B-lymphoid tissue present in mammals, the origin of B-cell precursors in fish is still under study. In unstimulated trout, the in vitro production of plasma cells appears to be restricted to kidney and spleen and of plasmablasts in blood, and after immunization >90% of the plasma cells are present in the kidney (Bromage et al. 2004). From the data obtained in trout, the kidney is considered to be the place where B-cell lymphopoiesis occurs, with diverse stages of development of lymphoblasts inside anatomic zones of the tissue (Zwollo et al. 2010). Recently, a structural analog of bursa of Fabricius has been described in Atlantic salmon and similarly to birds located in the terminal part of the intestine (Løken et al. 2020).

The increasing knowledge on the physiological activities of teleost B cells led to draw evolutionary relationships between fish IgM-B cells and IgM-secreting B1-B cells subpopulation of mammals, having conserved innate-like lymphocytes activities (Scapigliati et al. 2018).

5.4 IgM-B Cells

Among the antibody isotypes, IgM is the most ancient and the only isotype functionally conserved in all jawed vertebrates (Flajnik 2002), with the only exclusion of the coelacanth fish (Pallavicini et al. 2013). The similarity between the IgM antibody molecules present in fish and in other vertebrates including mammals appeared evident by means of conserved biochemical features (Acton et al. 1971). By taking advantage from this similarity, the biochemical isolation of purified IgM from sera was achieved, allowing the obtainment of polyclonal (pAb) and monoclonal (mAb) antibodies specific for the IgM of teleost species, the first of which was an antiserum directed to lymphocyte surface immunoglobulins in the goldfish (Warr et al. 1976). The use of these markers unvealed basical aspects of teleost antibody responses against pathogens and started the new field of fish immunity, boosted by the direct application of results to the protection of fish health in farming conditions. Due to consistent amount of antibody present in serum, the biochemical purification of IgM allowed its use as an antigen, and the number of antibodies directed against IgM-B cells have been continously increasing. In 1999, pAb/mAb specific for fish IgM and IgM-bearing cells was available for at least 10 species (Scapigliati et al. 1999a) and, at present, at least for 30 species. The knowledge accumulated on IgM immunobiology confirmed this class as the main antibody class in the blood of teleost species, and a high concentration of unspecific circulating IgM can be present and may increase with fish age/size (Scapigliati et al. 1997, 1999b). Upon immunization with bacteria and viruses, antigen-specific IgM is produced, a memory for the immunization antigen is mounted, and that upon secondary exposure to antigens an increase in affinity can be observed, although this point is controversial (Kaattari et al. 2002; Ye et al. 2011).

IgM-producing lymphocytes are mainly present in nonmucosal tissues, mostly present in kidney, spleen, blood, and at much lesser extent in thymus, intestine, gills, skin, and liver. The percentages of IgM B cells in unstimulated fish, measured by indirect immuno-fluorescence (IIF), can be greatly variable depending on the species, ranging from 12 to 30% in head kidney, 26–70% in spleen, 13–40% in peripheral blood, 6–20% in gills, 2–12% in the intestine, 3–6% in the thymus, and ca. 16% in skin (see Table 5.1). (DeLuca et al. 1983; Scapigliati et al. 2003; Rønneseth et al. 2007; Salinas et al. 2011; Nuñez Ortiz et al. 2014; Parra et al. 2016; Wu et al. 2020). From these data, it appears evident that IgM-B lymphocytes are principally located in internal tissues not in direct contact with the external environment, namely in the spleen, kidney, and blood. This evidence suggests that their interaction with antigens should be previously mediated by other immune cells present at mucosal surfaces that subsequently activate/stimulate B cells. Importantly, and with striking similarities with mammals, a large percentage of trout B cells coexpress surface IgM and IgD and down-regulate IgD after encountering an antigen (Tafalla et al. 2017). Other observed features are of importance to understand the physiology of B cells in an evolutionary context, such as the spontaneous phagocytosis and intracellular killing of bacteria, that makes teleost B cells acting as APC (Sunyer 2012), and the innate secretion of IgM when cells are directly activated by a number of PAMPs (Li et al. 2006). Thus, IgM-B

cells display classical lymphocyte-dependent acquired features, as they can be induced to produce antigen-specific IgM through MHC_{II} presentation and maintain a memory for encountered antigens, although the duration of the memory has not been fully ascertained yet. Available knowledge let us to speculate on the presence in fish of two IgM-B cell subpopulations with physiological activities comparable to B1- and B2- B cells of mammals, with the great majority behaving as B1-B cells, namely spontaneously active against nonself and secreting in serum large amounts of antibodies with poor affinity for random antigens. Another similarity with mammalian B1-B cells comes from recent findings that revealed the presence of functional IgM-B cells in the peritoneal cavity of investigated species that increase upon systemic antigen stimulation (Castro et al. 2017), although the site from which they are recruited and migrated into peritoneum is at present unknown. A knowledge on the presence of immunoreactive cells in the peritoneal cavity of fish is particularly important in the light of intraperitoneal vaccination, far the most employed route of antigen delivery/vaccination in fish.

Upon clonal selection, the IgM-B cells may acquire a memory for the immunization antigen, and this feature has been employed to produce cell-based immunoenzymatic assay platforms (ELISPOT) in which memory B cells present in PBL and/or head kidney, when restimulated in vitro with immunization antigen(s), secreted IgM that can be evidenced and quantitated (Lamers et al. 1985). With these immune-assay platforms, it is possible to evaluate the number of memory B cell colonies, or the amount of secreted IgM at defined timing after immunization, as shown in sea bass (Meloni and Scapigliati 2000).

5.5   Ontogenesis of IgM-B Cells

The ontogenesis of IgM-B cells during development has been investigated in several teleosts, and the results showed that the appeareance of first antibody-positive cells is variable depending on the species and, likely, depending on the environment in which embryos develop. In cyprinids, the first B cells can be detected in head kidney 14 days postfertilization (Rombout et al. 2005), in trout kidney at 12 days before hatching (Castillo et al. 1993), in sea bass kidney/spleen at 45 days posthatching (Rombout et al. 2005), and in Atlantic halibut kidney at 66 days posthatching (Patel et al. 2009).

5.6   IgD B Cells

B lymphocytes producing IgD are present in all jawed vertebrate classes excluding birds, and this confirms their ancient origin and fundamental importance in antibody responses (Ohta and Flajnik 2006), but the IgD gene features and physiological functions of the mature molecule are still under investigation. In mammals, B cells express IgD and IgM receptors with a similar antigenic specificity through alternative mRNA splicing, when an antigen is encountered, the proliferating clonal lines switch to express one of the two Ig

Table 5.1 Estimated percentages of B cells in leukocytes from fish tissues

Tissue	IgM	IgT/Z	IgD
Thymus	3–6	nd	nd
Head kidney[a]	12–30	6–10	69
Spleen[a]	26–70	4–20	1–7
PBL[a]	13–40	12	2
Intestine[a]	2–12	8–20	1
Gills[a]	6–20	10–18	1
Skin[a]	2–16	10	nd
Main references	In the text	Picchietti et al. (2017), Ji et al. (2020)	Ramirez-Gomez et al. (2012), Perdiguero et al. (2019)

[a]Leukocytes isolated from tissues

genes, mostly IgM. IgD-committed B cells are not reactive to self-antigens, are efficient against foreign antigens, and are involved in interactions with intestinal microbiota (Gutzeit et al. 2018). In teleost fish (rainbow trout), the physiology of B cells shows similarities with mammals, since they coexpress surface IgM and IgD (Ramirez-Gomez et al. 2012) and down-regulate IgD after encountering an antigen (Tafalla et al. 2017). Functional activities associated to trout IgD-B cells include interactions with commensal microbiota and, likely, modulation of microbiota-gut interactions (Ramirez-Gomez et al. 2012), together with possible interactions with the polymeric Ig receptor (Xu et al. 2016). Interestingly, in the sea bass, a chimeric gene encoding IgD/IgT has been recently found with highest transcription levels in kidney, gills, intestine, and consistently up-regulated in kidney leukocytes after LPS stimulation (Buonocore et al. 2020).

At present, a cellular marker for fish IgD is only available for rainbow trout (Table 5.1), where evaluation of the number of IgD-secreting cells has been achieved by employing a mAb, suggesting estimated percentages of cross-reacting cells of 69% in the head kidney, 7% in spleen, 2% in PBL, and 1% in gills (Ramirez-Gomez et al. 2012).

5.7 Ontogenesis of IgD-B Cells

No literature is currently available on the development of IgD-B cells, but it should be remembered that IgM and IgD are coexpressed on the surface of B cells before their committment to a single antibody class, and thus in the absence of specific information, it should be assumed a development of IgD-B cells similar to that of IgM-B cells.

5.8   IgT/Z B Cells

The more recently discovered Ig heavy chain gene family of fish was described in 2005 in rainbow trout (IgT) (Hansen et al. 2005), and zebrafish (IgZ) (Danilova et al. 2005). From the physiological features displayed by IgT/Z-bearing cells, it appeared soon evident their similarity and evolutionary relationships with mammalian IgA and IgA-bearing cells (Zhang et al. 2010). The IgT/Z gene family (from now referred as IgT only) is not present in all fish species investigated, being absent, for instance, in channel catfish and medaka (Fillatreau et al. 2013) and with important modifications in Antarctic species (Giacomelli et al. 2015). The molecular size of fish monomeric IgT is around 170–180 kDa, with the heavy and light chain masses of 70–75 kDa and 25 kDa, respectively.

Very importantly, the IgT gene was soon recognized as the possible evolutionary ancestor of mammalian IgA and, subsequently, the physiological activities associated to secreted IgT and IgT-bearing cells suggested these features as a bridge between innate and adaptive immunity of vertebrates (Macpherson et al. 2011). A support to evolutionary relationships between mammalian IgA and fish IgT came from the distribution of IgT gene products and IgT-expressing cells among fish tissues, showing a high enrichment in mucosal tissues, and from a peculiar physiological function of IgT, namely the coating of intestinal antigens and their subsequent phagocytosis (Zhang et al. 2010). IgT-producing cells are the principal B-cell subset in the intestine, gills, skin, and nasal mucosa, where they can proliferate and produce pathogen-specific IgT antibody (Xu et al. 2016). The antibodies obtained against IgT of fish permitted the first characterization of intestinal IgT-B lymphocytes in trout, showing their capability to engulf latex beads and bacteria, as well as an intracellular bacteria-killing activity (Zhang et al. 2010). Similarly to IgM-B cells, IgT-B cells display an in vitro proliferative activity upon stimulation with the polyclonal activator LPS and bacterial antigens, showing the ability to respond to pathogen-associated molecular patterns (PAMPs) and differentiated into antibody-secreting cells (Zhang et al. 2011). Mucosal IgT is also known for their capability of coating bacteria for their subsequent elimination, thus mediating relationships between host mucosa and microflora (Zhang et al. 2010). The expression of the IgT gene was modulated during in vivo infection with a viral fish pathogen (Buonocore et al. 2017). These evidences clearly show the evolutionary similarity is based on function and not seqence similarity between fish IgT and mammalian IgA, and a conservation of first-line defence mechanisms in vertebrate mucosal immunity. The availability of anti-IgT antibodies allowed the visualization of IgT-B cells in fish tissues (see Table 5.1) and the Mediterranean sea bass can be a good example of determination of cross-reacting cells in tissues by IHC, and live IgT-B cells isolated by tissues. By quantitative IHC, the relative number of IgT-B lymphocytes in section was: $9.5 \pm 0.7\%$ in gills, $8.4 \pm 1.2\%$ in the intestine, $6.2 \pm 1.4\%$ in head kidney, and $4.1 \pm 0.7\%$ in spleen (Picchietti et al. 2017). Interestingly, and likely related to intestinal microbiome content, the number of IgT-B cells showed a regionalization in the intestine, increasing from the proximal to the terminal part (Picchietti et al. 2017). On the other hand, by immunofluorescence and flow cytometry, the same

antiserum stained $34 \pm 11\%$ of GALT, $22 \pm 5\%$ in head kidney, $16 \pm 7\%$ in spleen, and $9 \pm 5\%$ in gills. From these observations, it is evident that IgT-B lymphocytes are abundant in fish lymphoid tissues. It remains to be elucidated if the presence of IgT-B cells in nonmucosal tissues (kidney and spleen) can be related to lymphocyte trafficking between tissues, as already postulated (Lange et al. 2019; Bakke et al. 2020), or to lymphocyte development/maturation.

The involvement of IgT in mucosal immunity is a key activity of IgT-B lymphocytes, and interactions with protozoan parasites of the intestinal tract have been investigated in trout, where fish infestated with the protozoan parasite *Ichthyophthirius multifiliis* showed enhanced IgT-B-cell proliferation and higher IgT content in mucosal tissues (Wang et al. 2019; Buchmann 2020). The antiprotozoan responses include rapid coating of the parasite surface with IgT and IgM molecules, and the induction of other innate activities (Von Gersdorff Jørgensen et al. 2011).

IgT-B cells are also involved in antibacterial humoral defence, as shown in trout immunized with *Flavobacterium psychrophilum* (Zwollo et al. 2017). This contribution elegantly showed that resistance to the pathogen had been shown to be related with the relative abundance of IgT-B cells in lymphoid tissues between two lines of naïve and resistant fish, as measured by flow cytometry. The gill epithelium is a site where IgT lymphocytes are present (Picchietti et al. 2017), and where they are involved in a first line of defence against pathogenic bacteria present in water (Tongsri et al. 2020). Being associated with mucosal surfaces, IgT-B cells are considered to play an important role during mucosal vaccination, that is of master importance for fish vaccination avoiding stress, and mucosal vaccination protocols in trout against *Flavobacterium psychrophilum* have been reported, describing an increased number of IgT-B cells in trout tissues (Hoare et al. 2017), as well as increased transcription of IgT gene in the gut (Makesh et al. 2015). In other experiments, mucosal (intestinal) vaccination in trout induced recruitment of IgM- and IgT- B cells in pyloric caeca tissue (Ballesteros et al. 2013) from which, likely, they move to the gut.

Interestingly, a peculiar involvement of IgT-B cells responses during natural or experimental infections has been recently observed in Atlantic salmon by ISH, where an enrichment of IgT-B cells in the heart of fish infected/vaccinated with salmonid alfavirus. In this study, a postulated transfer of cells from the spleen to the heart have been observed (Bakke et al. 2020). Viral antigens induce, as expected, clonal expansion of IgM and IgT lymphocytes, with studies in trout that showed a high complexity in the generation of B-cell clones (Castro et al. 2013) and the recombination of IgT gene repertoires (Magadan et al. 2018).

The nasopharyngeal associated lymphoid tissue (NALT) of vertebrates contains lymphocytes. The earliest occurrence on an evolutionary scale is considered in the teleost group (Tacchi et al. 2014), and in trout, the predominant B-cell population in NALT is represented by IgT-B cells. Although it is not surprising to find this predominancy in a fish mucosal tissue, the physiology of nasopharyngeal tissue still remains to be elucidated.

Fig. 5.1 Double staining of lymphocytes. The figure shows leukocytes from sea bass head kidney stained by IIF with a polyclonal antiserum against IgT (green) and a monoclonal antibody against IgM (mAb DLIg3, red), no double IgT/IgM staining can be detected
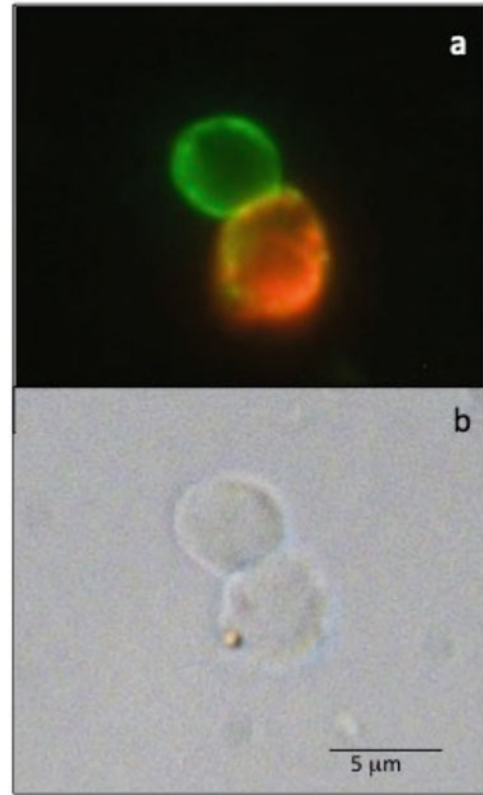

Figure 5.1 shows a double staining in sea bass head kidney leukocytes of IgT-B cells with a polyclonal antiserum and IgM-B cells by a mAb, confirming the absence of lymphocytes displaying both Ig classes on their surface, and Fig. 5.2a shows a snapshot of the number of IgM-B cells in the head kidney by IHC staining.

5.9   Ontogenesis of IgT-B Cells

The knowledge on the ontogenesis and deveopment of IgT-B cells are scarce, and at present a single study has been done in trout (Heinecke et al. 2014) showing by qPCR the expression of IgT in developing rainbow trout from day 32 posthatching.

Fig. 5.2 IgM and CD3 staining. A section of adult sea bass head kidney stained by IHC with a monoclonal antibody against IgM (mAb DLIg3), showing the high amount of cells in the tissue (2a). The thymus probed by ISH with antisense RNA for CD3ε is shown (2b), the low magnification shows the expression more present in thymus medulla
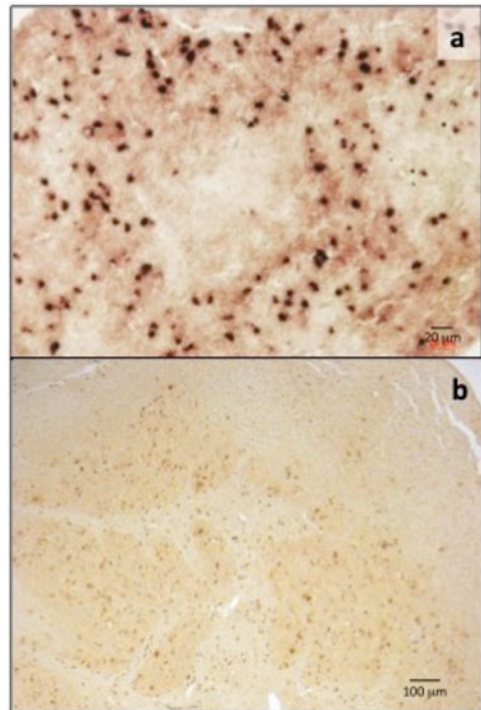
5.10   T Cells

The presence in fish of functional homologs of mammalian T lymphocytes was early deduced in carp by the observation of in vitro master activities associated to vertebrate T cells, like lectin-induced proliferation of leukocytes, mixed leukocyte reactions, and presence of proliferation upon stimulation by an IL-2-like factor (Caspi et al. 1982; Caspi and Avtalion 1984a, b). This early notion was supported by demonstration of functional T cells by the discovery of T-cell receptors as they are known in mammals, namely an αβTcR (Partula et al. 1995), and a γδTcR (Nam et al. 2003). The discovery of TcR, together with the already known presence of MHC_I/MHC_{II} and RAG genes suggested the presence of mechanisms of antigen presentation, epitope recognition-specific killing of nonself/infected cells, somatic recombination, and memory. Subsequently, clusters of TcR coreceptors and T-cell-associated master genes (e.g., GATA-3, Foxp3, t-bet) were identified, and from a gills transcriptome of Mediterranean sea bass, all the subpopulations of T cells like they are known in mammals, namely cytotoxic (CD8), helper (CD4), and regulatory (Treg, Th17) were identified (Nakanishi et al. 2011; Nuñez Ortiz et al. 2014; Takizawa et al. 2016; Kasheta et al. 2017). In addition, Th-specific signatures have been discovered, like genes coding for IFN-γ, Il-10, and IL-22 (Wang et al. 2011), and IL-2

(Wang et al. 2018). From an evolutionary point of view, this knowledge supports the notion that teleosts are the ancestors of the following vertebrate classes, with strong similarities in general traits of T-cell immune system organization and physiology. The first documented in vivo activity of T cells in fish, studied with an antipan T cells mAb, showed the rejection of an allotransplant by T cells infiltrating and killing nonself tissue, (Abelli et al. 1999). Subsequently, other classical T-cell activities have been documented in teleosts, including in vitro MHC-restricted and spontaneous cytotoxicity (Utke et al. 2007), lectin-induced and MLR-induced proliferation (Meloni et al. 2006; Nuñez Ortiz et al. 2014).

Fish T cells can be identified and studied by employing antibodies reacting with epitopes on TcR and to recognize TcR and T-cell coreceptors, but to-date these markers recognize diverse antigens scattered in various species, and a complete set of T-cell markers for a certain species is not available yet (see Table 5.2), and specific cell markers for fish αβTcR are still missing. Despite the lack of antibodies for TcRs of most teleost species, there is a reported knowledge on the germline usage and TcRs repertoires in naive and antigen-stimulated trout (Boudinot et al. 2001; Castro et al. 2011; Bengtén and Wilson 2015). Interestingly, TcRβ transcripts from unstimulated trout intestine were considerably different in sequences coding for complementarity-determining variable regions (CDR), at difference with the low diversity of repertoires described in mammals (Bernard et al. 2006).

From the initial evidence shown in sea bass (Scapigliati et al. 1999a), research performed in investigated species confirmed that T cells are principally located in mucosal tissues as in other vertebrates, with percentages depending on the TcR type and/or the coreceptor investigated, mammals T cells typically contain 95% of TcRαβ and 5% of TcRγδ. With respect to the canonical CD3 marker of T cells, the amount of T cells in fish peripheral tissues range from the lowest amount in blood to the highest in the intestine (see Table 5.2). The thymus is the main site of production and education of T cells and thus contains, obviously, the highest number of CD3^{+}-T cells (see Fig. 5.2b). The thymus of fish has been a subject of many studies investigating the production and education of lymphocytes (Langenau and Zon 2005), these studies supplied more information on the presence and trafficking of lymphocyte subpopulations inside thymus areas and toward the periphery (Picchietti et al. 2008, 2009). Figure 5.3 shows in sea bass, a view of TcR-β-expressing cells in juvenile thymus by ISH (Fig. 5.3a), and the richness of pan-T cells in the intestinal mucosa of developing fish by IHC (Fig. 5.3b).

Mucosal surfaces contain a large number of T-cell subpopulations, especially of spontaneously cytotoxic CD8^{+}-T cells. The fish intestine is particularly interesting, since it harbors the highest amount of T cells of the body, with the subpopulation CD3^{+}/CD8^{+} largely in excess with respect to CD3^{+}/CD4^{+}, and distributed differentially along the intestinal tract of sea bass (Picchietti et al. 2011). These observations suggest a differential capability of intestine regions for antigen/substances sampling, with differential leukocyte subsets involved. Intestinal CD3+ lymphocytes in the epithelium/lamina propria express both the αβ- and γδ-TcR genes at the highest level among peripheral tissues (Boschi et al. 2011), distributed in the epithelium and/or *lamina propria* (Picchietti et al. 2011; Buonocore et al. 2012), and this is particularly important by considering that also the

Table 5.2 Estimated percentages of T-cell content in fish tissues

Tissue	Pan-T/CD3	αβTcR	γδTcR	CD8	CD4–1/ CD4–2
Thymus	80–87	nd	nd	19–76	29–31
Head kidney[a]	4–40	nd	15	4–7	11–37
Spleen[a]	6-24	nd	14	2–5	11–18
PBL	1–2	nd	15	1–4	5–9
Intestine[a]	40–53	nd	nd	54	nd
Gills[a]	4–40	nd	nd	25	nd
Skin[a]	25–29	nd	nd	9–12	nd
Main ref.	Scapigliati et al. (1999a, b), Maisey et al. (2016), Boardman et al. (2012), Jung et al. (2017)		Wan et al. (2017)	Takizawa et al. (2011); Leal et al. (2016); Toda et al. (2011); Miyazawa et al. (2018); Chang et al. (2011); Tajimi et al. 2019	Maisey et al. 2016; Miyazawa et al. 2018; Xing et al. 2019

[a]Leukocytes isolated from tissues

Fig. 5.3 TcRβ and pan-T cells. A section of adult sea bass thymus probed by ISH with antisense RNA for TcRβ is shown (**a**), with the expression more present in thymus medulla. IHC staining of developing intestine with a pan-T-cell marker (mAb DLT15), showing the high number of T cells
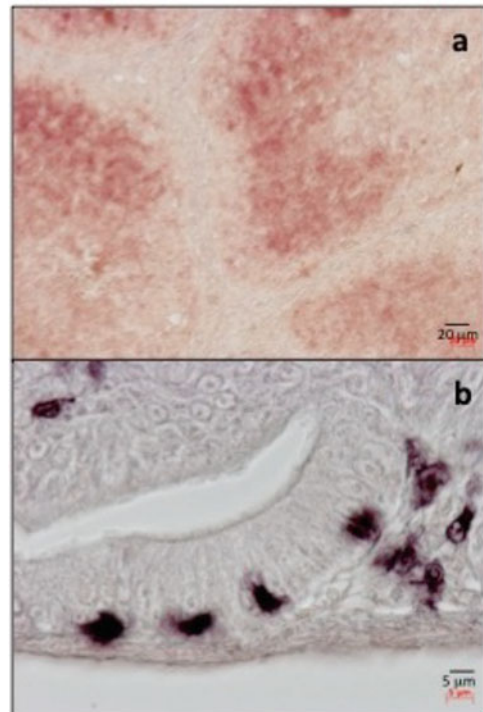
RAG-1 gene is expressed in the intestine. Spontaneous somatic rearrangement was noted for a given somatic rearrangement of a given V/C combination in the CDR3 junction length of TcRβ-chain/TcRγ-chain in the absence of antigen stimulation (Castro et al. 2011; Buonocore et al. 2012). From these observations, it can be assumed that the intestine of teleosts can be considered as a true lymphoid tissue for T cells. By considering the high amount of transcripts for γδTcR and CD8$^+$ cells and lower expression of CD4$^+$ cells, together with the spontaneous in vitro cytotoxicity of IELs, it can be speculated the presence of T cells having a phenotype γδTcR/CD8α and spontaneously cytotoxic without antigen presentation, as in the intestinal mucosa of mammals (Nonaka et al. 2005). The γδTcR-bearing lymphocyte population appears to be particularly represented in the sea bass intestine, since by immunopurifying pan-T cells from IELs, the γδTcR gene is greatly more expressed than the αβTcR gene (Boschi et al. 2011).

The gills are at direct contact with the aquatic environment and mediate the interactions with the the organism through a thin epithelium, that must be armed with potent immune defences. Master genes for lymphocytes classes and subclasses have been identified in a transcriptome of sea bass gills (Nuñez Ortiz et al. 2014), thus confirming that the whole immune armament is present at the forefront of a possible pathogenic invasion. Regarding T lymphocytes, the percent of pan T/CD3 cells ranges from 4 to 40%, with the CD8$^+$ subpopulation being the more abundant (see Table 5.2) and the CD4/CD8α genes expressed at high levels (Boschi et al. 2011). At difference with intestinal IELs, T cells from sea bass gills proliferate in vitro in response to lectins and viral antigens (Nuñez Ortiz et al. 2014), suggesting that T cells' activities could be diversified in gills and intestine, since in the intestine IELs display in vitro spontaneous cytotoxic activity, show low lectin-induced in vitro proliferation (unpublished), and display spontaneous somatic recombination At difference, T cells from the gills show fast lectin-induced in vitro proliferation and transcribe low amount of the RAG-1 gene. These data suggest that the intestine is also a site of production of T cells, whereas T cells of the gills appear more committed as effectors/helper cells.

Of particular interest is a T-cell-associated interbranchial lymphoid tissue (ILT) discovered in salmon (Haugarvoll et al. 2008) and, recently, reported in chondrichthyes and some teleost species (Rességuier et al. 2020). The salmon ILT has been found to express T-cell specific genes IL-2, IL-10, GATA-3, and Foxp3 (Aas et al. 2017). Although likely involved in T-helper cellular immune defences, the ILT is not present in all fish species investigated, and thus its physiology and the evolution among Actinopterygii are still under study.

The skin of most teleosts is carrying scales that are covered by mucosal tissue, nevertheless lymphocytes are present as scattered cells and T lymphocytes are present in measurable amount together with T-cell-specific transcripts, as shown in trout (Leal et al. 2016).

Looking at the distribution of T cells in nonmucosal tissues kidney and spleen, it can be observed they indeed contain quite variable amounts of pan-T/CD3 cells and substantial amounts of CD4$^+$-T cells (Table 5.2). The high variability of CD3 numbers could derive

either from the diversity in fish species and/or from the specificity of the antibody employed. Furher work is required to increase the panel of antibody reagents addressed to measure CD3 and TcRs, in order to establish in the highest possible number of species, a more precise evaluation of T lymphocytes.

The T-cell subpopulations Tc and Th are present in fish and display either some features known in other vertebrates, and others peculiar to fish. The CD8$^+$ cytotoxic T-cells population was discovered in fish in 2000 (Hansen and Strassburger 2000), and displays on the membrane the homodimer subunits αβ, and when the complex TcR/CD8 recognizes a nonself MHC$_I$, the CTLs secrete lytic enzymes that kill target cells. CD8 cells that are present in low amount in the blood and spleen and abundant in mucosal tissues, have been studied for their spontaneous and MHC$_I$-restricted in vitro cytotoxicity against allo/xeno-geneic cell targets (Dijkstra et al. 2001; Fischer et al. 2003; Toda et al. 2009; Picchietti et al. 2011). The CTLs accumulate in gills infested by a protozoan parasite (Olsen et al. 2011), and virus-specific CD8$^+$ cells can be generated by mucosal vaccination of ginbuna carp (Somamoto et al. 2014; Tajimi et al. 2019). Interestingly, catfish antiviral CTLs from blood have recently been found to have a phenotype CD4$^+$/CD8$^-$, and this observations needs to be further extended to other fish species to assess if the observed phenotype is species-specific or is a unexplored feature of fish CD4$^+$ lymphocytes.

T-helper lymphocytes have been identified in fish in 2004 (Suetake et al. 2004), and they are characterized by the CD4 surface glycoprotein, and the complex TcR/CD4 recognizes processed antigens bound to MHC$_{II}$. CD4 T cells, or T-helper cells, are involved in coordinating the immune response by stimulating other immune cells, such as macrophages, B lymphocytes, and CD8 T cells. T-helper lymphocytes exert their activities through Th1, Th2, and Th17 subpopulations each one characterized by a distinct pattern of cytokine production. In particular, Th1 produces IFN-γ, TNF-α, and IL-2; Th2 produces IL-4, IL-5, and IL-13; Th17 produces IL-17, IL-22, and IL-26. A substantial amount of CD4 T cells has been reported in kidney and spleen by employing specific antibodies, whereas a low amount is present in the intestine investigated by ISH (Picchietti et al. 2011). Overall, the modulation of the CD4 gene has been observed following various stimulations, confirming the pivotal involvement of T-helper activities in fish immune defences, includ-ing the above reported peculiar T-cell ILT tissue of gills, expressing mostly CD4 genes. The CD4 of teleosts occurs as two isoforms CD4–1 and CD4–2 (Takizawa et al. 2016). The CD4–1 molecular features, present in all fish species investigated as expressed genes and as investigated in ginbuna carp, display similarities with the classical CD4 counterpart of mammals (Toda et al. 2011; Kato et al. 2013). The CD4–2 gene has been found in several species and, where present, its functional features are not superimposable to that of CD4–1 isoform. However, information is lacking to better understand its physiology in fish. As above reported, a recently observed peculiar feature of catfish CD4$^+$ clonal leukocyte lines is a natural CD4-driven cytotoxic activity against alloantigens (Taylor et al. 2020), a feature that in mammals has been observed in myeloma tumor cells (Zhang et al. 2018).

The presence of natural killer cells-like lymphocyte population in fish was supposed in 1991 (Greenlee et al. 1991), and after discovering genes coding for NK-related secreted

lytic enzymes, novel immune type receptors (NITRs), and leukocyte immune type receptors (LITRs), a NK-like immunity has been described in trout (Fischer et al. 2013). NK cells of mammals are characterized by the absence of CD3 and CD8 from their surface and, similarly, NK-like cytotoxic leukocytes for virus-infected cells in ginbuna carp have been found to lack CD8 (Somamoto et al. 2013). However, a definitive characterization of fish NK-like cells based on functional activities and characterization of surface membrane phenotype is still lacking.

The CD45 antigen of mammals is a transmembrane protein tyrosine phosphatase playing important roles in T- and B-cell receptor and cytokine signaling. The CD45 gene has been identified in teleosts and a CD45 leukocyte population displaying features of lymphocytic CD45RO^{+}-T cells has been identified with a mAb in sea bass (Marozzi et al. 2012).

5.11   Ontogenesis of T Cells

Teleost fish have a thymus that displays a morphophysiology similar to mammalian thymus, and thus the development of thymic T cells, even in the absence of specific T-cell markers, was investigated by morphological studies (Chilmonczyk 1983). With the availability of specific molecular and cellular T-cell markers, the knowledge on T-cell development had a boost, with data confirming the regionalization of T-cell-related transcripts in thymic lobuli (Bajoghli et al. 2015), and negative selection processes by lymphocyte apoptosis (Romano et al. 2013). It is evident in the fish species investigated that T-cell ontogenesis precedes B-cell ontogenesis, since T cells are identified before than B cells (Rombout et al. 2005). The sea bass is the species for which much information is available on T-cell ontogenesis, obtained by employing a mAb specific for a thymus antigen (Scapigliati et al. 1995) that showed the presence of first positive cells in thymus by IHC at day 25 posthatching. Interestingly, at similar development time, T-cell-related cells were also identified in developing intestine of same species (Abelli et al. 1996). T-cell subpopulations αβTcR and CD8 also showed compartmentalization in thymus during development (Picchietti et al. 2008). In the absence of specific cell markers, the development of T cells has been investigated in other teleosts mostly by the analysis of T-cell-related transcripts (Corripio-Miyar et al. 2007; Øvergård et al. 2011) showing early expression of RAG-1, and late expression of CD3 and TcRs.

During rainbow trout development, CD8+ T cells were clearly shown by IHC and qPCR in the thymus of rainbow trout yolksac larvae at 17 days posthatch (Chettri et al. 2012), and transcription of TcR, CD4, and CD8 has been studied during the egg phase and the early posthatch period (Heinecke et al. 2014).

5.12 Summary and Perspectives

Studies on comparative immunology had a great boost in the last 20 years due to the request of information to be applied to animal farming and animal biotechnology. Fish farming is increasing worldwide becoming more and more important with respect to wild fish catches through the farming of already established and novel teleost species that are used as a food for humans.

The knowledge in fish immunology revealed close evolutionary relationships in the physiology of immune defence mechanisms throughout vertebrate species, resulting in the use of fish species as animal models, as clearly demonstrated by the zebrafish model.

Lymphocytes of jawed vertebrates represent a clear example of a long sought evolutionary marriage between two partners, RAG genes and Ig domain-containing lymphocyte receptors, and the ancestral cells gave rise to a rich variety of B- and T- lymphocytes. In turn, the progeny of lymphocytes acquired some new specializations among vertebrate classes, maintaining common features unaltered. In this view, it is possible to figure out that, starting from fish and arriving to mammals, new "layers" of specializations accumulated, producing the diversity of immune adptations as they are known.

Most of the lymphocyte and lymphocyte-like cell classes and subclasses present in mammals have been found in teleosts, and this review summarized available general knowledge on fish lymphocytes. Apart from the production of specific markers for lymphocytes of the many fish species of interest, much work is needed to better elucidate in vivo and in vitro functional immunity in fish models, among which investigations on NK cells, the possible presence of equivalents of NK-T cells, the functions of IgD in fish investigated both at molecular and cellular level, the innate activities of fish lymphocytes, and the relationships between the nervous and immune system in the intestine.

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Fish Macrophages

6

Geert F. Wiegertjes and Philip M. Elks

Abstract

Fish macrophages arise from haematopoietic progenitors in the head kidney and differentiate into tissue macrophage subtypes and/or self-maintaining resident populations. New insights into the 'forms' and functions of fish macrophages are provided by in vitro studies of macrophages purified by density gradients/adherence, as well as immortal macrophage-like cell lines and prolonged culture of primary macrophages. Polarisation states observed in mammalian macrophages, with associated changes in molecular and behavioural properties across a spectrum of two extremes, termed M1 and M2, also likely exist in fish macrophages. There is evidence that these different states are underpinned by immunometabolic changes. With the current advances in transcriptome sequencing, markers for macrophages and macrophage subtypes are slowly but definitively emerging in fish species. An ever-increasing toolbox of transgenic zebrafish lines allows for the elucidation of the multiple roles of macrophages in disease models, providing a more detailed insight into their in vivo function in fish.

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Abbreviations

angptl4	angiopoietin-like 4
arg	arginase
BCG	Bacillus Calmette-Guérin
cAMP	cyclic adenosine monophosphate
cfms	csf-1 receptor
CRISPR	clustered regularly interspaced short palindromic repeats
csf-1	colony-stimulating factor-1 (see also M-CSF)
cxcl11	C-X-C motif chemokine 11
dpf	days post-fertilisation
eGFP	enhanced green fluorescence protein
FACS	fluorescence-activated cell sorter
GAL4	galactokinase 4 transcription factor (see also UAS for combined system)
Hif1a	hypoxia-inducible factor 1 alpha
hpf	hours post-fertilisation
hsp70	heat shock protein 70
ifnγ	interferon gamma, cytokine
il-10	interleukin-10
il-1b	interleukin-1 beta, cytokine
il-4	interleukin-4, cytokine
il-6	interleukin-6, cytokine
inos	inducible nitric oxide synthase
irf8	interferon regulatory factor 8, transcription factor
irg1	immune-responsive gene 1
LPS	lipopolysaccharide
M1	type 1 macrophage
M2	type 2 macrophage
m-csf	macrophage-colony-stimulating factor (see also CSF-1)
mfap4	microfibril-associated protein 4
mm	*Mycobacterium marinum*
mpeg-1	macrophage-expressed gene 1
mrc1b	mannose receptor 1b
NO	nitric oxide
NOD	nucleotide-binding oligomerisation domain
nos	nitric oxide synthase
rag	recombination-activating gene
ROS	reactive oxygen species
spi1b	proto-oncogene b
TALEN	transcription activator-like effector nucleases
TB	tuberculosis
TCA	tricarboxylic acid cycle

tg   transgenic
tgfb   transforming growth factor beta
tnfa   tumour necrosis factor alpha
UAS   upstream-activating sequence (see also GAL4 for combined system)

6.1   Introduction

For a long time, immunologists made a clear distinction between cellular immunity and humoral immunity. Cellular immunity is mostly linked to nonspecific innate immunity. Humoral immunity is considered synonymous with antigen-specific acquired immunity, based on Emil Behring and Paul Ehrlich's identification of antibodies which neutralise microbial toxins. To date, these two distinctive arms of the immune system are considered as highly intertwined, partly overlapping, with mutually beneficial activities (Kaufmann 2019). Elias Metchnikoff laid the basis for innate immunity by identifying phagocytic cells which engulf and destroy invading pathogens and conceived the concept of phagocytosis as a process of uptake of particles or microbes rich in food after studying simple organisms such as starfish larvae. He described macrophages and microphages (now called neutrophils) as highly motile phagocytic cells which migrated to sites of foreign body insult. Whereas antibodies are only found in vertebrates, macrophages or macrophage-like cells are present in primitive animals such as starfish alongside vertebrates, and clearly pre-date the development of lymphocytes and associated acquired immune responses.

Macrophage polarisation is a process by which macrophages change their molecular and behavioural properties across a spectrum of two extremes; termed M1 and M2 (Xue et al. 2014). M1 macrophages are commonly associated with the presence of T helper-1 cytokines such as IFNγ, whereas M2 macrophages are commonly associated with the presence of T helper-2 cytokines such as IL-4. Macrophages are evolutionary conserved cell types that evolved more than 500 million years ago (Epelman et al. 2014; Barreda et al. 2016), predating the development of lymphocytes. It is therefore plausible that the initial trigger for 'macrophage polarisation' could rely primarily on sensing microbes and other innate danger signals, not requiring the presence of T-cell derived cytokines. It is possible that the M1-M2 dichotomy could be an evolutionary conserved, intrinsic property of macrophages associated with transitions from healing (M2) to inflammation (M1) (Mills and Ley 2014). The 'macrophage first' view is based on the fact that the ability of macrophage-like cell types to phagocytose foreign objects, and repair cellular damage, already existed in primitive animals.

Not surprisingly, fish macrophages with polarised profiles like mammalian M1 and M2 also exist. Common carp (*Cyprinus carpio*) macrophages can adopt an inflammatory (M1-like) phenotype, characterised by elevated nitric oxide (NO) production in response to lipopolysaccharides (LPS) (Wiegertjes et al. 2016). These macrophages can also develop an anti-inflammatory (M2-like) profile in response to the second messenger, cyclic

adenosine monophosphate (cAMP) (Joerink et al. 2006; Hodgkinson et al. 2017). The need for T-cell-derived cytokines to stimulate the polarisation of macrophages may be less obvious for fish than for mice (Forlenza et al. 2011; Wiegertjes et al. 2016). This takes us from the idea of dichotomous 'Th1 and Th2 driving' to the 'macrophages first' hypothesis.

Our knowledge of fish macrophages is rapidly advancing, but not at the same pace for all fish species of interest. Progress can be hampered by the diversity of fish, as well as the techniques employed that do not always address all aspects of the immune system. Evolutionary distant families, such as the Salmonidae (e.g. Atlantic salmon, rainbow trout), Cyprinidae (e.g. common carp, zebrafish), Ictaluridae (e.g. channel catfish) and Perciforms (e.g. sea bass, grouper) are commonly studied. It is therefore not surprising that macrophages can behave differently between these diverse groups. Zebrafish (*Danio rerio*) deepen our level of understanding of fish macrophages, especially with the advent of immune transgenic lines, allowing in vivo labelling of specific cell types/organ systems with fluorescent proteins. Investigation of innate immune cells is simplified in larval zebrafish, as before 5dpf there are only two immune cell lineages, the macrophages and the neutrophils. Rag-positive T cells appear at around 5dpf, but functional adaptive immunity, with antibody-producing B lymphocytes, does not develop until 2–3 weeks post-fertilisation (dependent on laboratory conditions) (Willett et al. 1997). The widespread use of zebrafish creates a level playing field, allowing for in-depth studies of macrophage function in a single fish species.

6.2   Macrophage Development

Macrophages arise from haematopoietic progenitors in the bone marrow, which differentiate into tissue macrophage subtypes, such as Kupffer cells in the liver, alveolar macrophages in the lung, microglia cells in the central nervous system and specialised macrophages in the spleen. It was originally proposed that tissue macrophages derive from circulating blood monocytes (van Furth et al. 1972). Now the evidence points to tissue macrophages being seeded during primary haematopoiesis, resulting in self-maintaining resident populations of macrophages (Ginhoux and Jung 2014). All macrophage subtypes are important for homeostasis and play an intricate role in the immune system.

In adult zebrafish, macrophages arise from haematopoietic progenitors in the head kidney and tissue macrophage development appears to be an evolutionary conserved process (Soza-Ried et al. 2010). Survival, proliferation, differentiation and functionality of most cells of the macrophage lineage are governed by colony-stimulating factor-1 (CSF-1), or macrophage-colony-stimulating factor (M-CSF). This cytokine, acting through its cognate receptor, is expressed almost exclusively on committed myeloid precursors and derivative macrophage populations. Goldfish (*Carassius auratus*) CSF-1 appears to be a proficient macrophage growth factor, although possibly with a different role in macrophage

differentiation (Hanington et al. 2009; Rieger et al. 2014; Hodgkinson et al. 2015; Barreda et al. 2016).

Early studies into zebrafish blood lineages were performed using light microscopy, immunohistochemistry and flow cytometry (Herbomel et al. 1999; Lieschke et al. 2001; Hsu et al. 2004). Monocytes and macrophages in adult zebrafish are primarily derived from the head kidney marrow, the site of haematopoiesis (Hsu et al. 2004). In embryos, macrophage precursors first develop at around 20 h post-fertilisation in the anterior lateral plate mesoderm tissue (Herbomel et al. 1999). These so called 'primitive macrophages' seed the macrophages resident in the body tissues; for example, by invading the brain tissue where they differentiate into microglia. Primitive macrophages are gradually replaced during embryonic development by those derived from definitive haematopoiesis, which begins at 24hpf in the caudal haematopoietic tissue (CHT) (Lieschke et al. 2001). Tissue-specific macrophages are also found in zebrafish larvae, most notably brain-resident macrophages, known as microglia (Peri and Nüsslein-Volhard 2008). Zebrafish microglia play a similar role in the homeostatic maintenance of the fish brain, as in mammals, and have the same ramified morphology when at rest. In zebrafish, the roles of microglial clearance of apoptotic cells (Hamilton et al. 2020), synaptic maintenance and regeneration (Kyritsis et al. 2012) and vascular maintenance (Fantin et al. 2010) can all be studied. It is reasonable to assume that many macrophage subtypes play an intricate role in fish immune systems.

6.3 How to Study Fish Macrophages

Traditionally, in vitro studies of fish macrophages are performed with primary cell cultures, enriched for macrophages by density gradients and/or adherence, in line with human primary blood studies. From the 1980s onwards, macrophages of many different fish species have been isolated using primary culture methods for in vitro studies on fish immunology (Villena 2003). Typically, (head) kidneys are gently passed through a sterile nylon mesh, after which cell suspensions are placed on top of a density layer (e.g. Percoll). After centrifugation, the interface layer is collected, washed, counted and seeded, at optimal concentrations, in cell culture medium. From such studies, it is clear that fish macrophages have the capacity to phagocytose and have microbicidal activity, in part mediated by oxygen and nitrogen radicals. Oxygen radical production is determined by colorimetric quantitation of the respiratory burst, using either single point measurements (e.g. by quantification of reduction of a yellow tetrazolium dye to a blue formazan) or real-time measurements (e.g. by quantification of luminol-enhanced chemiluminescent emission). Nitrogen radical production can be determined by a 'Griess' reaction, measuring the accumulation of nitrite over a period of a few days following initial stimulation. These in vitro studies contribute greatly to our knowledge of the mechanisms of antimicrobial immunity in fish macrophages (Grayfer et al. 2018).

6.3.1   Macrophage Cell Lines

One limitation of studying fish macrophages is the lack of available immortalised cell lines. For mammalian systems, these immortalised cell lines enable simplified in vitro assays to be used, without the need for primary cultures. Occasionally, the development of leukocyte cell lines is reported; however, this is not repeatedly cited in the literature, therefore it cannot be regarded as a regularly used and well-established tool. This is with the notable exception of cell lines developed from rainbow trout (RT) (*Oncorhynchus mykiss*) (Bols et al. 2017). The monocyte/macrophage cell line, RTS11, is one of several cell lines developed from rainbow trout and part of an informally shared 'invitrome'. In the early stages of establishment, RTS11 cells require 30% foetal bovine serum, which can be halved for routine growth, and it is one of only a few cell lines from rainbow trout that can be grown in suspension. RTS11 cells are used to study interactions with *Flavobacterium psychrophilum*, for example, showing that live bacteria alter cellular morphology, stimulate cytokine expression and impair phagocytic activity. This provides an example of how an in vitro macrophage-like cell line can help in understanding the pathogenesis of an important bacterium (Semple et al. 2020). In addition to rainbow trout, TO and SHK-1 are two cell lines derived from the head kidney of Atlantic salmon (*Salmo salar*). In the resting state, they express different immune relevant *genes* suggesting that they have potentially different functional properties: SHK-1 shares characteristics with dendritic cells, whilst the TO cell line shows characteristics more traditionally associated with macrophages (Collet and Collins 2009). The SHK-1 cell line is used to study interactions with *Piscirickettsia salmonis*, for example, showing that this bacterium, which infects and survives in its host cell, induces a global shutdown of translation during intracellular growth, resulting in decreased cell viability after 10 days (Zúñiga et al. 2019). Most recently, two macrophage-like cell lines were established from the head kidney of a marine fish species, the large yellow croaker (*Larimichthys crocea*) (Cui et al. 2020). Overall, although few in number, fish macrophage-like cell lines allow simplified in vitro assays to be developed to aid our understanding of cell-pathogen interactions.

### 6.3.2   In Vitro-Derived Macrophage Cell Cultures

Studies on the function of fish macrophages are greatly facilitated by standardised procedures for prolonged in vitro culture of primary cells. In particular, goldfish and common carp macrophages grown in the presence of relatively high serum concentrations can survive for at least 6–8 days. It is well known, also for mammalian macrophages that, macrophages but not lymphocytes selectively survive for longer in vitro (Carr 1973). The presence of progenitor/stem cells in primary kidney macrophage cultures from goldfish allows for studies of myelopoiesis, indicating the development of three distinct subpopulations in response to endogenous macrophage growth factors. Self-renewal is promoted by endogenous macrophage growth factors (Belosevic et al. 2006) in a culture

system, including three subpopulations of macrophage development, namely progenitor cells, monocytes and mature macrophages. These primary kidney macrophage cultures are particularly informative when it comes to the elucidation of the complex mixture of cytokines that regulate progressive and selective macrophage development, from progenitor cells to fully functional mature macrophages in vitro (Katzenback et al. 2016). Similar studies in common carp (Joerink et al. 2006; Wentzel et al. 2020a) show that, amongst head kidney leukocytes kept for several days in vitro, the lymphocytes die off and the remaining cells differentiate into head kidney-derived macrophages, with the ability to phagocytose and produce oxygen and nitrogen radicals.

6.3.3 Transgenic Zebrafish

Studies on fish macrophages are significantly advanced by the development of immune cell-specific transgenic lines in transparent zebrafish. These lines, in combination with genetic manipulation via clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 and drug treatments, allow investigation of macrophage phenotypes and function in vivo. The monocyte/macrophage lineage is one of the first blood cell types for which transgenic lines have been generated. The *Tg(spi1b:eGFP)* and *Tg(spi1b:GAL4, UAS:eGFP)* transgenic lines label the myeloid precursors of macrophages and neutrophils, but lack the specificity to distinguish between the two (Ward et al. 2003; Hsu et al. 2004; Peri and Nüsslein-Volhard 2008). The first transgenic line that specifically labelled cells of the macrophage lineage in larvae is the *Tg(mpeg.1:GFP)* driven by the *mpeg.1* promoter (Ellett et al. 2011). This line is extensively used in the field to label macrophage populations in disease model studies, despite the fact that we do not fully understand what the biological function of Mpeg.1 is. The *mpeg.1:GFP* line has been followed by a number of transgenic lines, using the promoters of *cfms* and *mfap4* (Gray et al. 2011; Walton et al. 2015) amongst others. The availability of multiple transgenic lines, with different promoters and fluorescent proteins, allows for outcrossing into transgenic lines of other cell types/organ systems. These are combined to produce powerful in vivo models for studying the roles of macrophages in diseases. These have been added to in recent years by functional transgenic lines of important macrophage signalling systems. These include transgenic lines of cytokines/immune stimuli, (including *il-1beta* (Nguyen-Chi et al. 2014; Ogryzko et al. 2019), *tnfa* (Marjoram et al. 2015; Nguyen-Chi et al. 2015), *nfkb* (Feng et al. 2012) and *irg1* (Sanderson et al. 2015)). This ever-increasing toolbox of transgenic lines is allowing for the unprecedented dissection of the roles of macrophages, in multiple in vivo models, throughout the course of disease pathogenesis (Fig. 6.1).

The genetic tractability of zebrafish allows for manipulation of macrophage populations in vivo. Morpholinos are antisense oligonucleotides that block translational start and splice sites, knocking down specific *gene* expression. The *spi1b* morpholino allows for knockdown of macrophage (and neutrophil) populations in the developing zebrafish larvae, though these effects are transient (Liongue et al. 2009). Morpholinos have been joined
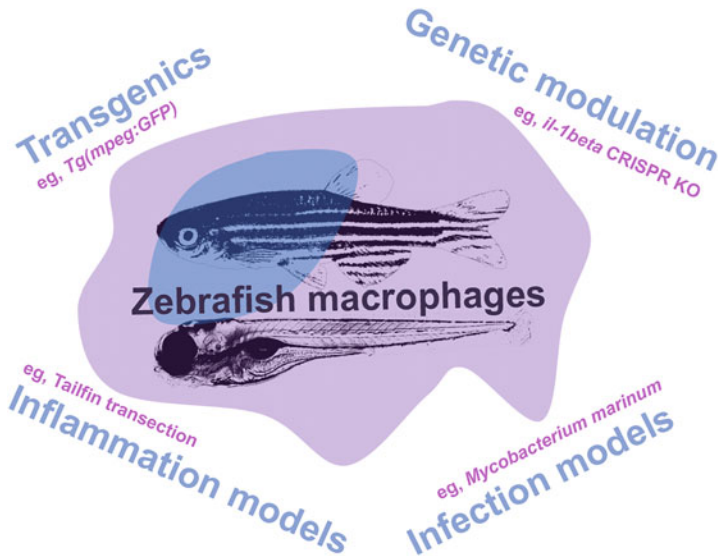
Fig. 6.1 The zebrafish toolbox to dissect the roles of macrophages in disease

recently by full zebrafish knockout lines that are now easier to generate due to TALEN and CRISPR-Cas9 technology. The *irf8* knockout line allows for depletion of macrophage populations, while stimulating neutrophil production (Li et al. 2011; Shiau et al. 2015). Mutants of important macrophage cytokines (eg *il-1beta*) are now available, to modulate macrophage behaviour (Ogryzko et al. 2019). Transgenic lines also allow for manipulation of macrophage and immune cell populations, in order to elucidate their effects on models of disease. Macrophage-driven *Gal4* transcription factor, in combination with a *UAS-nitroreductase-mCherry* expressing transgenic, allows for macrophage ablation and enables assessment of the macrophage contribution to disease (Gray et al. 2011; Prajsnar et al. 2012). Macrophage ablation can also be achieved by utilising their predisposition for high levels of phagocytosis/endocytosis. Liposome- encapsulated clodronate disodium (a bisphosphonate toxic to cells intracellularly), when injected into zebrafish larvae, allows for the temporal depletion of the macrophage population. These macrophage depletion and modulation tools can be used in combination with transgenic lines, to understand the roles of macrophages in development and disease processes.

6.4 Macrophage Polarisation

Macrophages are essential innate immune cells, involved in host defence that can play contrasting roles by initiating and sustaining inflammation. They can also play an important role in the resolution of inflammation and tissue regeneration. Under these conditions, different microenvironments drive macrophages to display a range of effector functions

which tailor immune responses to either combat pathogens, or repair damage. Small variations in microenvironments can lead to an array of macrophage phenotypes (Xue et al. 2014), with the most polarised termed M1 and M2. Traditionally, M1 macrophages are associated with microenvironments influenced by T helper-1 responses (hence M1) and produce pro-inflammatory cytokines, reactive oxygen species (ROS) and nitrogen species, such as nitric oxide (NO). In contrast, M2 macrophages are commonly associated with environments influenced by T helper-2 responses (hence M2) and produce anti-inflammatory products and factors associated with wound healing (Mills et al. 2000). Both M1 and M2 macrophages metabolise the same amino acid, L-arginine. M1 macrophages do so with the help of inducible nitric oxide synthase (iNOS, or NOS2), to produce inflammatory NO. In contrast, M2 macrophages use the enzyme arginase to produce proline and polyamines, important for wound healing processes such as cell proliferation and collagen production, during extracellular matrix regeneration (Vincendeau et al. 2003). The competition for L-arginine in macrophages, between iNOS and arginase is referred to as the 'arginine fork'.

6.4.1   M1 and M2 Macrophages of Fish

The arginine fork is used as a common readout for fish macrophage polarisation. The arginine forks' intrinsic capacity to polarise is determined by the differential activity of iNOS versus arginase, as measured by end products, such as nitrite (M1) and urea (M2). Such colorimetric assays typically measure accumulation of stable nitrite (NO_2-) in the culture medium by a Griess reaction, or urea in cell lysates as part of an arginase assay. Much knowledge of macrophage polarisation comes from studies in cyprinid fish, such as goldfish and common carp, aided by prolonged in vitro cultures.

   NO synthases display a large scale of parallel evolution (Moroz and Kohn 2011). Mammalian vertebrates have neuronal, endothelial and inducible isoforms of NOS, while fish only seem to express neuronal and inducible NOS, which they use to catabolise arginine to nitric oxide and citrulline (Andreakis et al. 2011). M1 macrophages abundantly express the enzyme iNOS. Simultaneous generation of NO radicals and reactive oxygen species, including the radical superoxide (O_2–), can lead to the formation of peroxynitrite (ONOO–), for example. Peroxynitrite is a powerful oxidant and the resulting tyrosine nitration can be considered as a hallmark of tissue injury and a (bio)marker for nitrosative stress, linked with inflammation, in common carp (Forlenza et al. 2008) and zebrafish (Elks et al. 2013, 2014). The combined generation of reactive nitrogen and reactive oxygen species helps define M1 as 'kill/fight type' of macrophage, with (*gene*) expression of *inos* (nos-2) as a marker for M1 macrophages in fish.

   Alongside its role in the urea cycle, arginase regulates cellular arginine and ornithine catabolism to deliver ornithine for processes of extracellular matrix synthesis and poly-amine synthesis, important for organogenesis and wound healing (Dzik 2014). Most eukaryotic cells have a polyamine transporter system on their cell membrane that facilitates

the internalisation of exogenous polyamines; a system highly active in rapidly proliferating cells (Wang et al. 2003). Mammalian vertebrates have two arginase isoforms that both catabolise arginine, but differ in cellular expression and cell-type-specific regulation (Jenkinson et al. 1996). In ureotelic animals that excrete excess nitrogen as urea (e.g. mammals), Arg-1 is expressed primarily in the liver and is a cytoplasmic enzyme central to the hepatic urea cycle, whereas Arg-2 is a mitochondrial enzyme that is expressed in almost all organs. Most fish species are ammoniotelic and thus do not excrete urea through the kidneys, instead they excrete ammonia mainly through the gills. Common carp also express two arginase isoforms and both appear to be mitochondrial enzymes (Joerink et al. 2006). The generation of arginase activity and downstream production of collagen and polyamines help define M2 as a 'healing type' of macrophage, with arginase as a robust marker for M2 macrophages of fish.

6.4.2 Studies into M1 and M2 Markers

Measuring increased *gene* expression of inos or arginase can be used as a first marker for macrophage polarisation in fish, but often more than one *gene* variant will need to be examined because of whole genome duplications (WGD). All teleosts are believed to have gone through three rounds (3R) of WGD. As a result of 3R duplications, zebrafish often, but not always, express two copies of the *genes* found in mammalian vertebrate genomes; e.g. zebrafish express two copies of *nos-2,* but only single copy of *arg1* and *arg2*. Some fish families have even undergone an additional round of duplication (4R), including the Salmonidae (Berthelot et al. 2014) and the Cyprinidae (Ohno 1967). An accurate interpretation of immune responses by measuring *gene* expression requires knowledge of the degree of functional divergence between duplicated *genes* (Petit et al. 2017). This is the case even if closely-related fish species, such as zebrafish and common carp, show extraordinary levels of synteny (Henkel et al. 2012). Next generation sequencing (NGS) increasingly assists with interpreting the expression of duplicated *genes,* thus defining their role in immune responses.

Syntenic analysis in zebrafish shows conserved synteny for *nos-2b* (Lepiller et al. 2009), whose function also appears well conserved (Poon et al. 2008; Hegedus et al. 2009). Head kidney-derived macrophages of common carp, when stimulated with LPS and polarised into M1 phenotypes are characterised by a high up-regulation of *nos-2b*, but not *nos-2a* (Wentzel et al. 2020b). This suggests that *nos-2b gene* expression might be a robust marker for M1 macrophages of (cyprinid) fish, while the role for *nos-2a* remains less clear. In mammals, Arginase-1 is located in the cell cytosol and is mainly expressed in the liver, taking part in the urea cycle and specifically ammonia detoxification. Arginase-2 is located in the mitochondria and could be considered most relevant for extracellular matrix regeneration. In fish, both *arginase-1* and *arginase-2* contain a mitochondrial targeting sequence making it uncertain as to which *gene* variant would be the best to measure with respect to M2 (Joerink et al. 2006). However, in common carp head kidney-derived macrophages,

arginase-2 seems to be most highly regulated, when challenged with cAMP (Wentzel et al. 2020b). There is further and compelling evidence of macrophage subtypes in zebrafish larvae, present before the development of adaptive immunity. In a tailfin transection inflammation model in *tg(tnfa:GFP)* transgenic zebrafish larvae, *tnfa*-expressing macrophages could be purified by fluorescence-activated cell sorting (FACS). Subsequent RNAseq analysis showed that the *tnfa*-expressing macrophages had a pro-inflammatory profile, suggesting an M1-like profile (Nguyen-Chi et al. 2015) (Fig. 6.2). Furthermore, at later stages of wounding, macrophages that were *tnfa*-negative showed signs of an anti-inflammatory profile, including expression of chemokine receptors that, at least in mammals, are linked with M2 macrophages (Nguyen-Chi et al. 2015). This provides in vivo evidence for the presence of M1 macrophages during early stages of inflammation, whereas M2 healing macrophages become more prevalent during wound healing stages.

Using well-described stimuli, LPS (M1) and cAMP (M2), NGS-based analysis of the transcriptional profile of M1- and M2-like polarised macrophages from common carp, shows that transcriptional profile stimuli display a high degree of affinity with those of polarised mammalian macrophages. Amongst well-known M1 marker *genes,* those highly expressed are interleukin-1β (*il1b*), inducible nitric oxide synthase (*inos*) and serum amyloid A (*saa*). Well-known M2 markers that are highly expressed include the tissue inhibitor of metalloproteinases (*timp2b*), transglutaminase (*tgm2b*) and arginase (*arg2*) (Wentzel et al. 2020b). Measuring increased *gene* expression of *inos* or *arginase* can be used as a first and indicative marker for macrophage polarisation in fish (Tables 6.1 and 6.2), but true evidence for different macrophage phenotypes may require more than one single marker.

It is possible that even more information can be obtained by determining those *genes* that are up-regulated or down-regulated in one macrophage subset and—at the same time— show opposite regulation in the other subset (Wentzel et al. 2020b). Based on such criteria, many more potential marker *genes* of M1 macrophages can be identified, for example, ROS-induced heat-shock protein 70 (*hsp70*), inflammatory response-linked immune response *gene* 1 (*irg1*) and the chemokine *cxcl11* (also identified as a M1 marker in zebrafish) (Lu et al. 2017). Based on the same criteria, additional marker *genes* for M2 macrophages include the mannose receptor (*mrc1b*) and the vascular growth factor angiopoietin-like 4 (*angptl4*). Putative marker genes, as listed in Table 6.1, may assist in defining a broader set of comprehensive markers for gene expression studies, as opposed to single genes (*inos, arginase*), to discriminate between polarisation states. No matter what, the correct interpretation of NGS-based information, such as these transcriptome profiles from in vitro polarised macrophage subsets, will require follow-up studies to understand the biological effects. These studies should preferably be in vivo, possibly using transgenic zebrafish larvae.

M2 macrophages can achieve their polarisation states by deactivation of a pro-inflammatory profile. Fish macrophages can be deactivated by cytokines, such as transforming growth factor-beta (*tgf-β*) or interleukin-10 (*il-10*). In fact, TGF–βsignalling, rather than microbial stimuli, could be the oldest evolutionarily trigger to initiate M1/M2
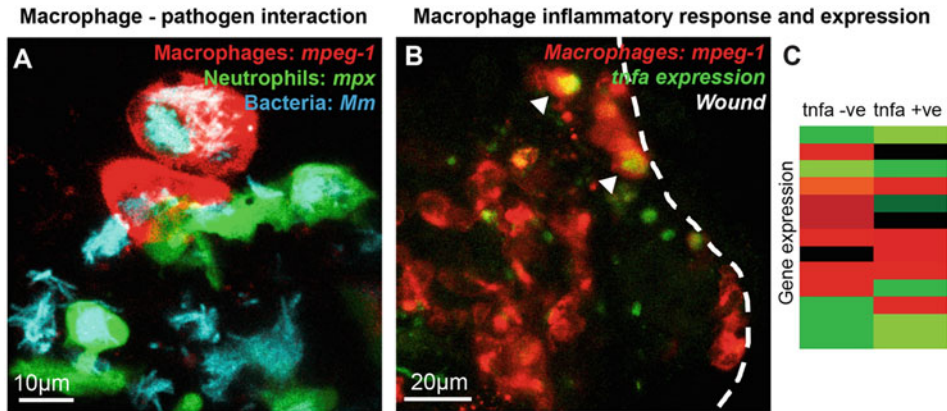
Fig. 6.2 Zebrafish disease models allow macrophage host-pathogen interactions and gene expression changes to be observed during challenge. (**a**) Fluorescent confocal micrograph of a *Mycobacterium marinum* infected transgenic that labels macrophages in red and neutrophils in green allowing observation of host-pathogen interactions in vivo. (**b**) Fluorescent confocal micrograph of red macrophages at a tailfin transection site (dotted line), with a population of them expressing *tnfa: GFP* (white arrowheads). (**c**) Hypothetical *gene* expression analysis that could be done with FACS-purified macrophages from B with or without *tnfa:GFP* expression, similar to that described in Nguyen-Chi et al. (2015)

macrophage polarisation (Dzik 2014). Both, recombinant Tgf-β and Il-10 attenuate LPS-stimulated inflammatory *gene* expression in monocytes/macrophages in grass carp (*Ctenopharyngodon idella*) (Wei et al. 2015). In vitro, recombinant Il-10 from goldfish (Grayfer et al. 2011) and common carp (Piazzon et al. 2015) reduces radical production and transcription of pro-inflammatory cytokines, pushing macrophages towards the M2 spectrum. These findings suggest that subtle differences in polarisation states exist in fish macrophages.

An interesting question is whether or not macrophages once polarised, can repolarise. Metabolic reprogramming has been linked to an inability to repolarise from M1 to M2. This is because mouse M1 macrophages do not regain their oxidative capacity upon repolarisation, whereas M2 are able to repolarise into M1 macrophages (Van den Bossche et al. 2016). In apparent contrast, an in vivo tracking experiment in zebrafish larvae, using transgenic *mpeg1+/tnfa+* macrophages recruited to an injury site shows that this M1 phenotype could be reverted to an intermediate phenotype at a later point in time (Nguyen-Chi et al. 2015). Whether the apparently different outcome in mice and fish is due primarily to differences in experimental set-up, or if it indicates more flexibility in the function of polarised macrophages in fish, remains to be determined.

Table 6.1 Gene profile associated with fish M1 macrophages. A list of 10 genes amongst the most highly expressed in LPS-stimulated (M1) macrophages of common carp (Wentzel et al. 2020b). Note the presence of typical M1 markers such as *nos2*, and the high number of pro-inflammatory cytokines

Gene	Gene description	Protein function
il12p35	Interleukin 12 subunit alpha (p35)	Pro-inflammatory cytokine.
il1β	Interleukin 1 beta	Pro-inflammatory cytokine. Also found in M2 macrophages of carp, but lower expressed.
agrn	Agrin	Extracellular-matrix protein involved in monocyte/macrophage survival and phagocytosis.
saa	Serum amyloid A protein	Acute phase protein, chemotactic to phagocytes and inducer of pro-inflammatory cytokines.
cox2	Prostaglandin-endoperoxide synthase 2a	Also known as Cox-2. Increased expression in M1 macrophages.
nos2b	Nitric oxide synthase 2b	Production of antimicrobial nitric oxide. Well-characterised M1 marker.
il6	Interleukin-6	Pro-inflammatory cytokine
irg1	Immune responsive gene 1	High expression in M1 macrophages contributes to metabolic reprogramming.
cxcl13	C-X-C motif chemokine ligand 13	Up-regulated in M1 macrophages
cxcl8l1	C-X-C motif chemokine ligand 8 like 1	Teleost-specific Cxcl8-like cytokine. Recruits neutrophils through Cxcr2.

6.4.3 Immunometabolism

Immunometabolism refers to changes in intracellular metabolic pathways in immune cells, including macrophages that alter their function in a complex interplay between immunity and metabolic reprogramming. Immunologically distinct macrophages may also be metabolically distinct and reprogrammed to enhance opposing pathways of energy (O'Neill et al. 2016). Whereas M2 macrophages rely primarily on oxidative phosphorylation, like many cells in homeostasis, in contrast M1 macrophages are the ones that are 'different' and show metabolic reprogramming towards glycolysis. In M1 macrophages, two 'breaks' in the tricarboxylic acid (TCA) cycle occur and this metabolic reprogramming towards glycolysis supports several inflammatory functions. The first break leads to a shuttling of citrate and succinate out of the mitochondria. Intracellular citrate contributes to the production of NO and ROS and to fatty acid synthesis for membrane and granule formation, whereas succinate contributes to ROS production and can stabilise hypoxia-inducible factor (HIF)1-alpha. Further release of succinate in the extracellular microenvironment can act as an alarmin. The second break in the TCA cycle leads to an inhibition of the electron transport chain in the mitochondria, mediated by both NO and itaconate, the latter produced from citrate with an enzyme encoded by immune response gene 1 (*IRG1*)

Table 6.2 Gene profile associated with fish M2 macrophages. A list of 10 genes amongst the most highly expressed in cAMP-stimulated (M2) macrophages of common carp (Wentzel et al. 2020b). Note the presence of typical M2 markers such as *arginase2*, and genes associated with angiogenesis and matrix remodelling

Gene	Gene description	Main Function
cyr61l1	Cysteine-rich angiogenic inducer 61 protein-like protein 1	Extracellular matrix protein involved in angiogenesis and regulation of matrix remodelling.
timp2b	Tissue inhibitor of metallo-proteinase 2b	Involved in extracellular matrix remodelling. Decreased in M1 and increased in M2 macrophages.
tgm2b	Transglutaminase 2b protein	Enzyme important in apoptotic cell clearance by phagocytosis. Well-known M2 marker.
arg2	Arginase 2	Arginase 1 is the canonical M2 marker in murine M2 macrophages.
vegfaa	Vascular endothelial growth factor Aa	Signalling protein involved in angiogenesis and tissue generation. Up-regulated in M2 macrophages.
hbegfb	Heparin-binding EGF-like growth factor b	Growth factor in early stages of wound healing. Promotes angiogenesis.
angptl4	Angiopoietin-like 4	Prevents the formation of lipid-laden giant cells. Associated with anti-inflammatory macrophages.
ppap2b	Phosphatidic acid phosphatase type 2B	Lysophosphatidic acid (LPA) inhibitor. Induced by VEGF and involved in angiogenesis.
tdh	L-threonine dehydrogenase	Converts L-threonine into glycine, which modulates macrophage activity.
crema	cAMP-responsive element modulator a	Binds cAMP response element and different splice variants. Modulates transcription.

(Van den Bossche et al. 2017; O'Neill and Artyomov 2019). First indications are that immunologically distinct macrophages of fish could also be metabolically distinct. M1 macrophages of common carp can alter their energy metabolism in a manner similar to those of mice, showing altered oxidative phosphorylation and glycolysis. Common carp M2 macrophages rely on oxidative phosphorylation, without reprogramming towards glycolysis (Wentzel et al. 2020a), with metabolic reprogramming appearing to be conserved.

6.5   In Vivo Disease Models

Zebrafish are a pivotal and widely used preclinical model of human and fish diseases, since the last 20 years (Renshaw and Trede 2012; Jørgensen 2020). Their transparent larvae and genetic tractability enable in vivo microscopy of intact organ systems, which twinned with their small size and high fecundity, have led to their expansive use as a model organism in laboratories across the globe. Originally used as a model for developmental biology in the

1980s and 1990s, they are now used as a model for a plethora of fish and human diseases. Thanks to the availability of transgenic lines and the ability to sort immune cells to assess *gene* expression, they are extensively used as models to understand the roles of macrophages in a wide range of disease settings (Fig. 6.2).

6.5.1 Zebrafish Models of Inflammation

Zebrafish are used as injury and sterile wound models, in part due to their highly regenerative properties, which enable visualisation of the complete healing process over just a few days post- challenge. Macrophages play important roles after tissue injury, caused by either exogenic factors or disease pathogenesis. Post-damage, macrophages protect against invading pathogens, but also play critical roles in tissue repair and regeneration. A widely used zebrafish model of inflammation is sterile transection of the tailfin to induce an inflammatory response (Renshaw et al. 2006; Yoo and Huttenlocher 2009). There are a number of variations in this model, (including a nick model (Yoo and Huttenlocher 2009), a more severe cut that dissects the end of the notochord (Renshaw et al. 2006), and combination with infection (Schild et al. 2020)), but all are common in their induction of an inflammatory response. Neutrophils are some of the first immune cells to reach the wound site after a few minutes, but they are closely followed by macrophages, recruited over the first 2–4 h (Loynes et al. 2010). Neutrophil numbers quickly reduce as inflammation resolves after 24 h post-wound (hpw), both via apoptosis in situ and reverse migration away from the site (Elks et al. 2011). However, macrophage numbers do not decrease, and persist at the injury site beyond 24hpw, playing important roles in efferocytosis (the clearing of apoptotic cells, including dead neutrophils) and subsequent wound healing (Loynes et al. 2010). When macrophage numbers are depleted, then the wound does not heal properly, mirroring the effects on wound healing observed in human diseases where macrophages are perturbed.

Wound models in zebrafish expand to other models beyond the tailfin, including heart, retina and somite injury to investigate the roles of immune cells in healing and regeneration (MacDonald et al. 2015; Gurevich et al. 2018; Marín-Juez et al. 2019; Bevan et al. 2020). The zebrafish is capable of injured heart regeneration, without scar formation, and the roles of macrophages in the regeneration process are becoming a field of intense study. Injury in the muscle block allows for the study of the important process of revascularisation and angiogenesis, a process in which macrophage involvement is critical, presumably in an M2 type response (Gurevich et al. 2018). Wound models are also used in combination with a wide range of disease models, including models of perturbed glucose signalling (diabetes), models of cystic fibrosis and foreign body response, to investigate the roles of immune cells in these processes (Olsen et al. 2010; Gurevich et al. 2019; Bernut et al. 2020).

6.5.2 Zebrafish Models of Infectious Pathogens

A critical function of macrophages is the handling of pathogens by phagocytosis and subsequent degradation, mechanisms that are subverted by many pathogens. As antimicrobial infections are on the increase worldwide, due to the emergence of antimicrobial resistance, there is a pressing need to understand and target these mechanisms. Understanding these processes in macrophages in vivo is a critical step towards new treatments. Some of the first disease models in zebrafish were infection models (Rougier et al. 1996; LaPatra et al. 2000; Neely et al. 2002; Davis et al. 2002; van der Sar et al. 2003). Zebrafish infectious disease models make significant contributions to our understanding of the macrophage response to pathogens and, in particular, shed light on how pathogens differentially interact with macrophages and evade their killing mechanisms (Rosowski 2020).

A widely used infection model in zebrafish is the use of *Mycobacterium marinum* (Mm) as a model of mycobacterial disease (most notably of human tuberculosis, but also as a model of Mm infection of commercial fish species) (Hodgkinson et al. 2019). The zebrafish Mm model was driven forward in the early 2000s due to a pressing need for accessible in vivo models of tuberculosis (Davis et al. 2002; Meijer et al. 2004). Unlike some other human infections, murine models of TB are hindered because mice are not natural hosts of human *Mycobacterium tuberculosis* (Mtb), and fail to produce all aspects of the hallmark granuloma response. Mm is the closest genetic relative of the human Mtb complex and can infect humans (leading to a condition called fishtank granuloma), where it causes granuloma formation in peripheral tissues, hindered only by its intolerance to temperatures above 28 °C. Mycobacteria are intracellular pathogens that can escape almost every step of the macrophage killing process in the right conditions (Chai et al. 2020). Macrophages are the primary cell type in the granuloma and as such have been a focus of zebrafish Mm research over the last 15 years plus (Davis et al. 2002; Volkman et al. 2004; Clay et al. 2007; Torraca et al. 2015; Rougeot et al. 2019). Antibody staining confirms the heavy involvement of macrophages in granuloma formation, showing that the presence of macrophages is sufficient for granuloma formation to begin, without the need for adaptive immunity (Davis et al. 2002). Genetic depletion experiments show macrophages to be critical players in Mm control, but also essential players in infection dissemination and granuloma formation (Clay et al. 2007). Zebrafish models show that there are several signalling pathways and factors involved in macrophage control of Mm, including Tnfα, Il-1B and autophagy components (Clay et al. 2008; van der Vaart et al. 2014). However, important findings in zebrafish models show that Mm can circumvent key macrophage defence mechanisms, including the production of reactive oxygen and nitrogen species (Elks et al. 2013, 2014; Roca and Ramakrishnan 2013). Elegant genetic work, where macrophages are FACS purified at different stages after infection and RNAseq performed, has identified a plethora of potentially new macrophage genes involved in TB pathogenesis (Rougeot et al. 2019). In combination with signalling transgenic lines, this demonstrates that although the macrophage pro-inflammatory response is initially present after infection,

this is circumvented by bacteria that allow for granuloma formation (Lewis and Elks 2019; Ogryzko et al. 2019). Then, at the granuloma stage the pro-inflammatory factors are once again activated in macrophages. These new lines of evidence are leading to the identification of novel therapeutic targets against TB. These include hypoxia inducible factors, which are able to increase the early pro-inflammatory response, including *il-1beta* and *tnfa,* to aid the clearance of bacteria by innate immune cells (Lewis and Elks 2019; Ogryzko et al. 2019). Aside from the natural fish pathogen Mm, zebrafish models of an increasingly growing list of pathogens including bacteria (e.g. shigella/edswardsiella/ staphylococcus/porphyromona/burkholderia), fungi (e.g. cryptococcus/candida/aspergillus), parasites (e.g. trypanosomes) and viruses (e.g. chikungunya/sindbis) are being utilised to uncover the roles of macrophages in disease pathogenesis (Torraca et al. 2014).

6.6   Future Perspectives

An interesting area of research is the assumed important role of macrophages in trained immunity. Trained immunity is a form of innate immune memory providing increased nonspecific immune responses to subsequent infection, which is based on enhanced inflammatory and antimicrobial properties of innate immune cells (Netea et al. 2016). In food animals, such as fish, the concept of enhancing the innate immune system is not new but few studies have purposefully investigated innate training (Petit and Wiegertjes 2016; Byrne et al. 2020).

Early studies in fish already indicate that injection of brook trout (*Salvelinus fontinalis*) with modified Freund's complete adjuvant containing killed *Mycobacterium butyricum,* increases protection and induces a long-lasting increase in phagocytic and bactericidal activity of peritoneal macrophages (Olivier et al. 1986). Further, vaccination of fish with BCG (the Bacillus Calmette-Guérin vaccine against tuberculosis based on an attenuated strain of *M. bovis* and frequently used to study trained immunity in humans (Kleinnijenhuis et al. 2012) provides cross-specific protection in several fish species, including Japanese flounder (*Paralichthys olivaceus*), amberjack (*Seriola dumerili*) (Kato et al. 2010, 2011, 2013) and zebrafish (Oksanen et al. 2013).

Purposely investigated studies in fish have used in vitro cultures of head kidney-derived macrophages from common carp. Following a resting period of 6 days, comparable to the experimental set-up used to study trained immunity in human monocytes (Bekkering et al. 2017), a 2 h in vitro exposure to a soluble nucleotide binding and oligomerisation domain (NOD)-specific ligand, or to soluble β-(1,3/1,6)-glucan, resulted in carp macrophages that displayed typical features of trained immunity for a period of at least 6 days (Petit et al. 2019). Unstimulated, but trained, macrophages displayed increased phagocytosis and elevated constitutive gene expression of the cytokines *il-6* and *tnfa* and a metabolic shift from oxidative phosphorylation towards glycolysis, which could be measured as increased production of lactate. The underlying mechanisms of trained immunity are as yet not fully understood in fish. In mammalian systems, these processes rely on long-lived epigenetic

modifications that persist for longer periods, even after removal of the training stimulus. For example, β-glucan-induced trained immunity of human monocytes has been associated with modifications of histone (H)3 activation and repressor markers at promotor sites of cytokine genes, such as IL-6 and TNFα, and appears key to the altered long-lived inflammatory response associated with trained immunity (Saeed et al. 2014). Areas of research which combine trained immunity with immunometabolic control (Riksen and Netea 2020) are of high interest for fish macrophages, because of the expected conservation of metabolic reprogramming (Wentzel et al. 2020a).

Last but not least, in vitro evidence for the effects of trained immunity alone is never easy to show in animals that also have adaptive immunity. Zebrafish could prove especially informative for such in vivo studies, owing to the availability of $rag-/-$ mutant lines that do not have a functional B- and T-cell response and therefore no functional adaptive immune response (Tokunaga et al. 2017). There are some data on $rag-/-$ zebrafish, showing that these fish have a constitutively heightened innate immune activity and increased survival 8 weeks post-exposure to an attenuated non-virulent strain of *Edwardsiella ictaluri* (Tokunaga et al. 2017). This could be interpreted as the first line of experimental in vivo evidence of trained immunity in fish.

6.7   Conclusions

In conclusion, fish macrophages are functionally similar to their mammalian counterparts, with the ability to polarise into different activation states, playing important roles in infection response and wound repair. The conservation of function is demonstrated in a wide range of fish species by in vitro immunology, performed on various primary cell cultures. Without validated specific markers for fish macrophages, it remains difficult to assess the purity of in vitro cell cultures. Although a repertoire of conserved genes for mononuclear phagocyte subsets seems to exist and thus could constitute a list of candidates for relevant markers (Vu Manh et al. 2015), this list is based on a meta-analysis combining cell sorting and comparative transcriptomic analysis and requires confirmation by functional evidence. Studies on the evolution of macrophages suggest that key processes, such as phagocytosis, contribute to the regulation of the inflammatory response since hundreds of millions of years of evolution (Barreda et al. 2016). While purification, differentiation and culture techniques vary across species, the importance of the arginine fork and nitrosative response as markers of activation states is demonstrated in most species studied. More recently, the expansion of zebrafish studies, with all the genetic and microscopy tools available in a single species, unifies groups around the world studying macrophages in fish to use similar techniques and tools. This illuminates further the roles of macrophages in homeostasis and disease, often in the absence of adaptive immunity. There is still much to understand in terms of the variety of macrophage phenotypes and function in fish species, especially in diverse tissue microenvironments.

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Immunoglobulins in Teleost

7

Manuel Mendoza and Susana Magadán

Abstract

Jawed vertebrate species (Gnathostomata) are all characterized by an adaptive immune system based on B and T cells along with the huge diversity and specificity of their antigen receptors, the immunoglobulins (IG) or antibodies and the T-cell receptors (TCRs), respectively. The availability of genome assemblies of many species has recently provided valuable information on the complexity and diversity of teleost germline IG loci. The development of deep sequencing technologies has also favored a growing interest for immunoglobulin repertoires, to address basic questions about immune mechanisms in teleost or applied concerns such as the identification of molecular markers of protection after vaccination. This work provides an overview on the germline configuration of teleost IG loci, IG repertoire studies, and recent findings on IG functional roles in this group of vertebrates.

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Abbreviations

IG   Immunoglobulin
TCR   T-cell receptor
BCR   B-cell receptor
IGH   Immunoglobulin Heavy chain
IGL   Immunoglobulin Light chain
C   Constant
V   Variable

7.1   Introduction

The emergence of vertebrates was accompanied by major morphological and functional innovations, such as the development of an adaptive immune system (Flajnik and Du Pasquier 2004). In jawed vertebrates (Gnathostomata), including bony fish (superclass Osteichthyes), the adaptive immune system relies on B and T cells and on the huge diversity and specificity of their antigen receptors, the immunoglobulins (IG) or antibodies, and the T-cell receptors (TCR), respectively (Cooper and Alder 2006; Flajnik 2018).

IGs are produced by B lymphocytes, either as a secretory form (antibody) or as a membrane form (B-cell receptor). As described in humans or mice, they constitute a key factor for fish-specific immunity and for the protection afforded by vaccines. The mechanisms underlying the humoral immunity mediated by IGs include opsonization of pathogens to be eliminated by phagocytes, neutralization of toxins and viruses, and activation of the complement cascade (Magadan et al. 2015; Mashoof and Criscitiello 2016). The canonical IG structure is a tetramer that consists of two identical heavy (H) chains and two identical light (L) chains. Both H and L chains contain one N-terminal variable domains (VH or VL) and one or more C-terminal constant domains that form the constant region (CH or CL) and define the isotypes. The variable region that engages the antigen is formed by one VH and one VL domain. So, one IG molecule has two antigen-binding sites, which can recognize the cognate antigen in its native form. Constant regions consist of constant domains of both IGH and IGL as well. While IGL have one CL domain, the number of constant domains of IGH chain vary among different immunoglobulin classes and species. Interestingly, the domains that comprise the constant regions mediate effector functions of the antibody molecule (Sun et al. 2020). As shown in this chapter, the availability of genome assemblies of many species has provided much information on the IGH and IGL chain gene loci in teleost fish.

Compared to mammals, teleosts have slower antibody-mediated responses, only three classes have been described (IgM, IgD, and IgT), and they do not present immunoglobulin class switching. Moreover, there is a considerable debate on whether the IG affinity maturation and clonal selection during B-cell responses in fish (extended to ectothermic vertebrates) are less efficient compared to mammals (Magor 2015), likely because

microenvironments do not allow the fast and powerful selection of cells expressing high affinity antibodies as in mammals (Fillatreau et al. 2013; Magadan et al. 2015). However, further studies are required to confirm this hypothesis. The teleost immune system is adapted to particular anatomical and physiological constraints. Therefore, features conserved in common ancestors of fish and mammals are likely to be essential, while other characteristics may represent teleost-specific original solutions. The aim of this chapter is to present an overview of the different IGs expressed by teleost, considering the germline configuration of teleost IG loci and the application of new approaches such as new generation sequencing to decipher the dynamics of the humoral adaptive immune response in teleost. Recent studies that shed light on the functional role of teleost IG classes are also discussed.

7.2   IG Loci

Bony fishes are classified into lobe-finned fishes (Sarcopterygii) and ray-finned fishes (Actinopterygii). Lobe-finned fishes include the coelacanth and lungfish, which are considered to be the closest living species of the land vertebrates (Shan and Gras 2011). Ray-finned fishes include the infraclass Teleostei, which comprises 95% of extant fish species (approximately 35,000 species) (Fricke et al. 2020), and they account for more than half of all extant vertebrate species. The success of this infraclass, along with their ability to thrive in a wide range of ecological niches, suggests that teleost species developed an immune arsenal to counter pathogen and environmental challenges.

Research on the immune system of fish has generally been limited by the lack of reagents suitable for classical cellular immunology research. However, complete genome sequences are now available for many species, allowing thorough analysis of immune mechanisms. In particular, a cycle of tetraploidization and rediploidization occurred during the early evolution of fish. The genome diversity was further increased by lineage-specific events of genome duplication and/or contraction. All these genome remodeling processes affected genes involved in the immune system, such as the major histocompatibility complex or immunoglobulin genes (Bradshaw and Valenzano 2020; Malmstrøm et al. 2016). A deep knowledge about the structure of IG loci (and T-cell receptor gene loci as well) is essential to comprehend the adaptive immune response developed against antigens in a given species. Furthermore, teleost fish can be considered a good model for comparative studies between distant living groups. They can provide clues about the primordial immune system of extinct vertebrates, and new insights into the functional development of adaptive immune systems throughout vertebrate evolution.

The structure of bony fish IGH loci follows the general pattern found in most vertebrates, i.e., the translocon organization, with a large region containing all IGHV genes in 5′, followed by several D, J, and then C region genes at the 3′ end (Fig. 7.1). However, the genomic organization of teleost IGL loci is quite different. They are arranged in multiclusters of VL, JL, and CL region segments: $(V_L–J_L–C_L)n$ or $(V_L–V_L–J_L–C_L)n$
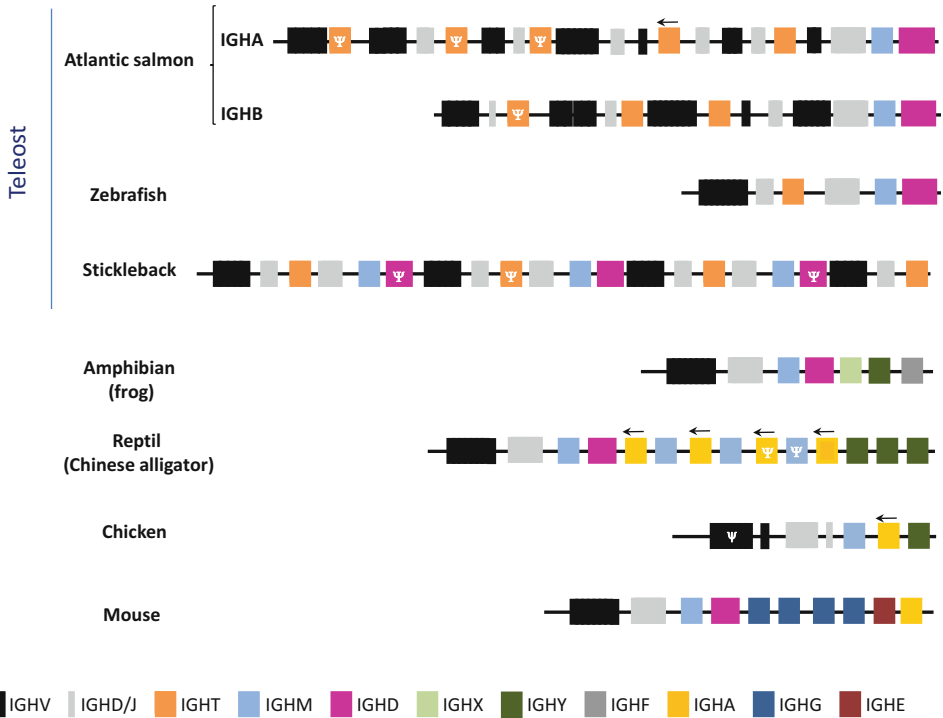
Fig. 7.1 Schematic structure of the genomic organization of IGH loci in representative vertebrate species. The schemes are not drawn to scale and depict only the genomic configuration of V (black boxes), D and J (light gray boxes), and CH gene segments. The number of IGHV, D, and J gene segments are not indicated, and except for chicken, functional V and pseudo V (ψV) segments are not distinguished. Different CH genes are represented as different colored boxes: IGHM (light blue), IGHD (purple), IGHT (orange), IGHX (light green), IGHY (dark green), IGHA (yellow), IGHG (dark blue), and IGHE (brown). The pseudo CH genes are indicated by a ψ. The CH genes with the opposite transcriptional orientation to the whole gene locus are indicated as an arrow. In Atlantic salmon, both iso loci (IGHA and IGHB) are represented. (Asterisk) IGHF has been only identified in *Xenopus laevis*

(Bengtén and Wilson 2015; Ghaffari and Lobb 1993, 1999; Magadán-Mompó et al. 2013; Rego et al. 2020a). Thus, bony fishes possess a chimeric gene organization for the H- and L-chain genes (Flajnik 2002; Sun et al. 2020).

Comparative analysis of IG loci structure in closely related species, and different lines or stock, provides information about IG loci variation that may have significant implications for practical issues in aquaculture. In addition, these analyses may also elucidate the mechanisms of gene conversion, somatic hypermutation, and memory in these species and during vertebrate evolution. Furthermore, the development of high-throughput DNA sequencing technologies has enabled large-scale characterization of functional antibody repertoires in human and mice. This way of understanding protective and pathogenic

immune responses has started to develop in teleost species, reflecting a growing interest for an accurate and comprehensive description of the antibody-mediated response.

7.2.1 IGH Locus

The immunoglobulin heavy chain determines the antibody effector function, and also contributes to the antibody diversity. Thus, the structure of IGH locus has a relevant effect on the adaptive immune response for any species. Previous works have characterized the IGH locus structure in teleost species, which are used as animal models in biomedicine, such as zebrafish (*Danio rerio*) (Danilova et al. 2005), or in ecological and evolutionary research, like medaka (*Oryzias latipes*) (Magadán-Mompó et al. 2011), three-spined stickleback (*Gasterosteus acuelatus*) (Bao et al. 2010; Gambón-Deza et al. 2010), turquoise killifish (*Nothobranchius furzeri*), and southern platyfish (*Xiphophorus maculatus*) (Bradshaw and Valenzano 2020). The IGH loci have been also elucidated in a number of fish species relevant for the aquaculture industry, i.e., rainbow trout (*Oncorhynchus mykiss*) (Hansen et al. 2005; Magadan et al. 2019b), fugu (*Takifugu rubripes*) (Fu et al. 2015), and Atlantic salmon (*Salmo salar*) (Yasuike et al. 2010). These characterizations have revealed remarkable diversity in the size and structure of teleost IGH loci (Bengtén and Wilson 2015; Bradshaw and Valenzano 2020). However, the number of studied IGH loci are very low compared to the total evolutionary diversity of teleost, and are mainly focused on major aquaculture species and established research models.

In all studied bony fish, the IGH locus is organized in translocon configuration. This IGH configuration is characterized by multiple tandemly organized IGHV, D, and J gene segments, followed by a series of IGHC genes, encoding diverse H chain isotypes or subclasses associated with different effector functions (Fig. 7.1). It was initially thought that the IGH locus in teleosts was a simplified version of the translocon IGH locus found in mammals. However, research in teleost fishes has revealed a remarkable degree of diversity in the length and organization (Bengtén and Wilson 2015; Fillatreau et al. 2013). After the discovery of the IgZ/T in zebrafish (Danilova et al. 2005) and rainbow trout (Hansen et al. 2005), the overall organizations of the IGH locus in teleosts have been better understood. Three sets of CH exons define the three Ig classes encoded by isotypes μ, δ, and τ/ζ. As in mammals, IgM and IgD are coproduced through alternative splicing of a long pre-mRNA containing the VDJ region, the Cμ exons and the Cδ exons. IgT/Z is encoded by an additional Dτ-Jτ-Cτ cluster located in the 5′ regions of the IGHD and IGHJ gene segments of Igμ and Igδ in zebrafish (Danilova et al. 2005) and stickleback (Bao et al. 2010; Gambón-Deza et al. 2010) or inserted within the IGHV segments close to IGHM-IGHD genes, as in rainbow trout (Hansen et al. 2005; Magadan et al. 2019a, b) and Atlantic salmon (Yasuike et al. 2010). This IGH configuration has led to the evolution of a distinct B-cell lineage in addition to IgM, which expresses IgT/Z (Danilova et al. 2005; Hansen et al. 2005; Salinas et al. 2011). However, all three classes (IgM, IgD, and IgT/Z) when

expressed from a unique IGH locus can use the same set of IGHV genes, which can rearrange either to DHτ or Dμ/δ. (Fig. 7.1).

The number of IGH loci are quite variable among fish species. Among salmonids, Atlantic salmon and rainbow trout possess two IGH isoloci (IGHA and IGHB), due to the tetraploidization of Salmonidae (Hansen et al. 2005; Yasuike et al. 2010). Two IgM isotypes were found in Atlantic salmon and brown trout (*Salmo trutta*), while it has been suggested through gel filtration analysis that rainbow trout possesses a single IgM (Hordvik 2002), suggesting that one of the two isoloci may be nonfunctional. However, the annotation of IGH loci using Atlantic salmon and rainbow trout assembly genomes resulted in the presence of two functional IGHM genes in both species (Magadan et al. 2019b). Authors found eight IGHT genes in Atlantic salmon, which were previously described by Yasuike et al. (2010), and three IGHT genes in rainbow trout, but only two of them are functional. These results are not in line with the three IgT subclasses previously reported in rainbow trout (Zhang et al. 2017). Nevertheless, it is important to note that different sequencing technologies (Illumina, PacBio long reads, etc.) can be used for genome sequencing, which likely affects the quality of the genome assembly and may generate sequencing gaps and artifacts that have to be clarified by further studies.

Only one IGH locus has been identified in zebrafish (Danilova et al. 2005), in torafugu (Fu et al. 2015), in turquoise killifish, and southern platyfish (Bradshaw and Valenzano 2020). In other species like channel catfish (*Ictalurus punctatus*), medaka (Magadán-Mompó et al. 2011), and three-spined stickleback (Bao et al. 2010; Gambón-Deza et al. 2010), tandem duplications of the IGH locus have been found (Fig. 7.1). Interestingly, no IGHT gene (or transcript) has been found in channel catfish, medaka (Bengtén et al. 2006; Magadán-Mompó et al. 2011). A recent comparative analysis performed in 12 genomes from species belonging to Cyprinidontiformes suggests that the IGHT gene has undergone duplication and convergent loss in the course of cyprinodontiform evolution (Bradshaw and Valenzano 2020). This work, in addition to confirm the IGHT absence in medaka, has identified other teleost that appears to lack IGHT constant regions, such as the *N. furzeri* and, two fresh water species used in aquariums, *Aphyosemion australe and Nothobranchius orthonotus*. All these results demonstrate the remarkable plasticity of the IGH locus across evolutionary time.

In addition to such diverse numbers of IGH loci, the structure of different IGH constant genes also shows a remarkable plasticity among teleosts. While the six-exon genomic structure of the IGHM constant region (four CH, and two transmembrane TM exons) is highly conserved across the jawed vertebrates, with similar configurations observed in mammals, teleosts, and elasmobranchs (Flajnik 2018; Sun et al. 2020), IGHD and IGHT genes have undergone repeated duplications and deletions in the course of teleost evolution. For instances, Cδ2-Cδ3-Cδ4 domains are repeated four times in zebrafish, three in catfish, and twice in Atlantic salmon and Atlantic halibut (*Hippoglossus hippoglossus*) (Fillatreau et al. 2013; Yasuike et al. 2010). A different structure has been reported in Atlantic cod (*Gadus morhua*) with two duplicated Cδ1-Cδ2 separated by a short exon, and followed by Cδ7 (Fillatreau et al. 2013). The rainbow trout IgD gene is also particular: the

configuration, Cδ1–Cδ2a–Cδ3a–Cδ4a–Cδ2b–Cδ7, seems to be the result of a first duplication of Cδ2–Cδ4 followed by deletion of the Cδ3–Cδ6 domains (Hansen et al. 2005). In other species, including Japanese flounder (*Olive flounder*) and three-spined stickleback, there is no Cδ duplication (Bengtén and Wilson 2015; Hirono et al. 2003). Interestingly, in catfish and takifugu, there are two different genes that encode the membrane IgD and the secreted IgD (Aparicio et al. 2002; Bengtén et al. 2002; Bengtén and Wilson 2015; Edholm et al. 2010). Based on these studies, we can speculate that the different IgD structures observed in a variety of species may have specific effector functions by adapting to the specific immune environments.

Different number of CH exons have been also identified in IGHT genes: four CH exons are found in most species (Danilova et al. 2005; Hansen et al. 2005; Yasuike et al. 2010), whereas the three-spined stickleback has three (Bao et al. 2010; Gambón-Deza et al. 2010) and fugu two (Aparicio et al. 2002). In carp (*Cyprinus carpio*), the secreted IgT/Z appears to be a chimera with a variable region and two constant domains, a Cμ1 domain and a Cτ/ζ domain (Savan et al. 2005). While the number of C domains differ among species, it is worthy to note that Cτ/ζ1 (Cμ1 in the carp IgT/chimera) and Cτ/ζ4 seem to be conserved (Bradshaw and Valenzano 2020; Gambón-Deza et al. 2010). If additional C domains may provide different functional proprieties, is a question to be resolved.

Alternative splicing of pre-mRNAs may contribute to an additional level of IGH isotypic variation in fish. In mice and humans, membranes versus secreted IgM H chains are typically produced from the same pre-mRNA through alternative splicing. A secreted Igμ transcript is produced when the mRNA is cleaved and polyadenylated between the constant region domain Cμ4 and the TM exons, and a membrane Igμ transcript is made if a cryptic splice site located within Cμ4 is spliced to the acceptor site of the TM1 exon. In fish, membrane Ig μ transcripts have the TM exons spliced to the donor site located at the 3′ end of the Cμ3 exon, hence lack the last Cμ domain that is present in the secreted IgM forms (Hikima et al. 2011). Exceptions to this rule have been found in different species, including Antarctic Notothenioids fish (Coscia et al. 2010). Transcripts which encode transmembrane IgM with only one or two CH domains have been reported in zebrafish (Hu et al. 2011) and medaka (Magadán-Mompó et al. 2011).

7.2.2   IGL Loci

To date, in all studied vertebrate species, with the exception of chickens, ducks, and snakes, more than one immunoglobulin light chain isotype can be found (Das et al. 2010; Gambón-Deza et al. 2012; Lundqvist et al. 2006). The conventional classification of IGL into kappa and lambda isotypes was initially designated as a means to classify mammalian IgL (Edelman and Poulik 1961). Over the years, additional isotypes have been described, and the classification system groups all vertebrate IGLs into four main ancestral branches: kappa (mammalian κ, elasmobranch type III/NS4, teleost L1/L3/F/G, Xenopus ρ), lambda (mammalian λ, elasmobranch type II/NS3), sigma (Xenopus σ, teleost L2, elasmobranch

type IV), and sigma-2 (elasmobranch type I/NS5, variant sigma-type in coelacanth). However, this classification can be modified when new vertebrate genomes are analyzed. A new clustering was proposed by Guselnikov et al. (2018), concluding that there were five ancient IGL isotypes (kappa, lambda, lambda-2, sigma, and sigma-2) which evolved differentially in various lineages of jawed vertebrates.

At first, it was very unclear to establish a relationship between teleost IgL isotypes and the mammalian κ and λ chains so, the nomenclature found in the literature is diverse and in some bony fish species they were named in order of discovery. Finally, a comprehensive phylogenetic analysis of different vertebrate VL and CL regions suggests that previously named L1 and L3 chains are κ orthologous (Criscitiello and Flajnik 2007) and the teleost L2 immunoglobulin light chains are σ orthologous (Partula et al. 1996). According to this, the teleost IGLs have been subdivided into σ-like (previous L2) and two groups of κ-like chains (L1/κG and L3/κF). The lambda chain isotype was thought to be lost in the teleosts until its characterization in channel catfish, Atlantic cod, and rainbow trout (Edholm et al. 2009). Furthermore, a recent phylogenetic analysis of extended IGL datasets indicates that teleostean lambda orthologs actually represent a distinct isotype designated as lambda-2 (Guselnikov et al. 2018). This isotype is also present in holostean and polypterid fish, which suggests its emergence before the radiation of ray-finned fish (see Table 7.1).

To date, the κ, λ (or λ-2), and σ isotypes identified in teleosts have been found to exist on different chromosomes in a cluster assemblage, it means multiple VL-JL-CL units (Bao et al. 2010; Daggfeldt et al. 1993; Edholm et al. 2011; Zimmerman et al. 2011, 2008). In a genome wide study, such clusters have been found in five different chromosomes in zebrafish (Zimmerman et al. 2011). Interestingly, recombination between clusters was reported in this species, which might offer a greater potential combinatorial diversity. A characterization of the fugu IGL genomic loci and southern blot analysis has shown a minimum of 12 clusters (classified as kappa and sigma) spread on three different chromosomes (Fu et al. 2017). Interestingly, a recent genomic analysis using nine rainbow trout lines revealed that kappa, lambda, and sigma light chain isotypes are present in all trout lines studied, with highly conserved constant region nucleotide sequences (Rego et al. 2020b). However, the authors detected differences between the trout lines in the number and size of clusters (mainly sigma-like) that could affect humoral immune responses. In addition, the presence of multiple clusters on one or more chromosomes also indicates that cluster duplication and expansions likely played a major role in the generation of antibody diversity in teleost fishes.

Regardless of the diversity in number or length of teleost IGL loci, the genomic organization is almost conserved. In the kappa locus (L1 and L3), the V segments are in opposite transcriptional orientation as for the J and C segments, which implies that the VJ arrangement happens rather by inversion than deletion. While the sigma (L2) and lambda gen clusters present V segments in same orientation. One exception is the stickleback (Bao et al. 2010), with σ clusters in which the 3′VL segment is in opposite polarity. This difference in genomic configuration would imply diverse mechanisms for generating antibody diversity.

Table 7.1 Vertebrate IGL chains. This table is adapted from Guselnikov et al. (2018)

Taxon	Species	κ	λ	σ	σ-2
Cartilaginous fish	Nurse shark	NS4	NS3	Type IV/σ	NS5/σ-cart
	HHorned shark	Type III	Type II	Type IV/σ	Type I/σ-cart
	EElephant shark	κ	λ	σ	σ′/σ-prime
Lobe-finned fish	African coelacanth	κ	λ	σ	σ-2
Teleosts	Rainbow trout	Type 1, type 3/L1, L3/κF, κG	λ[a]	Type 2/L2	
	Catfish	Type 1, type 3/F, G	λ[a]	Type 2/L2	
	Atlantic cod	Type 1, type 3/L1, L3	λ[a]	Type 2/L2	
	Zebrafish	Type 1, type 3/L1, L3		Type 2/L2	
	Common carp	Type 1, type 3/L1A, L1B, L3		Type 2/L2	
Amphibians	African clawed frog	L1/ρ	Type III	L2/σ	
Reptiles	Sea green turtle	κ	λ		σ-2
	Anole lizard	κ	λ		
	Asian glass lizard		λ		
	Chinese alligator	κ	λ		
Birds			λ		
Mammals		κ	λ		

[a]Guselnikov et al. propose a new isotype, the lambda-2

7.3 Immunoglobulin Repertoires in Teleost

An organism is exposed to a huge diversity of antigens, and the generation of different IG receptors required to specifically recognize them is the result of multiple processes that happen before and after antigen recognition. The germline structure of IGH and IGL loci provides the basic pieces from which the IG is assembled, and the diversity is generated. The theoretical diversity of the IG repertoire is a result of a number of different stages in the development of the B cell, and of the gene segments selection and assembly mechanisms therein. Briefly, the IG loci are subject to genomic rearrangements of variable (V), joining (J), diversity (D) gene segments, which along with the allelic exclusion, lead to the expression of a unique antigen receptor by each lymphocyte (Hozumi and Tonegawa

1976; Tonegawa 1983). The V(D)J junction is not exact, and the deletion and insertion of nucleotides at the joint region are commonly observed. This process, along with the pairing of heavy and light chains forming the IG, results in a vast repertoire of B cells bearing structurally diverse receptors (potentially $>10^8$ sequences in zebrafish, and $>10^{13}$ in humans) for specific antigen recognition. During the immune response, each lymphocyte produces a clone of cells specific to one antigenic motif and expressed IG receptors may diversify further by the process of gene hypermutation. Thus, the collection of IG sequences within an organism, also known as the immunoglobulin repertoire, reflect the current state of the humoral adaptive immune system of an animal. Moreover, the changes generated in response to external (i.e., vaccines or pathogens) or internal (i.e., aging) immune challenges, are also depicted.

The development of high-throughput sequencing protocols to analyze the immunoglobulin rearrangements expressed in a lymphocyte population overcomes the previous technical limitations that only allowed the analysis of small fractions of B-lineage lymphocytes (Chaudhary and Wesemann 2018; Pabst et al. 2015; Turchaninova et al. 2016). Sequencing of adaptive immune receptor repertoires (IGs and TCRs) may elucidate the highly complex adaptive immune response in teleost. The analysis of immune repertoire sequences and their quantitative composition on a nucleotide-level within and across individuals shed light on the dynamics of adaptive immune cells in healthy conditions or during an immune response.

The analysis of IG repertoire by Weinstein et al. (2009) reported that in adult naive zebrafish, where the total number of antibody-producing B cells are less than half million (a small number compared to an estimated 10^{12} cells in humans), a large portion of potential VDJ rearrangements is indeed present in the available IgM repertoire. The number of unique VDJ sequences for IgM were estimated between 1200 and 3500 per fish, most of them were presented at low frequency, and a few of them were found to be highly abundant. Using the same animal model and sequencing technology Jiang et al. (2011), described the structure of zebrafish IG repertoire across development. At early stage (2 weeks old fish), authors found a highly stereotyped state with preferential use of a small number of V, D, and J gene segment combinations, and frequent VDJ combinations shared between different fish. This stereotypy decreases dramatically as the zebrafish mature. Authors suggest that the main process that causes differentiation between adult repertoires is apparently random clonal expansion. In both studies, RNA was obtained from full organisms, but results should be treated with caution as it cannot be stated that the complete IG repertoire was characterized. Thus, RNA capture efficiency, sequencing depth (the platform used was 454 sequencing), the accuracy of IGH locus annotation, and the fact that there are no data about IGL chain or IGHD chain data, and further efforts are required to achieve this challenge.

Deeper sequencing platforms (mainly Illumina technology) with outputs ranging from 15Gb and 25 million reads (Illumina MiSeq) to 6000Gb and 20 billion reads (NovaSeq) are currently available. This technology is applied to decipher the humoral adaptive immune response in important species for aquaculture, such as Atlantic salmon, rainbow trout, and

takifugu (Castro et al. 2013; Fu et al. 2018; Krasnov et al. 2017; Magadan et al. 2018). A common feature is the presence of identical heavy chain sequences among several fishes, as described in mouse or human (Galson et al. 2015). This observation indicates that repertoires are not simply determined by equally likely random rearrangements of IG V (D)J gene segments. Thus, certain receptors might be shared between unchallenged controls simply due to their high generation probability.

The analysis of IG repertoires from virus-immunized rainbow trout showed modifications that persist at least for 5 months, with expanded public and private clonotypes (Castro et al. 2013; Magadan et al. 2018). Analysis of trout public responses suggested that repeated immunizations with a heterologous antigen do not lead to a significant attrition of pre-existing responding B cells specific for the primary vaccination (Navelsaker et al. 2019). Same technology was recently applied to characterize the nasal B-cell repertoire in rainbow trout, in which well-organized mucosal-associated lymphoid structures such as tonsils and adenoids are absent (Sepahi and Salinas 2016; Tacchi et al. 2014). In rainbow trout, nasal IgM and IgT repertoires comprise both low frequency and highly expanded clonotypes, being the nasal IgT repertoire dominated by expanded clonotypes Magadan et al. 2019a, b). Interestingly, these characteristics are reminiscent of those described for IgM and IgA mucosal repertoires in humans and mice (Holtmeier 2000; Lindner et al. 2012, 2015).

New teleost genome assemblies are coming, and they will provide a rich source of knowledge for the comparative immunology community, to address the mechanisms of gene conversion, somatic hypermutation, and memory in these species and during vertebrate evolution. In this sense, data available for Atlantic salmon have been recently used to establish a unique and consistent standardized nomenclature of salmonid IGH genes. These efforts led to the elaboration of a sequence reference directory at the international ImMunoGeneTics information system (IMGT, www.imgt.org) that allows the accurate annotation of rainbow trout and Atlantic salmon IG deep sequencing data (Magadan et al. 2019b). However, the full assembly and annotation of IG remain challenging, and more efforts are required to improve accuracy of annotation of adaptive immune receptor repertoire dataset generated by high-throughput sequencing, and to facilitate comparisons between studies and species. Furthermore, most bioinformatics tools for decoding immune receptor repertoires data have been developed for human and mice, and they have to be adapted to other models of interest, such as teleost.

7.4 Teleost Ig Classes: An Overview on Functional Roles

Compared to mammals, a smaller number of antibody classes have been reported in lower vertebrates. In cartilaginous fish, three heavy chain isotypes have been detected, which lead three IG classes: IgM, IgD/W, and IgNAR (Bengtén and Wilson 2015). In teleost fish, three different Ig classes have been identified: IgM, IgD, and IgT/Z. However, not every class is expressed in each fish species studied (Bengtén and Wilson 2015; Fillatreau et al. 2013).

Furthermore, the antibody classes expressed in the few extant species of lobe-finned bony fish seem to be different from those found in ray-finned bony fish. No IgT/Z has been identified in the African lungfish (*Protopterus annectens and Protopterus aethiopicus*), and both IgM and IgT/Z seem to be absent in the African coelacanth (*Latimeria chalumnae*) (Amemiya et al. 2013; Zhang et al. 2014).

7.4.1 IgM

The IgM class has long been considered the most ancient and was the first Ig class identified in fish. It can be expressed at the surface of B cells or secreted. Secreted tetrameric IgM is the most prevalent serum Ig in fish (Flajnik 2018, 2002), with a concentration between 800 and 9000 μg/mL (Uchida et al. 2000). While in mammals the IgM is mainly secreted as a pentameric form, teleost IgM is a tetramer apparently formed in the absence of the Ig joining (J) chains. These IgM tetramers are secreted at systemic and mucosal level (Elcombe et al. 1985). They are found in different oxidation states that depend on the degree of interdisulfide bond formation among the IgM monomers (Kaattari et al. 1998; Ye et al. 2013). Variability in the degree of inter-heavy chain disulfide polymerization has been observed in more than 15 species (Bromage et al. 2004; Dacanay et al. 2006; Kaattari et al. 1998; Morrison and Nowak 2001; Uchida et al. 2000).

In rainbow trout, the simultaneous structural analyses of mucosal and systemic antibodies (Bromage et al. 2006), suggested that mucosal IgM has considerably less disulfide polymerization than serum IgM. Additional studies also reported that antigen-binding affinity and the Ig half-life are associated with levels of polymerization (Kaattari et al. 2002; Ye et al. 2010). Via an in vitro antigen-driven selection experiment, Ye et al. (2011) shown that the BCR affinity for the inducing antigen affects to post-translational modification that results in both increased polymerization and glycosylation. These authors proposed an integrated model where the antigen-sensitive B lymphocyte operates as an affinity-based transducer, enabling each B cell to modulate the structure of its antibody in order to optimally suit its function with its affinity for the inducing antigen (Ye et al. 2011). The effector functions activated by fish IgM include complement activation, antibody-dependent cellular cytotoxicity (ADCC), and phagocytosis through effector cells (Lobb and Hayman 1989; Mashoof and Criscitiello 2016; Shen et al. 2003). Several studies also reported serum and mucosal noncovalently associated IgM monomers in different fish species, such as the grouper, *Epinephelus itaira*, the sheepshead, *Archosargus probatocephalus*, and rainbow trout, *O. mykiss* (Clem 1971; Clem and McLean 1975; Lobb and Clem 1981; Lobb and William Clem 1981). However, further research is required to shed light on monomeric IgM functionality.

7.4.2 IgT

In 2005, a new teleost fish immunoglobulin isotype was identified. In rainbow trout, this Ig was named IgT (Hansen et al. 2005), whereas in zebrafish it was called IgZ (Danilova et al. 2005). This Ig has been identified in most of studied teleost fish, except in medaka (Magadán-Mompó et al. 2011), in channel catfish (Bengtén et al. 2006), and in turquoise killifish (Bradshaw and Valenzano 2020). The recent analysis of genomes from 10 species belonging to Cyprinodontiformes order also suggests the absence of IGHZ/T exons in *A. austral* or *N. orthonotus* (Bradshaw and Valenzano 2020). These results need further confirmation when higher-quality genome sequences from these two species are available.

Most research on IgT has been developed in rainbow trout. In this host, at least three subclasses are expressed (Zhang et al. 2017). At transcriptional level, IgT1 subclass is expressed both in mucosal and systemic lymphoid tissues; IgT2 seems to be mainly expressed in systemic lymphoid organs. Zhang et al. (2017) reported IgT3 protein in rainbow trout serum level. Previous studies performed by Oriol Sunyer and collaborators (Zhang et al. 2010), provided a detailed characterization of rainbow trout IgT and revealed its function in mucosal immunity. It was shown that while plasma IgT is a monomeric Ig (~180 kDa), gut mucus IgT is polymeric (4–5 monomers) formed in absence of J chain. In the same study, a previously unknown IgT+ IgM- IgD- B-cell lineage was identified. This B-cell subset constitutes the first vertebrate B-cell lineage devoid of surface IgD expression. In addition, authors identified a polymeric Ig receptor in rainbow trout (tpIgR) whose putative secretory component was found associated with gut mucus but not serum IgT and IgM (Zhang et al. 2010). This result strongly suggested that like in mammals, pIgR in fish is involved in the transport of polymeric IgT and IgM from the mucosal epithelium into the gut lumen.

Although IgA is absent in lower-jawed vertebrates, a series of studies showed that amphibians and bony fish express specialized mucosal antibody isotypes (IgX in amphibian, and IgT/Z in bony fish), independently, by convergent evolution (Du et al. 2012; Mussmann et al. 1996). In this sense, IgT represents the most ancient vertebrate mucosal Ig identified to date. Its concentration in serum is much lower, approximately 1000 times less, than that of IgM, but in mucus the IgT/IgM ratio is almost 100 times higher than in serum. Results reported in different studies show that secreted IgT is highly induced at mucosal surfaces by pathogens and vaccines (Buchmann 2020; Magadan et al. 2019a, b; Tacchi et al. 2014; Xu et al. 2013, 2016; Yu et al. 2018; Zhang et al. 2010). In addition, secreted IgT seems to be the predominant Ig isotype coating a large portion of the fish microbiota (Xu et al. 2016, 2020). A recent study, Xu et al. (2020) have shed more light on the specialization of secreted Igs in protection of mucosal tissues from pathogens and, in the establishment of healthy microbiota. To address this question, an elegant teleost fish (rainbow trout) model was developed, in which the secreted IgT in adult fish was selectively depleted with the injection of IgT monoclonal antibodies and rainbow trout antiserum against mice IgG. Then, authors performed the analysis of microbiome composition and parasite infections, to evaluate the specific contribution of IgT on mucosal tissue

homeostasis, and in the protection of fish mucosal tissues against pathogens, respectively. In rainbow trout, the IgT depletion induced the loss of IgT-coated beneficial taxa, expansion of pathobionts, tissue damage, and inflammation. In addition, an increased susceptibility of fish to the mucosal parasite *Ichthyophthirius multifiliis*, without developing compensatory IgM responses, was observed. These results support further the notion that IgT and IgA are phylogenetically distant immunoglobulins that specialized in mucosal immune responses and reveal the existence of primordially conserved principles by which mucosal immunoglobulins control both pathogens and microbiota. Moreover, in mammals secreted IgA seems to be less essential in control of mucosal pathogens than IgT in rainbow trout. In patients with selective-secreted IgA deficiency, there is a modest increased susceptibility to respiratory and intestinal infections (Yel 2010). In mammals, the class switch recombination process, which is not present in fish, may allow different class antibodies (i.e., IgM, IgD, and IgA) to share their antigen-binding sites. So, in IgA-deficient individuals, there may be compensatory mechanisms mediated by IgM or IgD that would prevent disease manifestations in some IgA-deficient individuals (Choi et al. 2017; Yel 2010).

7.4.3 IgD

IgD was first discovered in human serum in 1965, and it was considered a recently evolved Ig class expressed in mammals. However, IgD was found in other vertebrates, including teleost, suggesting it may be as old as IgM (Mashoof and Criscitiello 2016; Ohta and Flajnik 2006; Wilson et al. 1997). In 1997, bony fish IgD was identified in the channel catfish, as Ig with a chimeric heavy chain containing a rearranged variable domain, the first constant domain of mu ($C\mu1$), and seven constant domains encoded by a delta gene ($C\delta$) (Wilson et al. 1997). A similar splicing pathway was retrieved in Atlantic salmon (Hordvik 2002; Hordvik et al. 1999), and in other fish species (Gambón-Deza et al. 2010; Susana Magadán-Mompó et al. 2011; Stenvik and Jørgensen 2000; Wang et al. 2016). The chimeric $C\mu1$-$C\delta$ structure has been also described in pigs and other Artiodactyls (Zhao et al. 2002, 2003) and may facilitate the formation of a covalent bond with the light chain. Fish IgD differs from eutherian IgD by a large number (7–17) of $C\delta$ domains and by the absence of a hinge. In contrast to IgM, IgD displays marked structural plasticity, which can be explained by different underlying mechanisms, such as diverse copy number of IgD-codifying gene, different number of germline $C\delta$ exons, and the expression of various splicing forms.

The specific functions of IgD have only recently begun to be elucidated (Gutzeit et al. 2018). Interestingly, secreted form of IgD has been identified in mammals, catfish, and trout, and its structure is different (Bengtén et al. 2006; Mountz et al. 1990; Ramirez-Gomez et al. 2012). In humans, secreted IgD can be detected in the circulation, nasopharyngeal, oral and lachrymal secretions (Chen et al. 2009; Gutzeit et al. 2018; Sun et al. 2020). Moreover, in the upper respiratory tract, the secreted IgD binds to basophils and

mast cells, and activates these cells to produce antimicrobial, opsonizing, proinflammatory factors. The cross-linking of basophil-bound IgD by an isotype-specific antibody triggers the IL4 cytokine and thereafter the stimulation of B cells (Chen et al. 2009). These results suggest that IgD orchestrates an immune surveillance system at the interface between innate and adaptive immune systems. In 2010, Edholm et al. (2010) found that all catfish-secreted IgD transcripts from IgM+/IgD+ and IgM−/IgD+ B cells lacked the variable domain, and began with a leader spliced to Cδ1. The authors concluded that IgM−/IgD+ B cells most likely expand in response to certain pathogens, and the secreted V-less IgD Fc region may function as an innate pattern recognition molecule. These results also support the dual nature of the IgD molecule, which may represent an early transition of the innate pattern recognition receptors to adaptive Ig molecules.

In contrast to what was found in catfish, rainbow trout-secreted IgD is expressed with a V domain (Ramirez-Gomez et al. 2012). The rainbow trout-secreted IgD transcripts were found at the highest levels in the spleen, followed by the blood and gills (Ramirez-Gomez et al. 2012). Moreover, rainbow trout-secreted IgD has been shown to coat gill microbiota, albeit at a significantly lower level than secreted IgT, and seems to have the ability to interact with the pIgR, which is required for its mucosal transport to the gill. This is the first description of a pIgR being implicated in IgD secretion in a vertebrate respiratory tract (Xu et al. 2016).

7.5 Conclusion

With very different lymphoid anatomical structures and microenvironments, teleost and mammals provide a very good subject for comparative approaches to distinguish fundamental conserved properties of adaptive immunity and convergent adaptations driven by the necessity to fight pathogens in critical tissues or at entry points. With the development of new reagents and deep sequencing technologies, there has been substantial advancement in our knowledge on the structural characteristics and effector functions of immunoglobulins present in teleost. We get more insight into the plasticity of teleost IG gene loci and repertoires. Comparative studies will provide important insights about the interplay between environment and the evolution of the adaptive immune system in vertebrates.

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B-Cell Responses and Antibody Repertoires in Teleost Fish: From Ag Receptor Diversity to Immune Memory and Vaccine Development

8

J. Oriol Sunyer and Pierre Boudinot

Abstract

This chapter reviews the particular features of teleost fish immunoglobulins, their B cells, and B-cell repertoires. We first review the current knowledge regarding teleost Ig genes, Ig proteins, and B-cell populations in the context of the diversity of this taxonomic group, the largest among vertebrates. We then describe how the development of sequencing technologies have fostered populational approaches in the field of B-cell biology, and how they have been used to start unraveling the complexity of teleost B cell and antibody (Ab) repertoires. Such approaches are particularly relevant to better understand the modifications of Ab repertoires after immunization, infection or vaccination. Moreover, application of these technologies will be instrumental in elucidating the mechanisms that determine induction of systemic versus mucosal B-cell responses. In this chapter, we also describe the current knowledge on the maturation of teleost B-cell responses and thus, provide a synopsis of the development of B-cell memory in different model species. Finally, we discuss unresolved key questions regarding B-cell populations and their respective responses in innate and adaptive immunity, and we propose critical future directions of research in this field.

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Abbreviations

Ab	antibody
Ag	antigen
CDR	complementary determining region
ERM	enteric red mouth
IFN	interferon
Ig	immunoglobulins
IPNV	infectious pancreatic necrosis virus
PMCV	piscine myocarditis virus
SRBC	sheep red blood cells
TNF	tumor necrosis factor
VHSV	Viral hemorrhagic septicemia virus

8.1   Introduction

B lymphocytes constitute a major arm of the immune system, with key roles both in innate and adaptive immune responses (McHeyzer-Williams 2013). As part of innate immunity, subsets of B cells in fish, amphibians, reptiles, and mammals have been shown to display a high phagocytic capacity (Parra et al. 2012), and express a number of important innate immune molecules, including antimicrobial peptides. As part of adaptive immunity, B cells are responsible for antibody (Ab) production and humoral immunity. B cells also produce "natural" polyreactive antibodies (Abs) that are capable of recognizing multiple distinct antigens. Moreover, B cells are also involved in the regulation of innate and adaptive immunity through the production of anti-inflammatory cytokines such as IL10 and IL35 (Fillatreau 2019), as well as proinflammatory, including TNF, IL-1, IL-6, IL-8, and IFN, among others (Lund and Randall 2010).

Ag-specific B-cell responses are mediated by their Ag-specific receptor, the Abs, which are encoded by the immunoglobulin (Ig) genes. Each Ab molecule is made of two identical light (L) and two identical heavy (H) chains. A large number of B cells—each of them bearing only one type of Ag-specific receptor—are present in the organisms of all jawed vertebrates (Flajnik and Du Pasquier 2013). After immunization, the Ag is recognized by a small fraction of B cells via their Ag-specific receptors. Subsequently, these lymphocytes get activated, proliferate, and differentiate into Ab-secreting cells, thus mounting an

Ag-specific response. The capacity of an individual to respond specifically to every possible Ag requires a large number of pre-existing Ag receptors, expressed by naive B cells. This large diversity of Abs is achieved through several mechanisms. First, the somatic recombination of Ig gene segments (V, J for the light chain or V, D, and J for the heavy chain) leads to the assembly of an Ig gene encoding the complete V domains of each Ab chain, during the differentiation of the lymphocyte. This mechanism, called V(D)J recombination, brings together targeted V, (D), and J genes from the germline DNA (Tonegawa 1983). The combinatorial association of a large number of V, D, and J genes present in Ig loci leads to a staggering number of combinations for genes encoding L and H Ig chains. There is also a junctional diversity at each V(D)J joint, resulting from the insertion or deletion of nucleotides (*i.e.*, N and P diversity). Across B-cell populations, these processes induce a very high diversity of the VDJ junctional region, which encodes a loop involved in the Ag binding. This loop is known as the complementary determining region-3 ("CDR3") and its diversity largely contributes to the immune repertoire (Wu and Kabat 1970; Lefranc and Lefranc 2001). The term "immune repertoire" was introduced in immunology by Niels Jerne to describe the diversity of Abs: "we must distinguish the potential repertoire of specificities that could arise given the genetic constitution of the zygote from which the animal develops, and the available repertoire embodied in the cells that can respond to antigens at a given moment in the life of the animal" (Jerne 1972). While in the 70s, the genetic basis of the diversity of Ag-specific receptors was still unknown; this dual concept of potential/available repertoire became later very useful to integrate Ab diversity at the genomic and molecular levels across lymphocyte populations.

Similar to B cells of all tetrapods, those of teleost fish also express Ig at their surface (i.e., BCR) and secrete immunoglobulins. While mammals produce five classes of Abs (i.e., IgM, IgD, IgA, IgG, and IgE), teleosts express only three (i.e., IgM, IgD, IgT). Fish Ig genes undergo VDJ rearrangements through similar mechanisms as those described in mammals and mediated by homologous enzymes (i.e., RAG1/2). Teleost Abs mediate protection against a number of pathogens, including parasites, viruses, and bacteria (Salinas et al. 2011). In addition, their antibodies recognize and coat a large portion of their microbiota and play a key role in their homeostasis (Zhang et al. 2010). However, there are a number of mechanisms that remain ill-defined in fish, including the details of fish B-cells differentiation and tolerance checkpoints. The mechanisms of allelic exclusion also remain unknown in these species. In mammals, the Ab class produced by a B cell is determined either by alternative splicing (for IgM/D), or by recombination events in the region of the IGH locus that encodes the constant domains of the different isotypes. In teleosts, the mechanisms are different: alternative VDJ recombination events typically determine that the B cells produce either IgM/D or IgT. IgM and IgT expressions therefore define two distinct B-cell lineages as have been experimentally demonstrated with the findings of B-cells subsets that specifically express either IgM or IgT (Hansen et al. 2005; Zhang et al. 2010). While systemic antibody responses in teleosts are overwhelmingly represented by the IgM Ab class, IgT is specialized in mucosal protection (Zhang et al. 2010; Salinas et al. 2011). It is worth pointing, however that some fish species such as the

Channel catfish (*Ictalurus punctatus*), do not seem to express IgT while using IgM and other mechanisms for mucosal protection. Well-defined subtypes of B cells have been described in mammals which express different Ab repertoires and Ig isotypes, and are found in various anatomical locations. Depending on their localization and Ig expression, these B-cell subtypes (e.g., B1/B2 cells, follicular B cells, marginal zone B cells) have become specialized in different roles (see Hardy 2013). However, no clear counterparts have been defined to date in fish, although there have been attempts to assign some fish B-cell subsets to those defined in mammals. Other fundamental issues that remain poorly defined in fish are the mechanisms of hypermutation, affinity maturation, and memory. While the enzymatic machinery for somatic hypermutation (i.e., AID) is present in fish genomes and Ig sequences are diversified after VDJ recombination to some extent (Jiang et al. 2011), the control and location of this process in fish have not been clearly described yet. Regarding B-cell differentiation, the main transcription factors involved in in this process, from the precursors to plasma cells in mammals, appear to have homologs in teleosts; thus, B-cell differentiation is anticipated to be controlled in a rather comparable manner to that of mammals (Zwollo 2011). However, the phenotype and the biology of teleost fish memory B cells remain elusive. Most critically, due to the apparent lack of specialised, organized lymphoid tissues (e.g., germinal centers, Peyer's patches), the mystery remains as to sites and lymphoid tissues in which the antibody responses are induced in these species.

Overall, here we will examine the particularities of teleost fish B-cell responses with a focus on repertoire analysis. We emphasize the complexity of the teleost B-cell system and its importance for immune defense, and discuss unresolved questions and future directions of research.

8.2 The Particularities of Fish Antibodies and Fish B-Cell Responses Across the Diversity of Teleost Fish Species

8.2.1 Teleost Fish Are Very Diverse

Bony fishes are the largest group of vertebrates with more than 26,000 extant species (Helfman et al. 2010), and one of the great evolutionary successes of this lineage. Teleosts represent 95% of bony fish species. They have colonized most aquatic environments including extreme habitats. This brought teleosts in contact with highly diverse pathobiomes, which constitute a major selection pressure on B-cells responses. It also induced a broad anatomical and physiological variation. Teleost fish are adapted to a broad range of temperatures, which are an important parameter for B-cell responses and their mechanisms. Temperature and its variations affect differentially B- and T-cell responsiveness, and regulate the efficiency of cell activation as shown in the channel catfish (Bly et al. 1988). Differential homeoviscous adaptation of the membranes of teleost B and T lymphocytes to temperature may actually explain seasonal variations of immunity

(Bly et al. 1988). Body size, total number of lymphocytes, ploidy level, and lifespan are other key parameters affecting clonal complexity and dynamics of their B-cell repertoires. From 8 mm gobies and *Danionella* to large sunfishes, swordfishes, and tunas, bony fishes recapitulate the tadpole/elephant paradox (Langman and Cohn 1987): the diversity of this very large zoological group raises the issue of the qualitative and quantitative differences between the Abs responses of the "units of protection" when comparing small with very large species.

Bony fishes also constitute the class of Vertebrates in which whole-genome duplications (WGD) have been the most frequent. While two rounds of WGD have largely expanded the gene repertoire of vertebrate ancestors as first proposed by Ohno (Ohno 1970), one more WGD occurred in the first teleosts (Jaillon et al. 2004), and several additional WGD events later affected several fish families including cyprinids and salmonids. Teleost fish inherited the basic components common to immune systems of jawed vertebrates, but how additional WGD and genome contractions did affect Ig gene rearrangements, somatic selection, and signaling pathways of lymphocytes in different fish groups remains an open question. The particularities of the fish Major Histocompatibility Complex (MHC) illustrate how WGD and genome recombinations could impact the genomics of immunity in these species (see Chap. 11). Strikingly, the MHC class II pathway has been lost independently in several distant groups, with a major impact on B-cell immunity: in cod and gadidae (Star and Jentoft 2012), in pipefish and seahorses with male pregnancy (Haase et al. 2013), and in anglerfish with male parasitism (Dubin et al. 2019). In anglerfish species, the degree of sexual parasitism from male/female temporary association to permanent fusion with several males is aligned with an increased range of immune gene loss: in the most extreme adaptations, the loss of key genes mediating Ig/TCR rearrangements and B/T cell function, including *igm, cd79, aicda, cd8, tcr,* and even *rag1* and *rag2* disrupted adaptive immunity (Swann et al. 2020). In fact, fish immune responses have mainly been studied in a few farmed species, with a focus on vaccination. Thus, more surprises likely await fish (B-cell) immunologists in the immense diversity of this group.

8.2.2 Teleost B Cells: Subsets and Specializations

8.2.2.1 Fish B Cells Have Typical Features Conserved Across All Jawed Vertebrates

B cells can be broadly defined as lymphocytes expressing immunoglobulins. As in mammals, fish B cells appear to differentiate from hematopoietic stem cells through a developmental program involving canonical transcription factors including Pu-1, E2A, EBF, and Pax5 (Zwollo 2011). After Ag encounters a B cell and binds its membrane Ig, the cell gets activated and can differentiate into a Ab-secreting cell/plasma cell, via a program that appears to be regulated by the transcription factors BLIMP and XBPL (Zhang et al. 2010; Zwollo 2011). Fish B-cell responses can be triggered by the recognition of repetitive Ags, or may require a cooperation with CD4^{+} T lymphocytes. In the latter case, antigenic

peptides presented by MHC class II molecules at the B-cell surface are recognized by the TCR of CD4$^+$ T cells, which in turn secrete soluble factors stimulating the B cell (see also Chap. 3). The first demonstration of B/T cell cooperation in fish was performed with hapten-carrier systems using purified IgM$^-$ and IgM$^+$ channel catfish leukocytes (Miller et al. 1985). Importantly, the capture and presentation of antigens by B cells are greatly increased by their phagocytic capacity, which was discovered first in fish (Li et al. 2006), but appear to be a property of some B-cell subsets across vertebrates (Parra et al. 2012).

8.2.2.2 Fish B-Cell Subsets Have Not Been Fully Characterized and Defined

In the mouse, phenotypic and functional properties define several subsets, including marginal zone B cells (mainly in the marginal zone of the spleen), follicular B cells (circulating in the blood and located in lymphoid follicles of secondary lymphoid organs), and B-1 cells (natural Abs producers, especially abundant on the peritoneal and other serosal cavities). In contrast, there are no such classifications of B cells in fish, partly due to the difficulty to obtain good mAbs against surface markers of these species. However, two fundamental fish B-cell lineages (Zhang et al. 2010) are defined by the expression of IgM/D versus IgT at the cell surface, and exert different functions: systemic (central) responses for IgM$^+$ B cells, and mucosal defense for IgT$^+$ B cells (see below). Finally, while orthologs of IL10, p35, and ebi3 are present in fish genomes, regulatory B cells producing these immunosuppressive cytokines have not been described thus far.

8.2.2.3 The Question of B1 Cells

In laboratory strains of inbred mice, B cells are classified into B1 cells (comprising CD5$^+$ B1-a and CH5$^-$ B1b) and B2 cells. B2 cells mount specific Ab responses against foreign Ags, and form follicles in secondary lymphoid organs while B1 cells produce for the most part natural Abs in a T-cell-independent manner (Fagarasan and Honjo 2000), and are particularly abundant in the peritoneal cavity. It has been proposed that B1 and B2 cells correspond to distinct lineages arising from different progenitor populations (Herzenberg et al. 1986). However, lineage commitment of B1 and B2 precursors before expression of their Ag receptor has remained controversial (Kreslavsky et al. 2018). Recently, an elegant transgenic mouse model in which the B-cell receptor specificity could be changed showed that mature B2 cell can redifferentiate into functional B1 cells when their surface Ig is replaced by the B-cell receptor from a B1 cell (Graf et al. 2019). These findings supporting a "selection model" were consistent with the lack of evidence of a true B1 lineage in other species, including humans, where the presence of such a lineage remains highly controversial. In fish, expression of natural Abs has been reported in several species (Sinyakov et al. 2002; Kachamakova et al. 2006; Magnadottir et al. 2009) but their characteristics, and the cells by which they are secreted remain undefined. It has been reported that trout IgM$^+$ B cells express a receptor related to CD5 (Abos et al. 2018), and the same report suggested that all fish IgM$^+$ B cells resemble mammalian B-1 B cells. In fact, it is important to note that the expression of CD5 alone does not define a B1-cell population. For instance, murine B1b B cells do not express CD5. Moreover, most outbred wild-derived mouse strains do

not have a CD5$^+$ B1a-cell population at all; in fact, besides B2 cells, a cell subset named Bw that is conserved during the evolution of the *Mus* genus, is clearly distinct from B1 from a functional point of view (Thiriot et al. 2007). In contrast, in rabbits essentially all peripheral B cells express CD5. Thus, the presence of CD5 on a B cell is not a good predictor of being a B1 B cell homolog. Cell size and complexity are not definitive criteria either for possible fish B1 cells especially in tetraploid species such as salmonids, since the size of these cells will be influenced by the ploidy of the species, with bigger cells correlating with a higher ploidy level. Finally, antibodies produced by B1 B cells are induced in a T-cell-independent (TI) fashion (Fagarasan and Honjo 2000), while teleost B cells typically respond against many antigens in a T-cell-dependent manner (Miller et al. 1985); also, teleost CD4 T cells have clearly been shown to proliferate in an antigen-specific manner (for example, Takizawa et al. 2016). Defining an equivalent of B1-like cells in fish would require the verification of several key features of these cells: first, the characterization of their Ag-specific receptors and their reactivity, to support the existence of a particular pathway of differentiation toward cells with "innate-like" functions; second, the localization of such cells mainly in serous areas, including the peritoneum, which are the areas in which such cells are overwhelmingly found in laboratory mice, the only vertebrate species in which B-1 B cells have been unambiguously defined so far; third, that this particular B-1-like cell subset produces IgM in a T-cell-independent manner. The argument of whether fish contain B-1 B cells may not be a relevant one as such cells might well be an oddity of laboratory inbred mice. Perhaps a more pertinent question is whether fish contain specific subpopulations of "innate-like" B cells both in the IgM$^+$ and IgT$^+$ B-cell lineages. Thus, future studies should address whether such subsets can be identified in these lineages, with the goal to define how these "innate-like" B-cell subsets are phenotypically and functionally different from those that are specialized in adaptive immune functions.

8.2.3 Fish Ig Isotypes, Their Physicochemical Properties, Functions, and the Lack of Switch

Most studied teleost species express three Ig classes, including IgM, IgD, and IgT, while a few appear to lack IgT (Fillatreau et al. 2013). IgM is by far the most abundant Ig in fish where its concentration in plasma is in the range of ~0.6–16 mg/ml, depending on the fish species (Salinas et al. 2011), as well as a number of biological and environmental conditions such as fish age, water temperature, seasonality, stress, infection, and vaccination, among others (Solem and Stenvik 2006). Teleost IgM, like that of mammals, is a high molecular weight (HMW) Ig of ~600–850 kilodaltons (kDa) (Salinas et al. 2011). However, unlike the pentameric mammalian IgM, that of teleosts is tetrameric, and under denaturing conditions is present in various redox forms that vary in the degree of interheavy-chain disulfide polymerization (Kaattari et al. 1998; Bromage et al. 2004a, b). Interestingly, an association between an increased disulfide polymerization and a greater

affinity of trout IgM to antigen has been found (Ye et al. 2010). It is worth mentioning that a low molecular weight (LMW) Ig has been described in the serum of several teleosts, although in most of these studies, the lack of sufficient biochemical tools makes it difficult to define to which Ig class these LMW Igs belong to (Salinas et al. 2011). It has been shown that the half-lives of both HMW and LMW Ig of teleost serum are ~12–16 days (Avtalion et al. 1973; Lobb and Clem 1981). IgD in teleosts was first identified in 1997 (Wilson et al. 1997), and has been shown to be present in all analyzed species (Mashoof and Criscitiello 2016). As for IgM, it is present both as membrane and soluble form. When secreted, IgD has been shown to display several molecular masses depending on the fish species (see Edholm et al. (2011) and Ramirez-Gomez et al. (2012) for the biochemical properties behind such MW differences). The serum concentration of IgD (~2–80 μg/ml) is much lower than that of IgM, although in the range of that found for IgT (Parra et al. 2016). The gene encoding the H chain for IgT was first discovered in 2005 in the genomes of rainbow trout (Hansen et al. 2005) and zebrafish (Danilova et al. 2005). This Ig has been identified in almost all studied teleosts except the catfish. Although IgT has been much less studied than IgM, serum IgT is a monomer with a molecular mass of ~180 kDa (Zhang et al. 2010). Interestingly, mucus-derived IgT has been shown to be a tetrameric or pentameric molecule in which all their monomeric units are bound through noncovalent forces (Zhang et al. 2010; Xu et al. 2013, 2016). Although very few studies have evaluated the biochemical properties of teleost IgT, it appears that the concentration of IgT in serum, is low (~3.7–11 μg/ml) (Zhang et al. 2010; Xu et al. 2013, 2016; Kong et al. 2019; Yu et al. 2019). In rainbow trout, it has been shown that the basal concentration of serum and mucus IgT increases several fold upon exposure of fish with different pathogens, while that of IgM only increases in serum (Zhang et al. 2010; Xu et al. 2013, 2016; Yu et al. 2018, 2019; Kong et al. 2019). In contrast, trout IgD levels remain unchanged in the serum or mucus upon stimulation with the same pathogens [for more in- depth physicochemical properties of fish Igs, please see (Salinas et al. 2011; Parra et al. 2016)].

With regards to the functional roles of fish Ig, up until recently it was thought that IgM was the only fish Ig responding to antigenic challenge (i.e., pathogens and vaccines). However, in 2010, IgT was reported to be an Ig specialized in mucosal immunity (Zhang et al. 2010) and responding also to antigenic challenge. More specifically it was shown that Ig responses against a gut parasite were overwhelmingly IgT-based (Zhang et al. 2010). In addition, and analogous to the role of IgA in mammals, it was shown that IgT was the main Ig coating a large portion of the trout gut microbiota (Zhang et al. 2010; Donaldson et al. 2018). Subsequent studies in several fish species supported the prevalent role of IgT in mucosal surfaces (see Chap. 12 for additional information on the IgT in mucosal sites). In these studies, it became also evident that IgM responses were prevalent in the systemic compartment (i.e., responses detected in the fish sera), albeit they could also be detected in some mucosal sites, although at a much lower level when compared to those of IgT (Zhang et al. 2010; Xu et al. 2013, 2016; Yu et al. 2018, 2019; Kong et al. 2019). In addition, these studies also showed that IgM coated a smaller, but significant portion of the microbiota when compared to IgT (Zhang et al. 2010; Xu et al. 2013, 2016; Kong et al. 2019; Yu et al.

2019). A recent study performed on fish transiently depleted of IgT confirmed the funda-mental roles of IgT both in the control of pathogens at mucosal sites, as well as in the homeostasis of the microbiota (Xu et al. 2020). Similar to mammalian IgM, fish IgM has been shown to have agglutinating and neutralizing activities against bacterial and viral pathogens respectively, and has been demonstrated to activate the classical pathway of complement upon binding to pathogens (Ye et al. 2013; Mashoof and Criscitiello 2016). Whether IgT have similar effector capabilities, remains to be demonstrated. The role of IgD in fish in immunity remains elusive, although in 2016 IgD was shown for the first time to coat a small but significant portion of the gill microbiota (Xu et al. 2016). Subsequent studies showed that IgD coated a portion of the microbiota in different mucosal surfaces (Kong et al. 2019; Yu et al. 2019; Xu et al. 2020), and thus, it has recently been hypothesized that it may play a role in the regulation of the IgD-coated fraction of the microbiome (Perdiguero et al. 2019). In contrast, several recent studies have shown the absence of IgD-specific titers both in serum and mucus of fish exposed to a variety of pathogens (Xu et al. 2016; Yu et al. 2018, 2019; Kong et al. 2019). Thus, such studies strongly argue against a role for IgD in adaptive immune responses to pathogens.

With regards to membrane Ig, fish B cells typically express either IgM/D or IgT. There is no class switch in fish, but alternative rearrangements of V to corresponding—isotype-specific—genomic DJ segments (see Chap. 7 for a genomic view of fish Ig genes). It is important to note that this pathway leads to populations of B cells expressing distinct IgM/D or IgT repertoires. Thus, and in contrast to mammals where class switch generates IgG or IgA repertoires from a primary IgM repertoire, fish IgM and IgT repertoires emerge independently through parallel VDJ recombination in different cells. This has critical implications for the functional specialization of IgM$^+$ versus IgT$^+$ lymphocytes.

The steps of early B-cell differentiation in fish remain ill-defined. No pre-B-cell receptor has been described, and it is still unclear whether heavy-chain and light-chain loci rearrange sequentially following an ordered model as in mice and humans. Importantly, mechanisms of allelic exclusion are also unknown. How such mechanisms guarantee the expression of a single Ig rearrangement per B cell is particularly intriguing in the case of salmonids and other quasi- tetraploid species, where the IGH locus is duplicated: four loci are available for rearrangement in each developing B cell in these species. Similarly, receptor editing and secondary rearrangements have not been described yet, and the validity of the two tolerance checkpoints described in mammalian models remains to be established in fish.

8.3 Clonal Complexity of Expressed B-Cell Repertoires in Fish at Steady State: A Population View of B-Cell Immunity

8.3.1 Methods Used to Analyze Clonal Complexity and Repertoires

In 90s, DNA sequencing systems running fluorescent Sanger reactions in acrylamide gels provided to immunologists the possibility to record the size profile of V/C or V/J PCR products. Immunoscope and CDR3-length spectratyping techniques were based on the aforementioned acrylamide methodology and were thus developed to produce global images of the diversity of Ig(/TCR) repertoires and their variations upon infections (Pannetier et al. 1993; Desravines and Hsu 1994; Gorski et al. 1994). Spectratyping studies could visualize IG or TCR responses, showing profiles of junction size distribution with quasi-monoclonal peaks, corresponding to clonal expansions (Pannetier et al. 1993). However, these techniques only provided information about CDR3 length distribution, that is, getting access to the sequence information behind the profiles required cloning and Sanger sequencing, which was very labor-intensive (Arstila et al. 1999). High-resolution and comprehensive analyses of expressed repertoires came with the development of "next-generation sequencing" (i.e., high-throughput sequencing technologies). A first report using pyrosequencing provided an inclusive description of the zebrafish IgH repertoire in zebrafish (Weinstein et al. 2009). The development of deep sequencing technologies led to a renewed interest for Ig (and TCR) repertoires, and to hundreds of studies in basic and clinical research. More recently, novel approaches of single-cell transcriptome analysis have led to targeted strategies providing both IgH and L (or TCRα and β) sequences expressed in a given lymphocyte, opening new avenues to repertoire analysis.

These new technologies have required new tools for data analysis at a large scale (Six et al. 2013), as well as new theoretical approaches (Mora et al. 2010). Modeling was also developed; for example, the suite IGOR allows generating a probabilistic model of the IgH VDJ, of which parameters can be estimated from experimental data (Marcou et al. 2018).

Importantly, all these approaches require a thorough annotation of Ig (or TCR) data. In mice and humans, Ig (and TCR) transcripts and loci have been extensively sequenced and analyzed for many years, leading to validated databases and well-defined nomenclatures (Lefranc and Lefranc 2001). In contrast, the lack of standardized annotation still hinders the development of repertoire analysis in fish models. This issue is complicated because many fish Ig loci can be particularly complex due to genome duplication in tetraploid species such as salmonids (Yasuike et al. 2010). In fact, IMGT standardized nomenclatures parallel to those used in mammals, have been established for very few species. In that regard, the recent nomenclature established for salmonids (Magadan et al. 2019b) should pave the way to similar developments for other fish groups in which repertoire immunology has to be developed (see Chap. 7 about these issues).

8.3.2   Diversity Structure of Expressed Antibody Repertoires at Steady State

The high-throughput sequencing study of the whole-body μ IgH repertoire of several adult zebrafish published by Weinstein et al. revealed that a large fraction of the possible combinations of V, D, and J genes are used in the available repertoire of each individual (Weinstein et al. 2009). The estimated number of unique μ IgH clonotypes expressed per fish was 1200–3500 (a clonotype being defined as a unique triplet combination [VH gene, JH gene, junction sequence] describing the IgH chain expressed by a B-cell clone). This relatively small number of clonotypes represented only a small fraction of the potential repertoire (i.e., of the possible clonotypes that can be produced). Importantly, the frequency of VDJ combinations was not random, but was correlated between different fish (Mora et al. 2010). The diversity of the expressed repertoire was therefore not limited by the number of VDJ genes present in the genome, but rather the number of lymphocytes and by the frequency distribution of clonotypes. Only about half of the μ IgH repertoire appeared to be specific to a given individual, the other half being shared between several fish and therefore likely produced with higher frequencies. An important issue is how this is determined, i.e., the respective contribution of the mechanistic constraints determining the rearrangements, and of selective processes influenced by the environment.

Another study of μ IgH showed that the repertoire expressed by 2-week-old zebrafish was highly stereotypic [i.e., with a preferential use of a small fraction of the potential VDJ combinations (Jiang et al. 2011)]. While these highly frequent VDJ combinations were shared between different individuals, this correlation decreased at later stages. These observations are reminiscent of the IgH repertoire modifications described in mice (Huetz et al. 1993), and underscore the importance of future studies of Ab repertoires along the fish development. Many pathogens are particularly dangerous at early stages of development. It is important to understand from what stage the expressed Ab repertoire allows successful vaccination, and to investigate which clonotypes are responsible for eliciting protective responses. Also, it will be interesting to understand if there is a link between the genomic structure of the Ig loci and the sequential use of VDJ combination during development.

When analyzing the structure of expressed Ab repertoires at steady state in the spleen of "naïve" adult rainbow trout, spectratyping and deep sequencing of μ, δ, and τ IgH repertoires showed a typical structure, with a few abundant clonotypes and a very large majority of clonotypes detected only once or a few times (Castro et al. 2013). As in the zebrafish, a large fraction of the potential repertoire was readily expressed. Later studies with increased sequencing depth confirmed these observations (Magadan et al. 2018; 2019a, b). Interestingly, the most expressed VH families were different in IgHμ and IgHτ transcripts, an observation consistent among individuals.

In Atlantic salmon, sequencing of Ig transcripts was performed at large scale from spleen/head kidney (Krasnov et al. 2017) at different stages of development. The repertoire diversity steadily increased from hatch to the onset of stimulation of smoltification, and

decreased shortly before smoltification while returning to the presmolt level one week after seawater transfer. The mechanisms behind these repertoire variations are still unknown. Another striking observation was the level of expression of clonotypes shared by several individuals: about 5% of clonotypes were found in more than three fish, but they represented more than one-third of all sequenced transcripts. Importantly, all abundant clonotypes were shared by several fish. This observation was likely explained, at least partly, by Ag-driven selection. Indeed, convergent rearrangements (i.e., clonotypes with the same junction sequence associated to distinct V and J genes) were more frequent in shared clonotypes, and clonotypes that were present in multiple fish at one research station were often missing from another possibly due to responses to site-specific antigens.

IgT is the fish Ig class specialized in mucosal defense, playing key roles in pathogen neutralization and local microbiota regulation (Zhang et al. 2010; Salinas et al. 2011; Xu et al. 2013, 2016). Magadan et al. compared rainbow trout IgM and IgT repertoires between spleen and a mucosal tissue, the nasopharynx-associated lymphoid tissue (NALT) (Magadan et al. 2019a, b). NALT is a diffuse network of myeloid cells and lymphocytes located in the fish olfactory organ, and contains both IgM[+] and IgT^{+} B cells. Rainbow trout NALT IgM repertoires exhibited a VH usage unique to each individual, in contrast to spleen. VH usage in the NALT IgT repertoire was more similar to that found in spleen, with lower interindividual variation. With small B-cell numbers, NALT repertoires were also less diverse and had a higher proportion of clonotypic expansions, compared to spleen repertoires. It seems possible that the constant Ag exposition of mucosa may favor B-cell expansions in these body sites. Alternatively, these clonotypic expansions may be induced by the microbiota inhabiting the nasal mucosa.

Overall, these initial results on Ig repertoires of "naïve" fish illustrate the power of the approach. Future studies will likely reveal other important features of fish B-cell biology. It will be particularly interesting to compare different stages of B-cell differentiation as performed in Greiff et al. (2017) to learn about allelic exclusion mechanisms and tolerance checkpoints. Repertoire variations with age, with environmental conditions or geographic origin, and across fish species will also be critical to understand the respective parts of genetics, accumulation of Ag-driven reactions, and natural Abs-producing cells in the composition of naïve repertoires. It will also be important to address how dysbiosis and changes in the microbiota influence the repertoire of the different fish Igs. Finally, it is important to note that an accurate knowledge of the genetic background (in particular, of Ig loci) of experimental fish is crucial. When available, the use of clonal fish can simplify the complexity of the data analysis, and eliminate a source of variability in the results (Castro et al. 2013; Magadan et al. 2018; Magadan et al. 2019a). More generally, standardized and comprehensive annotation of the Ig loci of key species will become a key resource for comparison of Ab responses at the molecular level. The extraordinary rise of new genomic technologies is already making it possible (Marks and Deane 2020).

8.4 Clonal Complexity of Expressed B-Cell Repertoires in Fish After Immunization or Infection by Pathogens

Detection of Ag-specific Abs from fish fluids (*i.e.*, serum, mucus) is probably the easiest strategy to follow B-cell responses. Ab titers provide a quantitative assessment of the strength of the response, and different assays using Abs can evaluate their functionality, such as virus neutralization capacity or agglutination activity, among others. However, these approaches do not reveal much of the molecular diversity of Ab responses or the corresponding B-cell clonal expansions. Comparing comprehensive repertoire sequencing data from naïve and immunized/infected individuals may therefore appear as a straightforward approach to identify responding B-cell clones. B-cell responses induce global modifications of the frequency and distribution of clonotypes, especially strong responses induced by infections. However, to assign a given clonotype to the Ag-specific response is not straightforward. Indeed, different individuals, even when they share the same genetic background, have their own immunological history and their own preimmune Ab repertoire. Hence, it is not straightforward to distinguish within the repertoire of an immune individual, between pre-existing abundant clonotypes and Ag-specific clonal expansions. Several approaches can be used to solve this problem and identify Ag-specific clonotypes:

1. Individual fish monitoring of the Ab repertoire at time intervals along the experiment would allow identification of emerging responses and show the dynamics of the response (Vollmers et al. 2013). However, this requires individual labeling—which is not always possible when using small fish—and repertoire sequencing from blood sampled at multiple time points, which may not be the most appropriate organ to capture the complete Ig repertoire.
2. New technologies have enabled single-cell analysis, giving access to the mRNA sequence of both heavy and light chains of the receptor expressed by each analyzed B cell and allowing to produce selected Abs and to test directly their Ag specificity. This remains difficult to establish, costly, and labor-intensive.
3. When the frequency of top clonotypes expressing a given VH (or VH/JH combination) is consistently low in nonimmunized controls, immunization or infection may lead to the appearance of abundant clonotypes expressing this VH (or VH/JH), which likely constitute Ag-driven clonal expansions.
4. Finally, some clonotype expansions are observed in all immunised individuals (ideally, genetically similar), constituting reliable markers of the response to the Ag. Such clonotypes are named recurrent, or "public". A public response was originally defined as a response consisting in a given Vβ-Dβ-Jβ rearrangement, which was found in anti-HEL response of all mice in an H2^d MHC context (Cibotti et al. 1994). Thus, the term "public" is used to distinguish responses found in all individuals with the same genetic background, from the "private" responses that are specific to each animal (see also (Magadan et al. 2015) for discussion of what is a public response).

While the analysis of fish Ab repertoires is at its infancy, it has already revealed interesting features of Ab-specific responses to pathogens in these species (see below).

8.4.1 Modifications of Systemic Antibody Repertoires After Immunization or Infection by Pathogens

In an early study, the spleen IgM and IgT repertoires of a fish species, the rainbow trout, were analyzed after vaccination against a virus, the Viral hemorrhagic septicemia virus (VHSV), that causes a systemic infection (Castro et al. 2013). In this study, isogenic fish were immunized twice at 3 weeks interval using an attenuated viral strain, and were analyzed one month later. Overall, the number and frequency of abundant clonotypes strongly increased after vaccination, both for IgM and IgT while no significant changes were observed for IgD. Modifications of the IgM repertoire were seen for all VH, indicating a wide perturbation of B-cell populations. Sharing analysis across individuals identified eight "public" expanded clonotypes using the same VH and JH genes, and having highly similar VDJ junction sequences (i.e., "CDR3" loop sequences), and coding for an IgM heavy chain. Although relative frequencies of these eight clonotypes varied from a fish to another, this combined public response was present and potent in every tested fish. Interestingly, no public response was found for IgT, which suggests that IgM and IgT evolved under different selection pressures. In a second study (Magadan et al. 2018), the same isogenic trout line was immunized once with the attenuated virus, and the spleen repertoire was characterized 5 months later. Repertoire modifications were then observed only for a few VH, suggesting that the wide reaction observed in the previous protocol was largely due to bystander responses. Large expansions of the eight public clonotypes identified in the first study were again found in all immunized fish, indicating that the public response is persistent over time. Importantly, a VDJ rearrangement model parameterized on experimental data predicted that these public junctions were generated by the recombination machinery with high probability. Accordingly, these clonotypes could be detected in the spleen of naïve animals, and their frequency was higher than for typical nonexpanded clonotypes. Hence, public rearrangements were favored at the recombination step, and were available at relatively high frequency among the naïve repertoire before vaccination. Interestingly, this origin of public responses against pathogens raises the question of a possible predetermination of Abs specific for important pathogens, which might be selected during evolution of the species to favor Abs directed against key pathogens. In this study, IgT responsive clonotypes could also be identified. In all immunized fish, the top IgT clonotypes expressing VH4 were 10 times more frequent than in controls. These abundant clonotypes were not shared between individuals, and their junction sequences were not highly similar. These "private" responses were also observed after boost, again based on unshared clonotypes. While public clonotypes typically target a common epitope, these private responses may recognize different antigenic determinants for which the VH4 is most suitable. Interestingly, anti-VHSV public clonotypes were
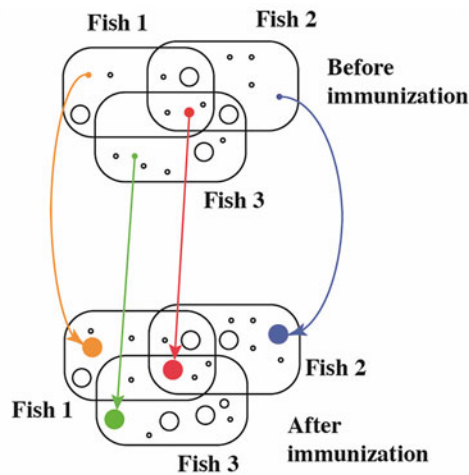
Fig. 8.1 Schematic model of trout IgM clonal response against VHSV. The diameter of circles reflects the size of clonotypes. Naïve repertoires include mainly clonotypes expressed at low frequency, as well as a few frequent clonotypes that are mainly specific to individuals or shared by a few. Among clonotypes present in all fish ("public" ones) at steady state, some are more frequent that most of the others (in red). During responses to the virus, both private clonotypes (represented in orange, green, and blue), and public clonotypes, are expanded. Public responses expanded in all fish originate from clonotypes initially shared and abundant (in red). The proportions of clonotypes of different sizes reflect schematically the global structure of the repertoires, but do not correspond exactly to the average distribution of real datasets

shown to correspond to neutralising Abs (Castro et al. 2021), as observed in a growing number of examples in mammals, further supporting a conserved role for public Ab responses across vertebrates.

Altogether, these studies on the spleen B-cell repertoire after infection with an attenuated VHSV showed that: (1) the main modifications were seen for IgM (involving a number of VH genes from several subgroups, and a strong public response) (see Fig. 8.1), (2) the anti-VHSV response comprised clonal expansions of IgT$^+$ spleen B cells, but they were much less prominent than for IgM, and serum IgT titers did not increase as IgM titers (Castro et al. 2013). It will be particularly interesting to revisit these observations when the circulation of B cells between spleen, head kidney, and mucosal tissues will be better understood.

The importance of shared clonotypes was also underscored in the work by Krasnov et al. in Atlantic salmon (Krasnov et al. 2017). This study investigated the response to infection with the piscine myocarditis virus (PMCV), the causative agent of cardiac myopathy syndrome. In the head kidney, but not in the spleen, a strong increase of the frequency of the most frequent clonotypes was observed 10 weeks after viral challenge, corresponding to clonal expansions and activated cells. Interestingly, these top clonotypes were all shared

by several individuals. A significant junction "convergence" (*i.e.*, the expression of the same junction sequence associated to distinct V and J genes) supported that they were expanded by Ag-driven selection. The contrast in spleen and head kidney responses suggested that, at the time-point considered, responding B cells were mainly located in the head kidney, which was consistent with the idea that Ag-secreting cells and memory cells accumulate in this tissue (Bromage et al. 2004b). Perhaps encounters between B cells and their cognate Ag also take place in the kidney, as in the spleen. In that regard, while the current paradigm dictates that fish do not contain organized lymphoid tissue, the sites where B-cell responses develop remain to be defined.

8.4.2 Particularities of Mucosal Responses

Repertoire sequencing is also a powerful approach to compare Ab responses among different tissues. IgM and IgT repertoires were compared in spleen and NALT after vaccination with a bacterial vaccine against the enteric red mouth (ERM) (Magadan et al. 2019a). *Yersinia ruckeri* bacterin was administered either intraperitoneally (i.p.) or intranasally (i.n.). I.p. immunization modified VH usage by IgM transcripts in spleen, and induced large clonotype expansions expressing members of several VH families. Importantly, NALT IgM repertoires were also modified, but in a different manner compared with spleen: the NALT IgM repertoire was enriched, with an increased clonotypic diversity for some VH. In contrast, the IgT repertoire was less altered after i.p. vaccination, both in spleen and NALT, suggesting that this systemic route of immunization has a lower impact on IgT compared to IgM. Intranasal administration also affected the spleen IgM repertoire, although it did not result in high levels of ERM-specific IgM titers in the serum. Interestingly, the prominent modification of the IgT repertoire in the spleen indicated that the intranasal route was particularly efficient to induce a response of the mucosal isotype in this tissue. However, it remains to be studied whether ERM-specific IgT titers were induced in either sera or mucus from vaccinated fish. Thus, the diversity of both NALT IgM and IgT clonotypes seems to increase slightly, suggesting that an influx migration of B-cell clones may occur as after i.p. immunization.

Altogether, these results unveiled an unexpected complexity of interactions between B-cell compartments in fish, showing that both IgM[+] and IgT^{+} B cells are responsive, albeit to significantly different degrees, to both i.p. and i.n. vaccination. Interestingly, mechanisms explaining the contrasted effect of intranasal immunization on IgM and IgT, remain to be discovered.

These first examples of Ig repertoire sequencing show how future repertoire studies will provide novel insights into the dynamics of fish Ab responses, the lymphoid organs, and potential microstructures where such responses occur, and the potential migration of B cells from inductor to effector lymphoid tissues.

8.5 Maturation of B-Cell Responses and Development of B-Cell Memory in Fish

8.5.1 Secondary B-Cell Responses in Fish

The kinetics of B-cell responses in fish remains poorly understood. Responses seem to be generally slow when compared to mammals and birds, especially in cold water species, thus indicating that such responses are temperature-dependent (Fillatreau et al. 2013) and references therein. The type of immunization (injection vs. mucosal exposure like immersion) also has a strong effect.

In rainbow trout, the Ab response against DNP-KLH peaked 50 days after immunization, and Ab titers remained high for at least until 150 days (Cossarini-Dunier et al. 1986). After vaccination with an attenuated strain of VHSV, expansions of public clonotypes against this virus were still observed 5 months later, indicating that the response can be persistent for a long time after the pathogen has been cleared (Magadan et al. 2018).

Fish secondary responses follow different patterns compared those known in mammals, although distinct primary and secondary responses have been reported in several fish species. Like in mammals, a faster and stronger secondary response (i.e., higher Ab titers) has been typically observed in several fish species upon repeated infections or immunizations. For example, faster and higher Ig titers were observed after immunization with bovine serum albumin and with sheep red blood cells (SRBC) in carp (Avtalion 1969; Rijkers et al. 1980a, b), as well as against *Yersinia ruckeri* and *Aeromonas salmonicida* in trout (Cossarini-Dunier 1986; Tatner 1986). Importantly, the induction of a secondary Ab response seems to be dependent on the dose of Ag administered at the primary immunization. When carp were immunized with 10^5 SRBC by intramuscular injection and challenged 1, 3, 6 or 10 months later with the same dose, a secondary response was observed at 3 months and at later time points. In contrast, a high dose of 10^9 SRBC did not produce a secondary response with enhanced titers (Rijkers et al. 1980a, b).

These data show that there are quantitative differences between primary and secondary responses. It is difficult to establish a general rule for fish from only a few studies. It is likely that the type of Ag, the dose used for immunization, but also the environmental conditions (especially temperature range) affect the kinetics and the maturation of Ab responses. Also, most experiments have been performed with a few species of farmed fish (salmonids, carp) and zebrafish, which does not represent at all the diversity of teleosts. In addition, for the most part, experiments have addressed only the IgM response, while thus far very few studies have investigated IgT and IgD primary and secondary immune responses. In that regard, recent studies show that in mucosal sites, secondary pathogen-specific IgT titers are higher than those observed upon primary pathogenic exposure, while IgD titers are absent both in serum or mucosal sites (see Chap. 12 for IgT primary and secondary responses).

8.5.2 Memory, Affinity Maturation, and Somatic Hypermutation

Long-term persistent B-cell responses and higher response to secondary immunization strongly suggest of the induction of immune memory in fish [for a discussion of the notion of immune memory, see Farber et al. (2016)]. In mammals, an important process associated to the improvement of secondary immune response is the process of affinity maturation of Ag-specific Abs. This process occurs through the selection of B cells with hypermutated Ig sequences, in germinal centers. While fish are thought to be devoid of germinal centers, affinity maturation of the Ab response has been observed, although the affinity increases in fish are much lower than those observed in mammals, and the mechanisms behind such affinity responses remain obscure. Monitoring of rainbow trout immunized with TNP-KLH for Abs titers and affinity throughout a 27 week period could detect a slowly occurring, but measurable (albeit low) affinity maturation response (Kaattari et al. 2002). Ab titers peaked at 10 weeks postimmunization, and started to decrease 22 weeks postimmunization, while affinity of the Ab response continuously increased until the end of the experiment.

Somatic hypermutation of Ig genes has also been observed in several fish species (Yang et al. 2006; Jiang et al. 2011; Magor 2015). However, its contribution to Ab affinity maturation remains to be established. In the absence of germinal centers in fish, the location and mechanisms for selection of hypermutated B cells expressing high affinity Abs remain elusive. Indeed, this selection step has to exert very strong pressures to expand the rare clones expressing high affinity Abs. Rather than occurring randomly in the spleen or other lymphoid tissues, this selection step likely requires a particular microenvironment in which Ag-presenting cells, as well as B and T cells can interact and induce the immune response (Wilson et al. 1992).

8.5.3 Lack of Attrition

Another factor that may affect long-term immune memory is successive infections with heterologous pathogens. Selective loss of LCMV-specific memory $CD8^+$ T cells was reported after sequential infections with heterologous viruses (Selin and Welsh 2004; Welsh et al. 2004). It was proposed that this cell loss—also referred to as "attrition"—was due to a combination of passive competition and replacement of pre-existing cells by new memory cells entering a compartment of defined size, and by deletion of pre-existing memory cells through apoptosis. Due to TCRs being highly cross-reactive, pre-existing memory T cells may then cross-react with newly encountered pathogens, and hinder the selection of potentially more protective clones against new pathogens (Welsh et al. 2010). Abs are usually more specific to their cognate Ag than TCR, although B-cell attrition has also been documented in mammals in several models (Radwanska et al. 2008; Ng et al. 2014; Fallet et al. 2016). Memory B-cell attrition was investigated in rainbow trout immunized using an attenuated VHSV vaccine (Navelsaker et al. 2019). Vaccinated trouts were reimmunized twice at 30- and 60-days post VHSV-immunization with live infectious

pancreatic necrosis virus (IPNV), a birnavirus that is immunologically distinct from VSHV. VHSV-neutralizing antibodies and VHSV-specific ELISPOT did not show any impact of the heterologous immunization. As the trout public response against VHSV had been previously characterized, it was possible to look for effects of the subsequent immunization with IPNV on the frequency or composition of public clonotypes. No significant modifications of the public response could be detected, and the response to IPNV (i.e., the second virus) did not appear to be affected by a prior vaccination against VHSV either. These observations suggested that repeated immunizations do not induce a strong attrition of memory B cells in teleosts. Such results will have to be confirmed by further work with different pathogens, and different fish species.

8.6 Conclusions: Perspectives of Basic and Applied Fish B-Cell Immunology and Repertoire Studies

In this chapter, we have reviewed how repertoire analysis is instrumental in understanding the Ag receptor diversity and clonal complexity in a few model teleost species. Importantly, these studies have so far unveiled the significance of public B-cell clonal against infections. Future studies will extend these approaches to multiple fish species (of variable size, physiology, lifespan, ploidy, etc), and integrate variations at the genomic level (potential repertoires) and adaptations of clonal composition of B-cell subsets during development and immune responses (i.e., available repertoires).

Fish Ab isotypes and some of their specialized roles have been well described in a number of species. It is now well-accepted that IgM and IgT, the main systemic and mucosal Igs respectively, are expressed by two different subsets of fish B cells. It remains to be defined however, which B-cell subset expresses soluble IgD across fish species. While the aforementioned IgT and IgM fish B-cell lineages have been clearly defined, the exquisite complexity of functional B-cell subsets found in mouse and in human is still lacking in fish. Regarding B-cell subsets, it is very complex to attempt to establish homologies between fish and mouse or human B-cell populations. Cell types being defined by a combination of transcription factors (Hobert 2016), such cell comparisons between taxonomic groups must disentangle evolutionary and developmental lineages (Arendt 2008), which can be particularly difficult when several whole-genome duplications have occurred during the evolution of one branch. In fact, it will be most important to identify functionally relevant B-cell subsets in fish, which may—or may not—play analogous roles as known mammalian B-cell subpopulations. Divergence, convergence, and homologies, between B-cell subsets of fish and other vertebrates will reflect the respective biology of these cell subsets, and the variations of the solutions selected during evolution to allow B cells play their defensive role against infections. Through the use of high-throughput sequencing technologies, Ab repertoire analysis will provide key insights into the biology and functionality of these B-cell subsets at the steady state as well as during infection and vaccination.

The genetics of Ig and genomic rearrangements, as well as the structure of Ig loci, have also been extensively studied and appear to be relatively similar in teleost fish and in mammals. However, fundamental questions about B-cell development remain unsolved in fish, such as the sequence of IgH and IgL recombination and the mechanism of allelic exclusion. In the apparent absence of organized lymphoid tissue in fish (e.g., germinal centers, Peyer's patches), the fundamental question remains as to where in lymphoid tissues are antibody responses induced, and which are the cells and mechanisms involved in the induction and development of an immune response. Also, the kinetics of responses and the mechanisms of B-cell selection will have to be evaluated in much further detail if we are to understand the structure and dynamics of the Abs repertoires after immunization or during infection. This knowledge is critical not only to understand how immune responses are induced and developed in fish, but also to establish a rational basis for developing better vaccines that induce both systemic and mucosal protective immune responses. In that regard, once this knowledge becomes available, it will be of paramount importance to establish signatures of response and protection that should be, ideally, predictive of vaccine efficacy.

A major feature of adaptive immunity is specific memory. In fish, the mechanisms of persistence of Ag-specific B cells remain ill understood, although the modalities of secondary responses appear to be different from what is known in classical mammalian models. Most importantly, the mechanisms of affinity maturation of Abs, and how B cells expressing hypermutated high affinity Abs can be selected, remain a major mystery of fish B-cell biology. Circulation of B cells between compartments (for example, different mucosa, spleen, and head kidney) constitutes still a black box in fish immunity. Equally important will be to characterize the specific phenotypes and roles of plasma-like cells in fish. In that regard, such cells appear to be present in teleost fish with similar phenotypic characteristics to those of mammalian IgM plasma cells (Zhang et al. 2010). Overall, the knowledge derived from all the points discussed above will be pivotal to comprehend the different modalities of responses to immunizations via distinct routes (i.e., systemic vs. mucosal routes). In turn, this knowledge will greatly impact key practical aspects of vaccine delivery in fish farming.

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Complement Activation in Fish with Emphasis on MBL/MASP

9

Per W. Kania and Kurt Buchmann

Abstract

The complement system in fish consists of a series of proteins interacting with both the innate and the adaptive immune systems. These distinct plasma proteins sequentially react with one another whereby they eventually directly kill the pathogens. The system is activated by binding of complement factors to surfaces of invading pathogens. During this process, complement factors and their cleavage products may opsonize invading microorganisms, induce a series of inflammatory responses and finally kill the pathogens. Activation of the complement system may take place through three pathways called the classical pathway (CP), the alternative pathway (AP) and the mannan-binding pathway (or lectin pathway) (LP). These activation routes depend on the different molecules acting in the initiation of the sequential reactions but they all converge to activate the same central effector molecule, C3. This is achieved by formation of a C3 convertase. Activation of C3 leads to the formation of two fragments C3a (an important factor, together with C5a, for inducing inflammation via its effect as an anaphylatoxin) and C3b which facilitates phagocytosis via its function as an opsonin. Activation of C3 also leads to the formation of a C5-convertase, leading to the assembly of the terminal C5b-C9 complex, otherwise termed the membrane attack complex (MAC). The MAC creates pores in the membrane of the invading pathogen, eventually leading to their cell-lysis and death.

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Abbreviations

AP	Alternative pathway
AS	Activating surface
B	Factor B
CP	Classical pathway
C1	Complement factor 1
C1q	Complement factor 1q
C1r	Complement factor 1r
C1s	Complement factor 1s
C2	Complement factor 2
C3	Complement factor 3
C4	Complement factor 4
C5	complement factor 5
C6	Complement factor 6
C7	Complement factor 7
C8	Complement factor 8
C9	Complement factor 9
CCP	Complement control protein
CRD	Carbohydrate recognition domains
CRP	C-reactive protein
CUB	Uegf/bone (complement-urchin-bone) morphogenetic protein
D	Factor D
GalBL	Galactose-binding lectin
GalNac	N-Acetylgalactosamine
GBL	Glucose-binding lectin
I	Factor I
IgM	Immunoglobulin M
LP	Lectin pathway
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MASP1	MBL-associated serine protease 1
MBL	Mannan (mannose)-binding lectin
MBLh1	MBL homologue 1
MASP2	MBL-associated serine protease 2

P   Properdin, Factor P
SAA   Serum amyloid A protein
utr   Untranslated region

9.1   Introduction

The complement system in vertebrates, including fish, is a series of proteins interacting with both the innate and the adaptive immune systems. Although a range of molecules, spanning from antimicrobial peptides, acute phase reactants, SAA, CRP, chemokines to cytokines, play a significant role in the humoral part of innate immunity the complement system is a major and substantial player. It consists of a large number of distinct plasma proteins that sequentially react with one another, to not only opsonize invading microorganisms and induce a series of inflammatory responses, but mainly to directly kill the pathogens (Fig. 9.1). Activation of the complement system may take place through three pathways called the classical pathway (CP), the alternative pathway (AP) and the mannose (mannan)-binding pathway (or lectin pathway) (LP). These activation routes depend on the different molecules acting in the initiation of the sequential reactions but they all converge to activate the same central effector molecule, C3. Activation of C3 leads to the formation of two fragments C3a (an important factor, together with C5a, for inducing inflammation via its effect as an anaphylatoxin) and C3b which facilitates phagocytosis via its function as an opsonin. Activation of C3 also leads to the formation of a C5-convertase, leading to the assembly of the terminal C5b-C9 complex or the so-called membrane attack complex (MAC) (Fig. 9.2) which creates pores in the membrane of the invading microorganisms, eventually leading to their cell-lysis and death. The mentioned complement factors have all been identified and partly described in teleosts and especially zebrafish *Danio rerio* has been well studied (Zhang and Cui 2014). The main organ of production in mammals is the liver. In fish the liver plays a prominent role for production but these factors are also produced in extrahepatic organs such as the spleen, the head kidney, the heart, the intestine, the skin, the gills and the gonads (Hu et al. 2010; Zhang and Cui 2014).

9.2   The Classical Complement Pathway

The generation of antigen-antibody complexes initiates the classical complement pathway. This pathway is therefore dependent on the existence of antibodies in the fish reacting with an antigen, e.g. invading bacteria or parasites. Antigen-antibody complexes are recognized by the C1 complex consisting of C1q and the two serine proteases C1r and C1s. The multiplicity of the C1q molecule is required for binding to the constant regions of the fish
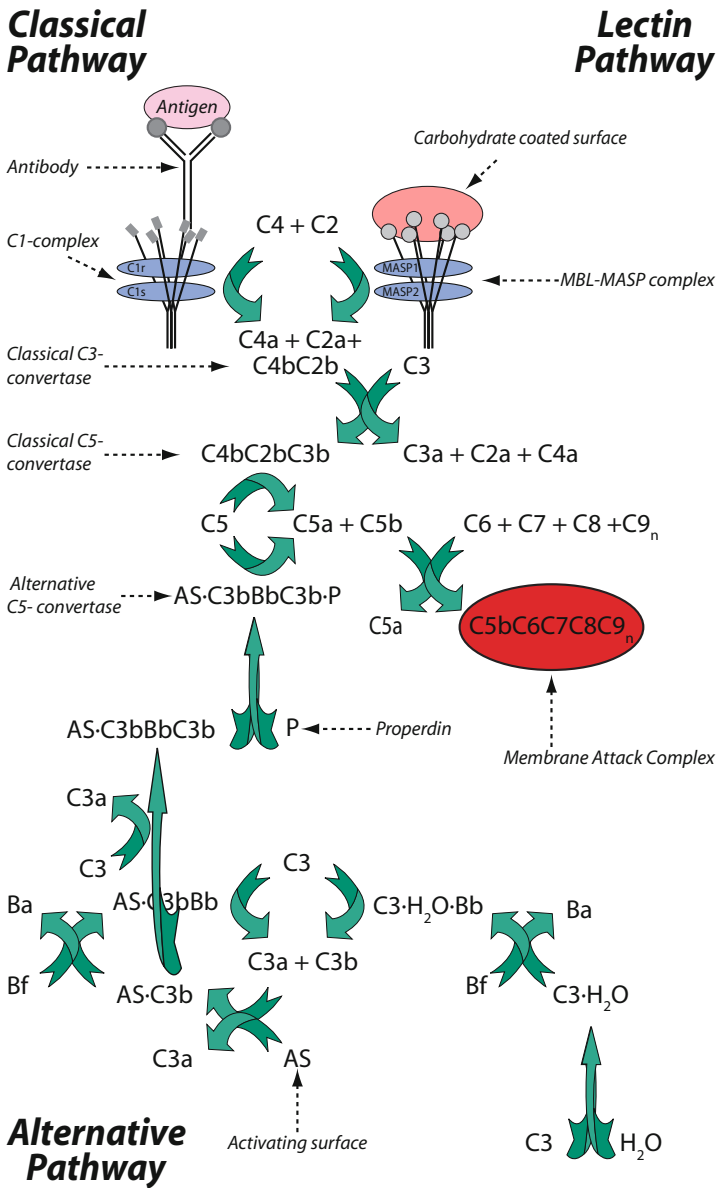
Fig. 9.1 Activation pathways of complement. The classical, the lectin and the alternative pathways for activation of complement. Three routes lead to formation of the membrane attack complex (MAC) which creates pores in the cell membranes of the intruding microorganism. This process will eventually lead to lysis and death of the pathogen
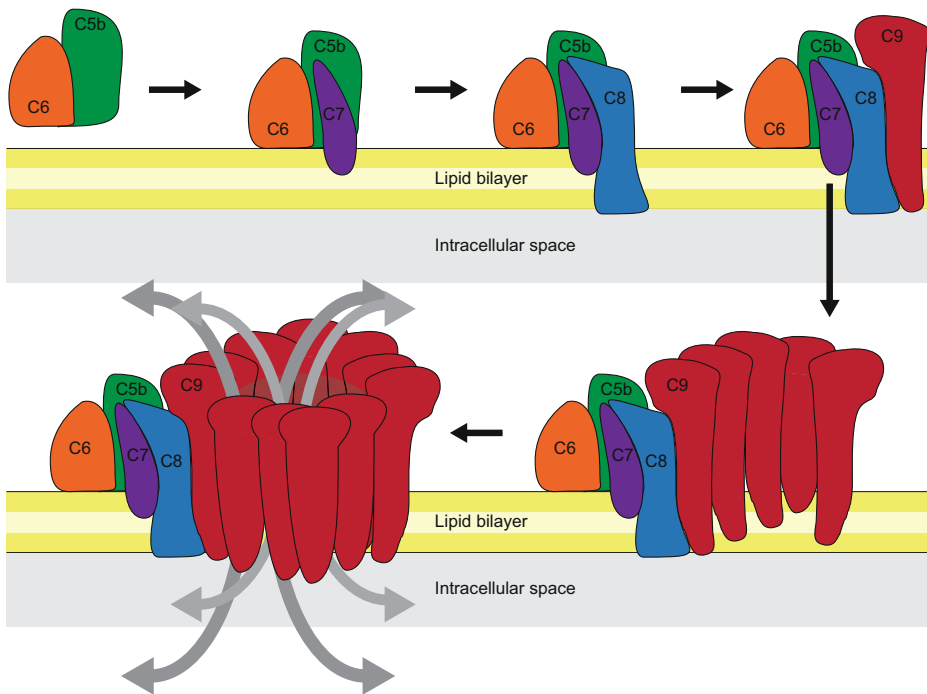
Fig. 9.2 Assembly of the membrane attack complex (MAC). The sequential binding of complement factor C5b and C6 followed by the binding of C7, C8 and C9 leads to formation of a MAC. Component 7 penetrates the lipid bilayer of the cell membrane whereafter C8 associates and acts as a transmembrane component joining with multiple C9 factors. The C9 components form a ring with a central pore perforating the cell membrane eventually leading to lysis and death of the cell

antibody. This multivalent binding of C1q to the constant part of the heavy chain of antigen-bound antibodies (e.g. IgM) activates first C1r and then C1s, which in turn first cleaves C4 into C4a (a weak anaphylatoxin) and C4b and then C2 into C2a and C2b. Subsequently, C4b and C2a combine to form the classical C3 convertase (C4bC2a), which cleaves C3 into C3a and C3b. The latter may subsequently bind to C4bC2a, resulting in the classical C5 convertase (C4bC2aC3b) which then cleaves C5 into C5a (the major anaphylatoxin). C5b serves as the anchor for the assembly of the MAC by binding the remaining complement factors (C6, C7, C8, C9) into a barrel-shaped pore in the membrane of the invading pathogen (Fujita et al. 2004; Matsushita et al. 2000a).

9.3   The Alternative Complement Pathway

In the alternative pathway, C3 (in low amounts in fish plasma) is spontaneously hydrolysed to $C3(H_2O)$ by a so-called tick-over mechanism, probably due to the presence of low amounts of active proteases, e.g. plasmin. Upon association of factor B to $C3(H_2O)$, factor

D (a serine protease) is induced to cleave factor B into Ba and Bb, forming a complex, C3 (H_2O)Bb, which functions as the alternative C3 convertase generating C3a and C3b fragments from C3. Normally, unbound C3b is quickly inactivated by factor I (another plasma protease). C3b may bind to the surface of "self" cells, but will then be inactivated by factor I, with factor H and membrane ("self") proteins as cofactors for factor I. Factor H may also regulate complement as it can displace factor Bb from the convertase. This is a control mechanism to avoid destruction of host cells by the alternative complement system. When C3b binds to a foreign activating surface, e.g. a microorganism (AS on Fig. 9.1), this AS-C3b will bind factor B, which is then activated by factor D and turned into the alternative complement C3-convertase C3bBb. Factor P, properdin, binds to the complex and stabilizes the C3-convertase. Association of another C3b now generates the alternative complement C5-convertase C3bBbC3b-P, and thus initiates the assembly of the MAC (Fig. 9.2) (Seeger et al. 1996).

9.4 The Lectin Complement Pathway

The lectin pathway is initiated by the binding of mannose (mannan)-binding lectin (MBL) to specific patterns of carbohydrates on the surface of various pathogens (Drickamer 1992; Weis et al. 1998). The MBL molecule, a so-called collectin, is a structural homologue of C1q as it contains a number of lectins, located on collagen stalks, which are able to bind carbohydrates present on pathogen surfaces. MBL-associated serine proteases (MASP) represent structural homologues of C1r and C1s. They are activated by cleavage resulting in two polypeptide chains linked by a disulphide bridge. Activated MASP2 now cleaves C4 and C2, whereby the classical C3-convertase C4bC2a is formed (Matsushita and Fujita 1992; Vorup-Jensen et al. 2000). The classical activation pathway (employing C1q, C1r, C1s) has then a corresponding reaction pathway in fish plasma represented by MBL and MASP molecules.

9.4.1 Collectins

The MBL molecule is a member of the collectin family which constitute the subgroup III of C-type lectins (Kilpatrick 2002). They are important components of innate immunity by binding to carbohydrate patterns on the surface of pathogens and subsequently interacting with the complement system (Drickamer 1992; Weis et al. 1998). C-type lectins all have globular carbohydrate recognition domains (CRDs), which bind to carbohydrates in a Ca^{2+}-dependent manner (Weis et al. 1991). Different types of domains can be attached to the C- and N-terminus of the CRDs including other CRDs (Kilpatrick 2002). The collectins (Fig. 9.3) are characterized by having a common structure starting with a cysteine-rich N-terminal domain involved in oligomerization of the molecule, followed by a collagen-like structure with every third amino acid being a glycine (Brodsky and Persikov 2005), then an α-helical coiled neck region and finally a globular carbohydrate recognizing
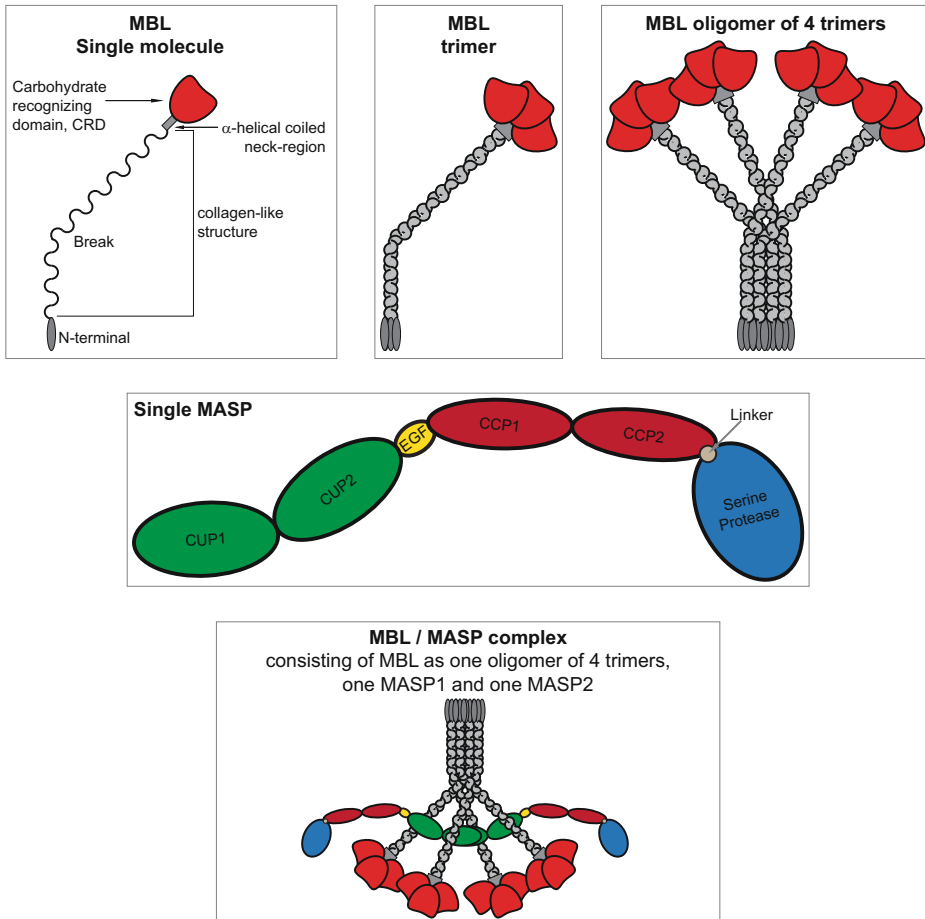
Fig. 9.3 Organization and tertiary structures of MBL. The cysteine-rich N-terminal region is followed by a collagen-like region, an α-helical coiled-coil structure (neck) and a carbohydrate recognition domain (CRD). The trimerization is initiated by three neck domains and continues along the collagen-like regions. Subsequently, the trimeric subunits assemble via cysteine residues in the N-terminal domain into a higher oligomer. This MBL oligomer of 12 MBL molecules, one MASP1 and one MASP 2 combine into a complex. The two serine-protease domains are auto-activated upon binding to an appropriate activating carbohydrate coated surface. Hereafter these serine proteases initiate the complement cascade by cleaving C2 and C4. CUB indicates the C1r/C1s/Uegf/bone morphogenetic protein 1. EGF indicates the epidermal growth factor. CCP indicates complement control proteins

domain, CRD. The collectins exhibit a high degree of hydroxylation of proline and lysine in their collagen-like domains (Colley and Baenziger 1987; Crouch et al. 1994; Kivirikko and Myllyla 1985; Mccormack et al. 1994; Phelps et al. 1986). The collectins can be divided into eight subgroups: conglutinin (Davis and Lachmann 1984), collectin

43 (CL-43) (Holmskov et al. 1993), collectin 46 (CL-46) (Hansen et al. 2002), lung surfactant protein A (Whitsett et al. 1985) and D (Persson et al. 1989) (SP-A and SP-D), collectin placenta 1 (CL-P1) (Ohtani et al. 2001), collectin liver 1 (CL-L1) (Ohtani et al. 1999) and mannose-binding lectin (MBL) (Kawasaki et al. 1978). They are all soluble and secreted proteins except for CL-L1 and CL-P1, which are found in the cytosol and membrane, respectively. Conglutinin, CL-43 and CL-46 have only been found in bovine animals (Hansen et al. 2002).

9.4.2 Ficolins

Ficolins are structurally homologous to MBL by having a short N-terminal region followed by a collagen-like region and ending in a globular domain (Harumiya et al. 1996; Ichijo et al. 1993; Lu et al. 1996; Matsushita et al. 1996, 2002). Their role in the fish immune system is not well elucidated but in mammals ficolins associate with MASPs and activate the complement cascade by the same route as MBL. The collagen-like region of ficolins trimerizes in the same manner as MBL resulting in the same bouquet-like structure. They also form higher disulphide-linked oligomers (Matsushita et al. 1996) and possess the same MASP binding motif (Girija et al. 2007). However, ficolins do not bind to pathogens by the use of a C-type lectin domain. Instead they bind via a fibrinogen-related domain (FReD) (Le et al. 1998, 1997) which is able to bind GlcNac (Liu et al. 2005; Matsushita et al. 1996; Sugimoto et al. 1998; Teh et al. 2000), GalNac (Liu et al. 2005; Sugimoto et al. 1998), sialic acid (Endo et al. 2005; Liu et al. 2005) and LPS (Nahid and Sugii 2006). Several reviews describe various aspects of ficolins (Bidula et al. 2019; Endo et al. 2006a, 2007; Fujita et al. 2004; Holmskov et al. 2003). Future studies should elucidate the role of ficolins in the fish immune system.

9.5 The Evolutionary Background for the Complement Components and Their Function

9.5.1 The Classical Pathway

Complement-like molecules and systems have been traced back to a number of invertebrate species (Sunyer and Lambris 1998) including echinoderms and tunicates (Endo et al. 2006b; Iwanaga and Lee 2005; Yoshizaki et al. 2005). In these animals, the complement system may have emerged as a simple system comprising a smaller number of the components (as found in vertebrates) and with limited functions such as facilitation of phagocytosis. It can be speculated that the classical pathway may be the most recent one to have appeared due to its connection to the adaptive immune response and interaction with antibodies. Thus, functional immunoglobulins evolved as functional entities late in evolution with the emergence of the jawed vertebrates (the class Gnathostomata) (Fujita et al.

2004; Zarkadis et al. 2001). However, it cannot be excluded that C1q has an even earlier origin as it is not activated solely by antigen-bound antibodies, but can also be activated by other molecules, such as CRP.

9.5.2 The Alternative Pathway

The alternative complement cascade is believed to have an ancient origin. Factors with the central molecule C3 have been traced to early metazoans and are found in molluscs and arthropods. They function in echinoderms and ascidians, mainly as opsonins, but without involving a cytolytic cascade (Nakao et al. 2011). These factors are readily expressed in both zebrafish (Zhang and Cui 2014) and rainbow trout (Köbis et al. 2015).

9.5.3 The Lectin Pathway

This activation system may play an important role in the ascidians since it has been shown that this group of tunicates possess a glucose binding lectin, homologue to MBL, together with ficolins, MASPs, C2/factor B, C3 and C3 receptor (Fujita et al. 2004; Matsushita et al. 2004). However, in the sea urchin an atypical factor B, having five CCP-domains instead of the normal three in the C2/factor B family, has been found (Smith et al. 1998; Terwilliger et al. 2004). This could be interpreted as an "older" alternative pathway. On the other hand, two collectin-like genes have been identified in the ascidian *Ciona intestinalis* (Skjoedt et al. 2010), one with supposed affinity for mannose and one with supposed affinity for galactose in the most primitive of the living vertebrates; and the lamprey, a jawless fish belonging to the class Agnatha, have genes encoding MASPs (Endo et al. 2003). In addition, MBL has been isolated from sera together with a protein showing homology to mammalian C3/C4, described as a C3-related protein (Matsushita et al. 2004; Zarkadis et al. 2001). This C3-related protein was shown to possess opsonizing activity, and MASP from the same species showed C3 cleaving activity. Complement in the lamprey may thus consist of a form of the lectin pathway where lectins recognize and bind to structures on the surface of intruding microorganisms. MASP is activated and cleaves C3 into C3a and C3b followed by the binding of C3b to the microorganism. The microorganism is now opsonized and a C3b receptor on phagocytes facilitates phagocytosis of the pathogen (Fujita et al. 2004; Zarkadis et al. 2001). Furthermore, the lamprey also possesses a common factor B/C2 ancestor-like molecule, indicating the possible existence of an early alternative pathway (Zarkadis et al. 2001) (Kuroda et al. 1996). As the immunoglobulins appeared with the jawed fish, the basis for the emergence of factors (C2 and C4) in the classical and the lectin pathway in vertebrate species from their homologue components in invertebrates was a combination of gene duplications and exon shuffling, including the sequential additions or deletions of several types of modules or domains from various proteins. The vertebrates (e.g. mammals) thus seem to have developed a more multifaceted

complement system by taking advantage of the cascades in invertebrates, but they have added new components. This has enabled the mammalian vertebrates to take full advantage of adaptive immunity. The vertebrates retained complement pathways from the invertebrates, but also established the classical complement pathway by exploiting the invertebrate model (Dodds and Law 1998; Sunyer and Lambris 1998; Zarkadis et al. 2001).

9.5.4 Multiple Complement Isoforms

All three pathways are able to trigger the complement system of teleost fish, but the complement system here is distinct from other higher vertebrates by having multiple active forms of C3 as products of individual genes. So far, the rainbow trout (Sunyer et al. 1996, 1997a, b), the sea bream, the carp (Nakao et al. 2001), the medaka (Kuroda et al. 2000) and the zebra fish (Gongora et al. 1998) have been reported to express multiple isoforms of C3 with different binding efficiencies to complement-activating surfaces. In rainbow trout four genes encode complement factor C3 of which three (C3-1, C3-3 and C3-4) are functionally active whereas the fourth (C3-2) apparently lacks the ability to induce haemolysis (Sunyer et al. 1996, 1997a, b). In mammals, two isoforms of C4 have been described (Belt et al. 1984; Yu et al. 1986), and the same is seen in carp (Mutsuro et al. 2005), zebra fish (Gongora et al. 1998) and trout (Boshra et al. 2004), whereas in shark only one isoform is recognized (Terado et al. 2003). In contrast to mammals which have only one C5, two isoforms were isolated from the carp where expression level increased when stimulated with LPS or beta-1,3-glucan (Kato et al. 2003). In contrast to the findings in fish, mammals display only one or two forms of MBL. These show affinity towards mannose whereas the common carp MBL occurs in multiple forms, one with affinity to mannose (carp MBL) and two with affinity to galactose [carp GalBL1 and 2 (Nakao et al. 2006)]. Analysis of the zebra fish genome indicates the presence of three GalBLs and one MBL. The rainbow trout possesses a collectin with supposed affinity for mannose together with two highly (98.3%) homologous collectins with a cryptic carbohydrate-binding motif (Kania et al. 2010). The multiple pathogen recognizing MBL homologues and the multiple C3 and C5 isoforms with different binding specificities expand the immune recognition repertoire against a wide range of pathogens. This provides support for the hypothesis that complement diversity may be an evolutionary strategy for bony fish to compensate for a less developed adaptive immune system (Sunyer and Lambris 1998; Sunyer et al. 1998; Vitved et al. 2000). The primary structures described above are organized as trimers (Holmskov et al. 1994; Holmskov 2000; Sheriff et al. 1994; Wallis and Drickamer 1997) which further oligomerize (Jensen et al. 2005). The trimerization seems to be initiated at the neck regions and continues N-terminally as a zipper (Sheriff et al. 1994). Collectins interact with various ligands present on a large array of bacterial, viral and fungal surfaces. The structural patterns of different oligosaccharides, present as part of glycoproteins, on the surface of the microorganisms as compared to glycoproteins on the surface of host cell surfaces allow for the discrimination between self and non-self carbohydrates. Binding of collectin to the

surface of a microorganism may then initiate different effector mechanisms, such as agglutination, neutralization, lysis or opsonization of the invading microorganism (Van De Wetering et al. 2004).

9.6   Mannose-Binding Lectin (MBL)

9.6.1   The Primary Structure of MBL and Homologues

MBL has been isolated from several mammalian species (Drickamer et al. 1986; Holt et al. 1994; Jensenius et al. 1985; Kawasaki et al. 1978; Sastry et al. 1995), from the chicken (Laursen et al. 1998) and from fish (Kania et al. 2010; Nakao et al. 2006; Vitved et al. 2000). Sequence analysis of an MBL-homologue from a tunicate revealed that the CRD domain of this is homologous to C-type lectins recognizing glucose and was designated GBL (Sekine et al. 2001). Even though a collagen-like domain was lacking in this molecule, an ascidian MASP able to activate C3 was associated with this ascidian GBL (Sekine et al. 2001).

In general, C-type-lectins can be divided into two distinct groups according to their ability to bind different kinds of carbohydrates. The diversity of specificity is based on the orientation of hydroxyl groups at carbon three and four of the carbohydrate. The amino acid triplet Glu-Pro-Asn, also designated the EPN-motif, recognizes mannose-like carbohydrates with equatorial hydroxyl groups on the third and fourth carbon, whereas the Gln-Pro-Asp or the QPD-motif recognizes galactose-like carbohydrates with equatorial/axial hydroxyl groups (Iobst and Drickamer 1994).

Analysis of the amino acid sequence deduced from cDNA of MBL homologues isolated from three members of the carp family indicated galactose specificity instead of mannose specificity (Vitved et al. 2000), and they have now been designated galactose-binding lectins (GalBL). The teleost family Cyprinidae members produce collectins with affinity for galactose-like carbohydrates. The common carp possesses in fact 2 highly homologous but clearly distinct GalBLs (Nakao et al. 2006). In addition, an authentic carp MBL has been described (Nakao et al. 2006). Two transcripts in rainbow trout were designated MBL1 and 2 (Nikolakopoulou and Zarkadis 2006) despite the lack of the structural requirements of a collagen-like region and a neck. Furthermore, these CRDs formed an outgroup, which separated from the other CRDs very early in evolution when they were subjected to phylogenetic analysis (Kania et al. 2010). However, three *bona fide* collectins have been identified in rainbow trout (Kania et al. 2010), one with a structure common to MBL in general (designated MBL homologue 1 or MBLh1), and two with a normal short break and an additional prolonged break of 16 amino acids (MBLh2 and MBLh3). The latter two have a rather cryptic carbohydrate-binding motif consisting of Glu-Pro-Lys (EPK). The specificity of this motif is not clear but a macrophage mannose receptor has such a motif in one of its CRD domains (Taylor et al. 1992; Taylor and Drickamer 1993).

9.6.2 Expression of MBL

MBL is mainly produced by the liver and secreted into the bloodstream (Van De Wetering et al. 2004). Two MBL genes have been identified in mammals. In rodents (Drickamer et al. 1986; Hansen et al. 2000), rabbits (Kawasaki et al. 1978), pigs (Bezouska et al. 1992), and rhesus monkeys (Mogues et al. 1996) both genes are transcribed and translated producing two proteins called MBL-A and MBL-C. MBL-A is the dominant serum protein among rodents, while MBL-C is mainly found in liver cells. The human gene for MBL-A is transcribed, but results in a non-functional truncated product (Guo et al. 1998; Mogues et al. 1996). In addition to expression in the liver, minor but significant expression of MBL has been noted in the kidney and small intestine of mice (Uemura et al. 2002).

In contrast to mammalian MBL, fish MBL homologues seem to have a quite diverse expression pattern. Carp MBL is expressed primarily in the intestine, whereas the two carp GalBLs seem to be complementarily expressed as the GalBL1 was observed in the spleen, the posterior intestine, the gills and the skin and as GaBL2 was observed in the liver, the anterior intestine, the kidney and the head kidney (Fig. 9.4) (Vitved et al. 2000).

In rainbow trout, the three studied MBL homologues were differentially expressed although not as diverse as in the carp (Kania et al. 2010). MBLh1 with supposed affinity for mannose had the highest expression level in the anterior intestine followed by relatively high expression levels in the gill, the pancreas and the skin. Low levels of MBLh1 expression were observed in the head kidney, the kidney, the gill, the posterior intestine,
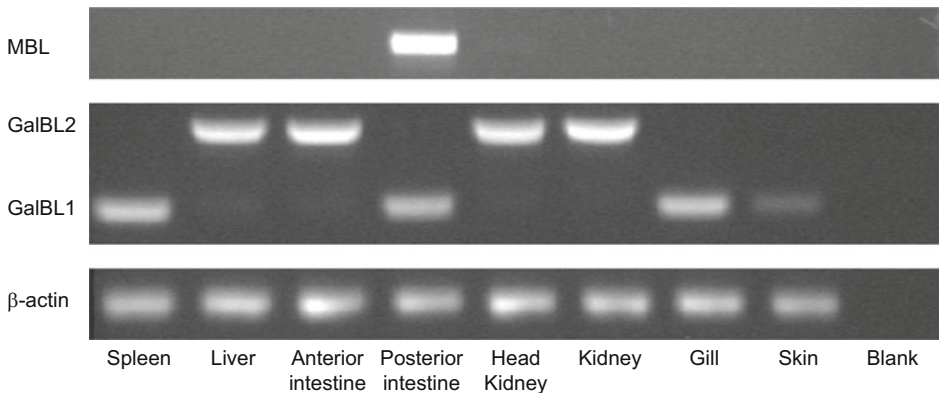
Fig. 9.4 Expression of collectins in the common carp. Purified RNA from organs of a naive was reverse transcribed into cDNA and used as template for PCR. Three genes MBL (fwd. primer: atggcgctgttaatgcatcg, rev. primer: cagttcacacaccacat, annealing temp. 60 °C, product length 735 bp), GalBL1 (fwd. primer: gccagagtcccatactgtt, rev. primer: gcgtatatcaccacaactgc, annealing temp. 56 °C, product length 660 bp), GalBL2 (fwd. primer: gctgttcctcaaggct, rev. primer: gccagagtcccatactgtt, annealing temp. 56 °C, product length 337 bp) and the housekeeping gene β-actin (fwd. primer: ggagaagatctggcatcacacct, rev. primer: ggagaagatctggcatcacacct, annealing temp. 60 °C, product length 264 bp) were analysed

the liver and the spleen. The heart and the muscle do not appear to express MBLh1. The other two MBL homologues possessing the cryptic EPK-motif were quite equally expressed in the analysed tissues. The highest level of expression was observed in the anterior intestine, followed by relatively high levels of expression in the pancreas, the spleen, the posterior intestine, the liver and the skin. A low degree of MBLh2 expression was observed in only the kidney and the skin, whereas a low level of MBLh3 expression was observed in all of the remaining tissues. Immunohistochemical analyses supported the molecular expression study of rainbow trout MBL homologues (Kania et al. 2010). The minute expression of murine MBL-C biosynthesis detected in endothelial cells of the small intestine (Uemura et al. 2002; Wagner et al. 2003) may be a reminiscence of the more profound synthesis observed in fish.

9.6.3   Organization of the Human MBL Gene and a Deduced Genomic Structure of a Homologous Region in a Cyprinid, *Danio rerio* (Zebra Fish)

In humans the genes encoding the collectins reside in a cluster on the long arm of chromosome 10 (q21–24) (Kölble et al. 1993) including the MBL, the SP-D and two SP-A genes. The human MBL consists of 5 exons (Sastry et al. 1989; Taylor et al. 1989; Taylor and Drickamer 2003) spanning approximately 7 kb (Fig. 9.5). The genomic sequence (GenBank accession no. AL954692) from the cyprinid fish *Danio rerio* shows that genes encoding four collectins are arranged as beads on a string (Vitved et al. 2000) (Fig. 9.5). The 3′utrs were estimated from the presence of a supposed poly-adenylation site of the type AATAAA. Three had the QPD-motif (GalBLs) and one the EPN-motif (designated MBL) at the carbohydrate-binding sites. The MBL seems to be organized in the same manner as the human one, taking into account that fish in general have shorter introns. The three GalBLs all have one exon less as the neck regions are joined with the CRD-3′utr exon. Another difference is that exon 1 contains the entire 5′utr.

9.7   MBL-Associated Serine Protease (MASP)

The MBL-associated serine protease, MASP, was discovered when it was co-purified with MBL (Matsushita and Fujita 1992; Takada et al. 1993; Takayama et al. 1994). MASP is homologous to C1r and C1s by sharing the same primary, secondary and tertiary structure. The proenzyme (Fig. 9.3) consists of an N-terminal C1r/C1s/Uegf/bone (complement-urchin-bone) morphogenetic proteins 1 (CUB), an epidermal growth factor (EGF)-like protein, a second CUB, two complement control proteins (CCPs) or short consensus repeats, a short linker and finally a serine protease domain. The MBL binding site on MASP is located at the distal end of the CUB1 domain (Gregory et al. 2004). Two trimers of MBL and a dimer of MASP constitute the smallest functional unit capable of activating
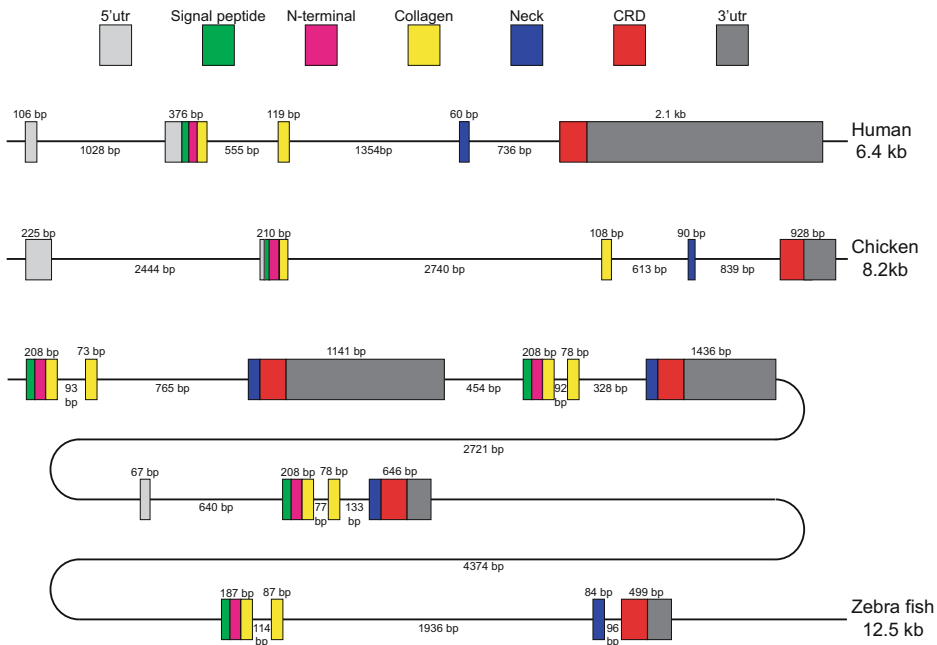
Fig. 9.5 Genomic structure of human, chicken and zebrafish MBL and homologous molecules. The overall genomic structure is well conserved from fish to mammals and birds. In all cases, the signal peptide, the N-terminal region and the 5′ end of the collagen region are part of the same exon; the remaining 3′ end of the collagen region are in a separate small exon

complement through the MBL pathway (Childs et al. 1989; Feinberg et al. 2003). The existence of such complexes in carp serum has been indicated by two-dimensional SDS-PAGE (first dimension, non-reducing conditions; second dimension, reducing conditions) and subsequent N-terminal sequencing of the protein (Nakao et al. 2006). Upon binding of the MBL-MASP complex to activating surfaces MASP is activated by auto-cleavage of the dimer (Ambrus et al. 2003; Gal et al. 2005; Kardos et al. 2001) between the linker and the serine protease domain resulting in two chains, the heavy chain (CUB1→CCP2) and the light chain (linker + the serine protease domain), joined together by a disulphide bridge (Schwaeble et al. 2002; Thiel et al. 1997). MASPs have been identified in all mammalian species investigated so far. In carp (*Cyprinus carpio*) two MASP3-like products have been identified and denoted MASP A and MASP B (Nakao et al. 2001). Truncated forms of these two exist as well (Nagai et al. 2000). Carp also expresses a MASP2, which has been found associated with MBL homologues and has the ability to cleave C4 (Nakao et al. 2006). In rainbow trout a MASP3 gene was identified (Kania et al. 2010). In the jawless lamprey (*Lampetra japonica*) a MASP was found to associate with MBL and the complex was able to cleave C3 (Takahashi et al. 2006). The

origin of MASP has been traced back to invertebrates and two MASPs were cloned from the chordate amphioxus (*Branchiostoma belcheri*) and the solitary ascidian *Halocynthia roretzi* (Endo et al. 2003; Sekine et al. 2001).

9.8 Activation of Complement by MBL and MASP

Oligomers of MBL (Fig. 9.3) are essential for activating complement through C4 and C2 (Lu et al. 1990). Both carp GalBL (Nakao et al. 2006) and rainbow trout MBLh2 and 3 (Kania et al. 2010) have the ability to form higher oligomer complexes comparable to the ones observed in mammals. MASP2 cleaves C2 and C4 (Ambrus et al. 2003; Chen and Wallis 2004; Rossi et al. 2001; Schwaeble et al. 2002; Thiel et al. 1997; Vorup-Jensen et al. 1998, 2000) (Fig. 9.1). Carp MASP2 has been shown to cleave human C4 (Nakao et al. 2006). MASP1 is cable of cleaving C2 (Chen and Wallis 2004; Ikeda et al. 1987; Kawasaki et al. 1983; Matsushita et al. 2000b) and maybe C3 (Matsushita et al. 2000b; Wallis 2002), although the cleavage of C3 has been questioned due to low affinity of MASP1 for C3 (Ambrus et al. 2003). It has been shown by ELISA, using microtiter plates coated with *Salmonella* oligosaccharides, that MBL can activate the alternative pathway in the absence of C2, C4, MASP1, or MASP2 as indicated by deposition of C3 (Selander et al. 2006).

9.9 Interactions Between Complement, Environment and Pathogens

The complement system in fish is one of the most powerful elements of the innate immune response. Chronic stress may affect and depress immunity and immune factors in many ways and thereby complement production. However, acute stress, in contrast, may in fact increase the production of a central complement factor such as C3 (Demers and Bayne 2020) which provides the host with an immediate protection during a short-lived critical situation. In general, the invading microorganisms activate the complement cascade but several of the pathogens are nonetheless able to resist the host response by inactivating one or more complement factors. One example is the bacterium *Aeromonas hydrophila*, which in some cases survives in fresh fish serum containing active complement. This serum resistance has been shown to result from cleavage of complement factor C3, as seen in grass carp, by a bacterial secreted metalloprotease (Chen et al. 2019).

9.10 Conclusion

The complement system of fish plays a prominent role both in innate and adaptive immune responses. The activation of the complement system in fish may follow one or more pathways, the classical, the alternative or the lectin pathways. Activation and the

subsequent formation of the membrane attack complex (MAC) is a major player in host defence, which can act immediately upon the encounter of a pathogen. Fish seems to have a much more complex innate immune system in the context of the lectin pathway compared to mammals. Fish have MBL-like collectins recognizing both mannose and galactose like carbohydrates, whereas mammalian collectins, including MBL, all have affinity for only mannose like carbohydrates. In mammals, the expression is mainly restricted to the liver. MBL forms in fish have a broader and more complex expression pattern. This together with the higher diversity of individual complement factors gives the opportunity for a more complex innate immune system in fish, with the potential to generate a broader diversity both in recognition and immediate elimination of pathogens.

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Cytokines and Immunity

10

Christopher J. Secombes

Abstract

In this chapter the cytokine molecules and pathways present in teleost fish are described, with a focus on the different types of immunity with which they are associated. Whilst there are many molecules conserved between teleosts and mammals, differences do exist. Some novel cytokines are present, some absent (evolved later?) and often multiple copies/paralogues are found due to the extra rounds of whole-genome duplication that have occurred relative to most other vertebrates. In each section the cytokines present in zebrafish are outlined first, since this is an important model species that is increasingly used for in vivo functional analysis. The molecules present in other teleost groups, such as the salmonids, gadoids and perciforms, are then discussed. It will become clear that some important differences exist between these fish groups and this may influence how their response to a particular pathogen is regulated.

Keywords

Teleosts · Cytokines · Antiviral cytokines · Cytokines of type 1–3 immunity · Regulatory cytokines

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Abbreviations

aa	amino acid
AMP	antimicrobial peptide
BAX	BCL2-associated X protein – apoptosis regulator
BCL2	B-cell lymphoma 2 – apoptosis regulator
BP	binding protein
CCR	CC chemokine receptor
cDNA	complementary DNA
Chr	chromosome
CKAP2L	cytoskeleton-associated protein 2 like
ConA	concanavalin A
COX2	cyclooxygenase 2
CXCL	CXC chemokine ligand
DC	dendritic cell
EST	expressed sequence tag
GH	growth hormone
GM-CSF	granulocyte-macrophage colony-stimulating factor
HK	head kidney
HPI axis	hypothalamus–pituitary–interrenal axis
ICE	interleukin-1 converting enzyme/caspase 1
IFN	interferon
Ig	immunoglobulin
IHNV	infectious hematopoietic necrosis virus – a rhabdovirus
IL	interleukin
ILC	innate lymphoid cell
IL-1R	IL-1β receptor
ILT	interbranchial lymphoid tissue
iNOS	inducible nitric oxide synthase
IPNV	infectious pancreatic necrosis virus – a birnavirus
JAK	Janus kinase
LEAP	liver-expressed antimicrobial peptide
LPS	lipopolysaccharide
LT	lymphotoxin
LTi	lymphoid-tissue-inducer
MCSF	macrophage colony-stimulating factor
MoAb	monoclonal antibodies
α-MSH	α-melanocyte-stimulating hormone
NADPH	nicotinamide adenine dinucleotide phosphate
nIL-1F	novel IL-1 family member
NK	natural killer

NLRP   NLR family pyrin domain containing
NO   nitric oxide
ORF   open reading frame
PAMP   pathogen-associated molecular pattern
PBL   peripheral blood leucocytes
PHA   phytohemagglutinin
PMA   phorbol 13 acetate 12 myristate
Poly I:C   polyinosinic:polycytidylic acid
PTEN   phosphatase and tensin homolog
PURB   transcriptional activator protein Pur-beta
ra   receptor antagonist
RAG   recombination activating gene
Rel   related
ROR   RAR-related orphan receptor
ROS   reactive oxygen species
SMG   salmonid Mx group
SOCS   suppressor of cytokine signalling
STAT   signal transducer and activator of transcription
SVCV   spring viraemia of carp virus – a rhabdovirus
TGF   transforming growth factor
TNF   tumour necrosis factor
TSLP   thymic stromal lymphopoietin
UTR   untranslated region
WGD   whole-genome duplication

10.1   Introduction

The bioactivity of teleost cytokines has been studied for many decades but it is within the last two that most progress has been made in characterising them at a molecular level. This was aided by extensive EST libraries followed more recently by genome sequencing, allowing the loci to be mapped quite comprehensively in many species. At first sight, the cytokine network of teleost fish looks quite similar to that of the well-studied mammalian network. There are many molecules conserved between teleost and mammalian species, and where data is available for the teleost cytokines they typically elicit responses akin to the known function of their mammalian equivalents. However, differences do exist, with some novel molecules present, some absent (evolved later?) and often multiple copies/ paralogues are found in fish. The latter phenomenon results from the extra rounds of whole-genome duplication (WGD) that have occurred relative to most other vertebrates, firstly at the base of the teleosts (the so-called 3R WGD) and again in particular lineages (e.g. salmonids, some cyprinids – a 4R WGD). Hence two or four copies of a cytokine present in other vertebrates can exist, giving added complexity to their responses. In this

chapter the cytokine molecules and pathways present in teleosts will be outlined, with a focus on the different types of immunity with which they are associated (Table 10.1). The main exception will be the chemokines, a complex subgroup that control the movement of leucocytes to lymphoid tissues and sites of infection. They have received several recent reviews (Chen et al. 2013; Nomiyama et al. 2013; Bird and Tafalla 2015; Liu et al. 2020a) and so will not be reported here. The cytokines present will be described initially for zebrafish, as an important model species that is increasingly used for in vivo functional analysis using reporter fish and mutants. Studies in other cyprinids will then be outlined, followed by a review of what is known of the genes in other major teleost clades, especially the Euteleostei that include many important species for aquaculture and fisheries, such as the Protacanthopterygii (e.g. salmonids), Paracanthopterygii (e.g. cods) and Acanthopterygii (e.g. perches, flatfish, pufferfish, etc.). It will become clear that some important differences exist between these fish groups and this may influence how their response to a particular pathogen is regulated.

10.2 Antiviral Cytokines

The key cytokines involved in antiviral immunity are the interferons (IFN), present in all jawed vertebrates and released from host cells to effect an antiviral state (Redmond et al. 2019). Three broad categories are known, termed simply type I–III. Type I and III are primarily involved in antiviral defence whilst type II IFN (IFN-γ) have a broader function and contribute to cell-mediated immunity against a variety of pathogens during adaptive immune responses. Within the teleost fish type III IFN are absent, as in other ray-finned (actinopterygian) fish but there has been an expansion of type II IFNs, which will be discussed under "Cytokines of Type 1 Immunity" below. Hence the focus of this section will be to outline the type I IFN that are present in different teleost groups.

In zebrafish 4 type I IFN are present, three on Chromosome (Chr) 3, linked with growth hormone (GH) (amongst other genes) and one on Chr 12 linked with CD79b (Liu et al. 2020b). This seems to be a typical situation for cyprinids (Li et al. 2020a) that have not undergone a further genome duplication (i.e. 2R fish). However, some cyprinids, such as the common carp have undergone an allotetraploidization event (Zhu et al. 2015; Xu et al. 2019), and the IFN loci are duplicated giving two loci with three genes both linked to GH and two with a single gene linked to CD79b (Liu et al. 2020b). Many functional studies have been performed with cyprinid IFNs and show that indeed they have potent antiviral activities, can induce more than 400 genes, termed IFN-stimulated genes (ISG), and help reduce mortality following viral challenge (López-Muñoz et al. 2009; Xia et al. 2018; Zhou et al. 2019; Levraud et al. 2019). However, whilst only four IFN genes are present, they show differential regulation (Chen et al. 2015; Feng et al. 2016) and even use different receptors (Aggad et al. 2009), the latter finding is unusual in that all IFN within a "type" in tetrapods use the same receptor. The two receptors identified in zebrafish are composed of a common short chain (CRFB5) and a longer private chain (CRFB1 or CRFB2).

Table 10.1 Grouping of teleost cytokines by function

Function	Cytokines involved
Antiviral responses	Type I IFN: group 1 (IFNa,d,e,h), group 2 (IFNb,c), group 3 (IFNf)
Pro-inflammatory responses	IL-1β, TNFα, IL-6, IL-11, M17
Type 1 immunity	IFN-γ, IFN-γ-rel, IL-2, IL-15, IL-15L, IL-12 family, IL-18
Type 2 immunity	IL-4/13A, IL-4/13B, IL-20L
Type 3 immunity	Il-17A/F, IL-17B, IL-17C, IL-17D, IL-17N, IL-21, IL-22, IL-26
Regulatory responses	nIL-1F, IL-10, IL-35, TGF-β1

The cytokine molecules in teleost fish are grouped in this chapter by the predominant type of immunity with which they are associated, as listed above. However, chemokines are not reported here

Compared to other teleost groups, zebrafish/cyprinids have relatively few IFN genes in terms of subgroups present and gene number (Liu et al. 2020b). To date 7 subgroups have been discovered for the teleost type I IFN, termed IFNa-IFNf and IFNh, although no fish are known to possess all subgroups. Curiously in zebrafish the four genes present include three different subgroups: IFNa and IFNc (x2) linked to GH and IFNd linked to CD79b. The largest number of subgroups exist within the Protacanthopterygii that include the well-studied pike and salmonids. These fish possess six subgroups (IFNa-IFNf), typically with multiple copies, especially in the salmonids, although the exception is IFNd that remains as a single copy and the only gene present at the CD79b locus (as in zebrafish/cyprinids) (Liu et al. 2020b). The salmonid whole-genome duplication event (Lien et al. 2016) gave rise to two IFN loci linked to GH but only a single locus exists linked with CD79b. Interestingly the two GH-linked IFN loci are diverging, in that (1) IFNb has been lost at locus 1 (present as pseudogenes), (2) at locus 2 there has been an expansion of IFNe and (3) the IFNc genes from both the loci cluster separately in phylogenetic tree analysis (Liu et al. 2020b). In addition, relative to pike there is a reduction of genes at locus 1 but an expansion at locus 2. Currently rainbow trout has the largest IFN repertoire known in teleosts, with some 34 IFN genes at these three loci.

In the neoteleostei that include the Paracanthopterygii and Acanthopterygii, quite different IFN loci are found although still linked to GH and CD79b. In these fish a new subgroup has arisen, IFNh, at the GH-linked locus. In Atlantic cod (gadoids) IFNd has been lost at the CD79b locus, and only a single IFNb and IFNh are present. This is also true in haddock although the linkage has not been confirmed. Similarly in the perciforms typically a single IFNh is present at the GH-linked locus (although 2 are present in tilapia), sometimes together with a single IFNc, as in the flatfish (e.g. flounder, turbot), large yellow croaker and tilapia (Gan et al. 2020a). However, in some species the IFN genes have been lost at this locus, as seen in tetraodon and icefish (Liu et al. 2020b). Interestingly, there has been an expansion of IFNd at the CD79b-linked locus in the Acanthopterygii, with many species having 2–4 genes but some have as high as 12–18 IFNd.

From an evolutionary perspective, IFN genes arose early in vertebrate evolution and are found in cartilaginous fish as well as non-teleost actinopterygians (Chondrostea, Holostea)

(Redmond et al. 2019; Liu et al. 2019). Three major groups of type I IFN can be identified in the actinopterygians, with group 3/IFNf having relatedness with the cartilaginous fish IFN genes and is thus an ancient IFN. A single type of group 1 (IFNe) and group 2 (IFNb/c) genes are present in the Chondrostea (e.g. sturgeon), with the latter having diverged into IFNb and IFNc in Holostea (e.g. gar). Subsequent expansion of the group 1 genes occurred with the emergence of teleosts, when the locus was duplicated (Boudinot et al. 2016) and initially IFNa (group 1) appeared (at the GH-linked locus). Curiously IFNd (group 1) has not been found in early teleosts such as eels and bonytongues (Huang et al. 2019a; Liu et al. 2020b), where IFNc or IFNc and IFNf are present at the CD79b-linked locus. Hence, during teleost evolution there has been a progressive gain or loss of IFN subgroups, and in some cases complete loss of IFN genes at one of the two loci.

Whilst many functional studies have now been performed in a wide range of fish species/groups (Pereiro et al. 2014; Zou and Secombes 2016; Milne et al. 2018; Laghari et al. 2018; Ding et al. 2019; Huang et al. 2019a; Stosik et al. 2021), as outlined above for the cyprinids, there is still much to be learnt about the function of the different IFN genes. In the case of rainbow trout where 6 IFN subgroups are present, it is clear that differences exist in the speed and magnitude of expression following viral infection (Zou et al. 2014) and that one of the subgroups (IFNa) is able to produce intracellular forms of the IFN as an additional means to control the spread of infection (Chang et al. 2013).

Recent evidence in channel catfish suggests that the loss of IFNb, IFNe and IFNf in cyprinids did not occur in the siluriformes, that also have an expanded IFNd repertoire (Drs Sylvie Quiniou & Pierre Boudinot, personal communication). Thus, this teleost group also have six IFN subgroups present, as in the Protacanthopterygii (e.g. pike and salmonids).

10.3   Pro-inflammatory Cytokines

Inflammation is the reaction that occurs following wounding and/or infection, or exposure to other harmful stimuli. It functions to try to eliminate any potential pathogens at the site of damage, to remove dead and dying cells and begin the process of tissue repair. It is initiated as part of the innate immune defences and involves the influx of granulocytes and macrophages to the site, directed by the release of chemokines. These cells trigger further downstream antimicrobial mechanisms and cell activation, via the release of a cascade of cytokines that are thus considered to be pro-inflammatory. These cytokines include IL-1β, TNFα, IL-6, IL-8 (a chemokine also called CXCL8), IL-12, IL-18 and IFN-γ. The cytokine response to Gram-positive and Gram-negative bacteria is similar, although Gram-positive bacteria stimulate a larger release of IL-12, IFN-γ and TNFα whilst Gram-negative bacteria induce higher levels of IL-6 and IL-8 (from human blood mononuclear cells) (Hessle et al. 2005; Skovbjerg et al. 2010). These differences are linked to the PAMPs in the bacterial cell walls that trigger cytokine release following binding to pattern recognition receptors on the phagocyte cell surface. In addition, the cytokines appear in a sequential manner, with IL-1β and TNFα released first and considered proximal cytokines, that help augment the

response by promoting the secretion of distal cytokines such as IL-6 and IL-8. These cytokines are all present in teleost fish. IL-1β, IL-6, TNFα and some of their cytokine family members are discussed in this section, and are commonly examined together in fish studies looking at inflammatory responses to infection (Roy et al. 2017). IL-12, IL-18 and IFN-γ will be described under "Cytokines of Type 1 Immunity".

10.3.1 Interleukin-1β

IL-1β was one of the first cytokines to be cloned in teleosts (Zou et al. 1999a, b). It is one of three IL-1 family cytokines in fish, which also include IL-18 and nIL-1F (see later). In zebrafish the IL-1β gene is found on Chr 10, and has a 7 exon/6 intron organisation, with 6 coding exons, as found in tetrapods and cartilaginous fish (although the first non-coding exon can be missing in some species) (Husain et al. 2012). It is produced as a precursor molecule without a signal peptide that is cleaved to release a bioactive mature protein. This process has been studied in detail in zebrafish, where it is linked to the activation of a classical inflammasome that is responsible for IL-1β secretion of the 18 kDa mature protein (Li et al. 2020b). Functional studies have taken advantage of the ability to generate transgenic zebrafish expressing the mature IL-1β under the control of various promoters (Hasegawa et al. 2017; Delgadillo-Silva et al. 2019), the generation of reporter fish where a reporter gene (e.g. GFP) is inserted under the control of the IL-1β promoter (Hasegawa et al. 2017; Ogryzko et al. 2019) and fish that have had the IL-1β gene disrupted using the CRISPR approach (Tsarouchas et al. 2018; Ogryzko et al. 2019). An example is the visualisation of IL-1β up-regulation in macrophages early after challenge with *Mycobacterium marinum*, with IL-1β stimulation of neutrophils triggering NO production that can lead to reduced infection (Ogryzko et al. 2019). In contrast, inhibition of IL-1β (in null mutants) reduces the NO response and increases the bacterial burden. Epithelial cells have also been shown to be important producers of IL-1β following tissue injury (Hasegawa et al. 2017), in addition to macrophages (Ogryzko et al. 2014), and that IL-1β-induced inflammation is an important aspect of fin regeneration.

In carp two IL-1β genes are present, likely a consequence of the WGD in this lineage, with 74% amino acid (aa) identity between IL-β1 and IL-1β2 (Engelsma et al. 2003). Multiple copies of IL-1β2 appear to exist, thought to be due to one or more duplications of the IL-1β2 gene in this species. IL-1β1 is expressed more highly but both are up-regulated by infection and stimulation in vitro with PAMPs. Transcripts that are not fully spliced have been detected, containing introns 5 and 6 or intron 5 only (Engelsma et al. 2001). These partially processed RNAs have also been seen in salmonids (Zou et al. 1999b) and may represent one means of post-transcriptional regulation of the mature protein product. Involvement of IL-1β in the HPI axis has also been suggested in carp, where following stress (restraint) hypothalamic IL-1β expression increases together with IL-1R1 expression in head kidney and the pituitary pars intermedia (Metz et al. 2006). In addition, carp rIL-1β, in vitro, increases the release of α-MSH and β-endorphin from the pituitary gland.

Interestingly, in barbel steed rIL-1β stimulation of monocytes/macrophages at low concentrations increases IL-1R1 but inhibits IL-1R2 expression whilst at high doses both IL-1R1 and IL-1R2 increase (Zhou et al. 2020a). IL-1R2 lacks a signalling domain and is considered to be an endogenous inhibitor of IL-1β action, thereby helping to prevent host tissue damage from IL-1β overexpression. Lastly, in catfish two IL-1β genes have also been described (gene 1, gene 2), that appear to result from a tandem duplication on the same chromosome (Wang et al. 2006a). The genes are highly similar, with 94.3% aa identity of the encoded proteins, suggesting a recent duplication event. Both exhibit a wide tissue distribution, with gene 1 more highly up-regulated following bacterial infection.

In salmonids three functional IL-1β genes (IL-1β1–3) and a pseudogene (IL-1β4) have been discovered, and appear to have been generated by the teleost wide WGD, followed by the salmonid (4th) WGD (Husain et al. 2012). This suggests that some teleost groups have lost one of these loci. A clue comes from gene synteny and gene organisation analysis. For the type I IL-1β genes (IL-1β3/4), IL-1β is next to PURB and the genes have the classical 7 exon arrangement (as in zebrafish). For type II IL-1β genes (IL-1β1/2), CKAP2L is inserted between IL-1β and PURB, and the trout/salmon genes have a 6 exon organisation (5 coding exons), with apparent loss of a coding exon at the N-terminal relative to type I genes. Interestingly, CKAP2L is at the end of the IL-1β locus on human Chr 2. The two type II paralogues share a higher aa identity (75–79%) than with IL-1β3 (30–32%), with a predicted Atlantic salmon IL-1β4 sequence showing a similar trend. A second allele of IL-1β1 has also been reported in trout, where a SINE insertion into intron 3 gives a 211 bp difference that can be easily distinguished by PCR (Wang et al. 2004). In trout monocytes/macrophages, IL-1β1 and IL-1β2 are more highly induced by PAMPs than IL-1β3. However, all three are induced by the mitogen PHA, with IL-1β2 the highest, but after viral infection it is IL-1β1and IL-1β3 that are induced (Husain et al. 2012). Many studies of the bioactivity of the trout protein and derived peptides have been undertaken (Zou and Secombes 2016). An example is the microarray study of Martin et al. (2007a), where trout rIL-1β was shown to up-regulate a discrete set of genes in a macrophage cell line (RTS-11 cells), which have a clear role in the inflammatory response. In vivo effects have also been studied, with an increase in disease resistance seen in the short-term following injection (Hong et al. 2003), and vasoactive effects on the cardiovascular system (Costa et al. 2015). Interestingly peptides derived from the IL-1β sequence have clear stimulatory effects but fail to induce cortisol release, seen when injected IL-1β activates the HPI axis (Holland et al. 2002).

IL-1β has also been found in gadoids, such as haddock and cod, but only a single type II IL-1β is present (Corripio-Miyar et al. 2007; Seppola et al. 2008; Hu et al. 2018). It has the same gene organisation as seen with salmonid type II molecules, namely 6 exons/5 introns, and is highly induced by PAMPs and infection as expected. However, in a wide range of perciform species both a type I and type II gene are present (Husain et al. 2012; Angosto et al. 2014; Eggestol et al. 2020). The type I molecules have retained the 7 exon arrangement (although the first non-coding exon is not always apparent), with the exception of lumpfish where a highly truncated 5′ end is present (Eggestol et al. 2020). However, the

type II molecules have a further reduction in exon number, to 5 exons (4 coding), due to a possible fusion of exons 2 and 3 as defined by the salmonid and gadoid equivalents. Nevertheless, some perciforms appear to lack the type I molecule, such as the pufferfish. Comparative expression analysis of the two paralogues has shown that type II IL-β is the main form induced following in vitro PAMP stimulation of lumpfish head kidney (HK) cells (Eggestol et al. 2020) and in spleen of turbot injected with LPS or bacteria (Chen et al. 2019a). The recombinant type II IL-1β protein has been made in gilthead seabream and orange-spotted grouper, and shows typical bioactivity for phagocytes/leucocytes (Lu et al. 2008; Angosto et al. 2014).

10.3.2 Tumour Necrosis Factor

TNFα is a member of the TNF superfamily (TNFSF) that are mostly type II transmembrane proteins that function as trimers, and many can be cleaved to release a soluble cytokine. Their presence and genomic loci have been well studied in teleosts (Glenney and Wiens 2007; Biswas et al. 2015; Marín 2020; Li et al. 2021a), and several novel genes/clades have been described. In zebrafish there are 19 known genes that include two TNFα genes (on Chr 15 and Chr 19), as well as a novel TNF termed TNF-new (TNF-N) on Chr 15 adjacent to TNFα and in the same orientation (Savan et al. 2005; Glenney and Wiens 2007). This is similar to the situation in tetrapod genomes where TNFα is adjacent to TNFβ/LTα (same orientation) and LTβ (opposite orientation). There has been some debate as to the relatedness of TNF-N to other TNFSF, especially as it has some features similar to LTβ in being a transmembrane protein without a clear enzyme cut site, and that the LTβR exists in fish. However, more robust analysis (Marín 2020) suggests this is an independent duplication of TNFα that arose in the clupeocephala (otomorphs and euteleosts) and has diverged rapidly (less than 20% aa identity between TNFα and TNF-N). In addition, functional studies have revealed that teleost LTβR binds a different TNFSF member, LIGHT, as also occurs in mammals (hence LIGHT was probably the original ligand) and does not bind TNF-N (Maeda et al. 2018).

The two TNFα genes in zebrafish are likely due to the teleost WGD, with large blocks of conserved genes at both loci. The gene on Chr 15 has been termed a fish type I TNFα, and that on Chr 19 a type II TNFα (Hong et al. 2013). The proteins differ in that the stalk between the transmembrane domain and the enzyme (TNFα converting enzyme – TACE) cut site is longer in type I TNFα and there is a 2 aa insertion between beta-strands 9 and 10 of the mature protein in type II TNFα (Hong et al. 2013). Both genes show a similar expression profile in different tissues and are induced to similar levels in HK cells stimulated with LPS, although expression of the type II TNFα increases more rapidly (Kinoshita et al. 2014). Zebrafish double reporter lines, for TNFα and macrophage expressed gene 1 (MPEG1), have shown the rapid recruitment of TNF[+] macrophages to the wound site following caudal fin amputation (Nguyen-Chi et al. 2017). Similarly, in a spinal cord regeneration model, it was shown that macrophages are the main cells

expressing TNFα at the injury site and that TNFα is necessary for axonal regeneration as revealed by TNFα knock down (by CRISPR) (Tsarouchas et al. 2018). Following infection of embryos with *Mycobacterium abscessus*, double reporter fish (for TNF and macrophage or neutrophil markers) again confirmed that macrophages are the major source of TNFα, initially at the injection site and later in the *M. abscessus* containing macrophages and within granulomas (Bernut et al. 2016). Knock-down of the TNFR1 with morpholinos, which has no impact on embryo survival, increases severity of infection verifying the crucial protective role of this pathway. However, in the case of viral infection, studies with rhabdoviruses and birnaviruses suggest that TNFα can have a negative impact. For example, zebrafish injected with rTNFα together with SVCV show enhanced mortality relative to fish given SVCV alone (Roca et al. 2008), and this effect appears to be due to blocking of the host autophagic response that is needed for viral clearance (Espín-Palazón et al. 2016). In the case of IPNV, infection of the zebrafish ZF4 cell line results in both apoptosis and secondary necrosis, as seen in vivo, and this can be blocked by RNA interference (siRNA) of TNFα production (Wang et al. 2011a). Such results demonstrate that, dependent upon the cellular context in which the signal is received, TNFα can mediate survival or apoptosis of cells.

Many copies of TNFα have been found in carp. Initially, three were found that were all type II molecules (Saeij et al. 2003; Savan and Sakai 2004). The proteins differ in size, being 237 aa for TNF1, 231 aa for TNF2, and 227 aa for TNF3. They show relatively high aa identity (72–80%) but their origin is not clear. TNF2 has a polymorphism at position 24 of the mature peptide, which codes for a proline (TNF2pro) or serine (TNF2ser). Following the full-body transcriptome of carp being sequenced (Kolder et al. 2016), two further copies (240 aa, 245 aa) have been found that are type I molecules (Milne et al. 2017), which in multiple aa alignment have a longer stalk and the 2 aa deletion (Wang, SFIRC, personal communication) as outlined above. Only the function of the type II molecules has been studied to date. TNF1 and 2 are induced in HK phagocytes by LPS and *Trypanoplasma borreli* lysate, with expression of TNF2 higher (Saeij et al. 2003), as also seen after *T. borreli* infection (Forlenza et al. 2009). This effect is ablated when the cells are treated with an inhibitor of NF-κB, as also seen with grass carp rTNFα-stimulated HK cells (Zhang et al. 2012). Interestingly, in a carp line susceptible to the parasite *T. borreli* only TNF2pro is present, whilst in a trypanotolerant line both isoforms are found. Knock down of TNFα in vivo, with specific neutralising antibodies, has been shown to impact behavioural fever in response to infection with cyprinid herpesvirus 3 (CyHV-3) and reduces survival (Rakus et al. 2017). Indeed, CyHV-3 is able to express a soluble decoy receptor homolog (ORF12) for TNFα to manipulate the host immune response. rTNF1 and rTNF2 have been studied comparatively and both can induce the expression of a range of proinflammatory genes in endothelial cells, with rTNF2 being more potent (Forlenza et al. 2009). They are also directly trypanolytic but have no effect on phagocyte activity or leucocyte migration. In contrast, goldfish rTNFα is able to enhance monocyte/macrophage phagocytosis, respiratory burst activity, NO production and chemotaxis (Grayfer et al. 2008). Using a variety of catfish cell lines, TNFα expression was shown to occur in T cells and macrophages but not B cells or fibroblasts (Zou et al. 2003a).

In salmonids four copies of TNFα exist that consist of a pair of type I (TNF1, TNF2) and type II (TNF3, TNF4) genes (Hong et al. 2013; Veenstra et al. 2018). The proteins share 80–88% aa identity within each pair but only 44–47% aa identity between the pairs, typical of genes duplicated by the teleost WGD and salmonid WGD events. TNF4 is relatively understudied, with one of the few comparisons of the four genes being in adipose tissue after *Aeromonas salmonicida* challenge of rainbow trout, where TNF1 and 2 are induced the most and TNF3 the least (Veenstra et al. 2018). However, several studies have compared TNF1, 2 and 3 in trout. Analysis of constitutive expression in a range of tissues (14) showed that all are expressed, with TNF1 highest (Hong et al. 2013). In a time-course study of LPS-stimulated RTS-11 cells TNF3 increases first, with TNF2 and TNF3 having a similar magnitude of induction. Similarly using flagellin to stimulate RTS-11 cells, TNF3 is up-regulated first and by the greatest magnitude (Wangkahart et al. 2016). Differential kinetics of induction is also seen in vivo after infection. However, following vaccination against Enteric Redmouth Disease (caused by *Yersinia ruckeri*), all three genes are induced rapidly in spleen (Wangkahart et al. 2019). Curiously PHA stimulation of HK cells preferentially increases TNF3 expression, perhaps reflecting induction in T cells (Hong et al. 2013). The function of the three proteins has also been studied, where in general similar bioactivity is seen across a range of assays (Zou et al. 2003b; Hong et al. 2013). Visualisation of TNFα-producing cells in tissues of infected salmon and trout has begun to be undertaken with the development of anti-TNFα antisera (Yousaf et al. 2012; Powell et al. 2014). The use of anti-TNFα antisera to inhibit trout T-cell growth in macrophage-derived conditioned media suggests a role for TNFα in T-cell development (Hino et al. 2006).

Most perciform fish have a type I and a type II TNFα molecule, although in some species only a single gene has been identified to date. Expression of the two genes has been studied comparatively in a few species (Lam et al. 2011; Pleić et al. 2014; Milne et al. 2017; Cui et al. 2020a; Huang et al. 2020), with many examples of differential expression detected. For example, in orange-spotted grouper PBL stimulated with LPS the type II TNFα is induced rapidly (within 2 h) whilst the type I transcript increases later (24 h) (Lam et al. 2011). In general, similar bioactivities of the two proteins have been found (Cui et al. 2020a; Huang et al. 2020), although in large yellow croaker only the type I rTNFα is able to increase phagocytosis by monocytes/macrophages (Huang et al. 2020). Other notable findings in these fish are the ability of mandarin fish rTNFα to induce apoptosis of epithelial (HeLa) cells (Xiao et al. 2007), the use of TNFα as an oral adjuvant in sea bass (Galindo-Villegas et al. 2013), the visualisation of TNFα[+] macrophages in tissues of *Enteromyxum scophthalmi* infected turbot by immunohistochemistry (Ronza et al. 2015), and the demonstration of circadian oscillation of TNFα expression in medaka (Onoue et al. 2019). Curiously an eel TNFα has been characterised that appears to have features of type I and II molecules (Feng et al. 2014). Similarly, in phylogenetic analysis of teleost TNFα sequences, the more basal groups (eels, herring, bony tongues) separate as a single discrete clade (Eggestol et al. 2020), and it is possible that the divergence to type I and type II TNFα happened later in teleost evolution.

10.3.3 Interleukin-6 and Related Cytokines

IL-6 is considered a key cytokine of the acute phase response but is also involved in B-cell differentiation into antibody-secreting cells. It is a member of the IL-6 family that are long type I cytokines with four α-helices, which mostly use gp130 as a common signal-transducing component of their receptors (Metcalfe et al. 2020). IL-6 can signal in three ways, (1) classically by binding the IL-6R and gp130, (2) by trans-signalling via binding to a soluble IL-6R (known to occur in teleost fish, Wang et al. 2019a) and subsequently to a membrane-bound gp130, and (3) by trans-presentation, where IL-6 binds IL-6R on one cell and gp130 on a different cell which undergoes signalling. IL-6 was first discovered in teleost fish by genome analysis (Bird et al. 2005a), and in zebrafish is located on Chr 19 and translates into a 231 aa protein (Varela et al. 2012). It is highly expressed in kidney and spleen and induced following injection of PAMPs, and during caudal fin regeneration following amputation (Li et al. 2021b). Similarly, in grass carp, in vitro stimulation of HK cells with killed *Aeromonas hydrophila* rapidly enhances IL-6 expression, as does rIL-6 treatment (Wen et al. 2020). rIL-6 also transiently increases IL-10 expression, and this effect is dependent upon STAT3 phosphorylation. However, oral administration of LPS to common carp down-regulates HK IL-6 expression (Kadowaki et al. 2013).

Within salmonids two IL-6 genes are present, termed IL-6A and IL-6B, likely due to the salmonid WGD event (Veenstra et al. 2017; Eggestol et al. 2020). They have been studied comparatively on only a few occasions, as seen in HK of Atlantic salmon post-vaccination (Beckmann et al. 2020), and in adipose tissue of rainbow trout following vaccination, infection with *A. salmonicida*, or injection of flagellin (Veenstra et al. 2017, 2018), where similar levels of induction are seen. The rainbow trout rIL-6A has been studied and shown to enhance macrophage growth, AMP (cathelicidin 2, hepcidin, LEAP2), SOCS (SOCS1-3) and IRF1 gene expression but to down-regulate macrophage IL-1β and TNFα expression (Costa et al. 2011). In addition to its effects on macrophages, trout rIL-6 can induce (IgM[+]) B-cell proliferation, Blimp1 expression and increase IgM secretion, suggesting it is an important differentiation factor for B cells (Abós et al. 2016). Similarly in perciforms rIL-6 affects antibody secretion in fugu IgM^{+} B cells (Kaneda et al. 2012) and Ig levels after injection into orange-spotted grouper (Chen et al. 2012), the latter associated with increased GATA3 expression – a transcription factor associated with Th2 cells. In flounder (Guo et al. 2017) and humphead snapper (Huang et al. 2019b) IL-6 has been used as a vaccine adjuvant, giving better serum antibody levels and protection whether used in protein- or DNA-based vaccines. Lastly, in lumpfish two isoforms (i1, i2) of IL-6 have been found, with the transcript for IL-6i2 retaining intron 2 which translates in-frame into the signal peptide region of the protein, making it unclear if it can be secreted (Eggestol et al. 2020). This region is unusual even in IL-6i1, due to a relatively long coding region in the first exon (94 bp vs 16 bp in other teleosts). Typically the IL-6 gene is composed of 5 exons, as also seen in the human gene, although an intron insertion appears to have happened in exon 5 of the tilapia gene (Eggestol et al. 2020).

Other members of the IL-6 cytokine family of note in relation to inflammatory responses are IL-11 and M17. IL-11 is a pleiotropic cytokine, with functions (in mammals) ranging from promoting haematopoiesis, bone formation and the acute phase response, to regulating T cells and inhibiting pro-inflammatory cytokine expression. It was first discovered in teleosts by analysis of cDNA libraries, from bacterial-challenged fish, that underwent suppression subtractive hybridisation (SSH) (Wang et al. 2005). It was quickly realised that two types of IL-11 exist in teleosts (IL-11a, IL-11b), with the two zebrafish molecules sharing just 27% aa identity (Huising et al. 2005). Only IL-11a has been found in carp to date, where it is induced by ConA and LPS in HK macrophages. In crucian carp, IL-11a is one of the most highly up-regulated genes in CyHV-2-infected fish (Podok et al. 2014). It is also induced following bacterial (*A. hydrophila*) infection, where a much faster increase is seen in kidney relative to virally challenged fish. In trout, IL-11a is highly expressed constitutively in intestine and gills, and can be induced in immune tissues after bacterial infection (*A. salmonicida*) and in vitro after stimulation of macrophages with PAMPs or rIL-1β (Wang et al. 2005). Whilst both IL-11a and IL-11b are known in pufferfish only one has been found in other perciform fish to date, e.g. IL-11a in large yellow croaker and redlip mullet (Kasthuriarachchi et al. 2020; Zhu et al. 2020) but IL-11b in Atlantic halibut and Japanese flounder (Santos et al. 2008; Øvergård et al. 2012). IL-11b expression is broadly similar to that of IL-11a in other species, in being induced by PAMPS in vitro and by infection in vivo. However, the only functional data on IL-11 comes from the study of IL-11a. Over-expression of IL-11a in a yellow-croaker kidney derived cell line (LCK) results in up-regulation of Mx and TNFα, whilst in contrast treatment of kidney cells with redlip mullet rIL-11a down-regulates IL-1β and TNFα expression and inhibits (entirely or partially) LPS stimulation of these genes. Down-regulation of BLC2 and BAX by rIL-11 was also found, and suggests a possible anti-apoptosis role in fish.

A novel IL-6 family member was discovered in carp SSH cDNA libraries and given the clone name M17 (Fujiki et al. 2003). The gene organisation is very similar to oncostatin M (OSM) and leukaemia inhibitory factor (LIF), two IL-6 family members present in mammals, with which it clusters in phylogenetic tree analysis (Secombes et al. 2016). Since LIF has been identified in all other jawed vertebrate groups, and LIFR is present in teleosts (Hanington and Belosevic 2005), it is possible it is a divergent teleost LIF or closely related gene. In zebrafish M17 is found on Chr 5, in a region with synteny to the locus containing LIF and OSM on human Chr 22. It is expressed from 24 h post-fertilisation and is constitutively expressed in adult kidney and brain (Hanington et al. 2008). It is induced in zebrafish retinal ganglion cells after optic nerve injury, as well as LIFR and other IL-6 family members, and may contribute to optic axon regeneration (Elsaeidi et al. 2014). LIF knockdown with morpholinos impairs axon sprouting in vitro and in vivo delays functional recovery from optic nerve injury (Ogai et al. 2014). In carp M17 expression is detectable in brain and PBL, and in peritoneal cells elicited by injection of sodium alginate (Fujiki et al. 2003). The recombinant goldfish protein can induce NO production from macrophages, and this effect is increased in the presence of LPS (Hanington and Belosevic 2007). In addition, goldfish rM17 induces the differentiation

of monocytes into macrophages and enhances the proliferation of macrophage progenitor cells seen with macrophage conditioned medium, and clearly has a role in myelopoiesis. Rainbow trout M17 is highly induced in spleen after bacterial infection (*Y. ruckeri*) and in vitro by LPS, poly I:C or rIL-1β stimulation of RTS-11 cells (Wang and Secombes 2009). In perciforms the M17 gene has been identified in the genomes of pufferfish and stickleback but expression levels have only been studied in the Japanese flounder (Hwang et al. 2007). M17 is up-regulated in flounder HK cells by poly I:C, LPS and peptidoglycan, although with different kinetics (faster induction with poly I:C) (Hwang et al. 2007), again suggesting a role in the early response to infection.

Overall, the key cytokines involved in eliciting pro-inflammatory responses are present in teleost fish and show a high level of conservation with their counterparts in other vertebrate groups. The additional paralogues do show some functional differences, especially in terms of where and when they are expressed, whilst the encoded proteins tend to have similar bioactivity (where known). Zebrafish models of inflammation are proving extremely useful to elucidate the function of these genes, with the information from target species allowing generalised network pathways to be established.

10.4 Cytokines of Type 1 Immunity

The responses that protect against intracellular infection (from viruses, bacteria and protozoa) and cancer cells are termed type 1 immunity/cell-mediated immunity (Annunziato et al. 2015; Yamaguchi et al. 2015). The crucial cytokine associated with such responses is IFN-γ, which as part of its pro-inflammatory function activates macrophages to more effectively kill such pathogens/cells. IFN-γ is released from several major cell types, including CD4$^+$ Th1 cells, type I CD8$^+$ cells (Tc1) and their functional counterparts in the innate immune system, namely classical Tbet$^+$ ILC1 cells and NK cells (NKp461 cytotoxic lymphocytes) that are Tbet$^+$/Eomes$^+$ ILC1 and represent the innate equivalent of CD8$^+$ Tc (Messing et al. 2020). Th1 cells are also known to produce TNFα/TNFβ and IL-2 as further signature cytokines. IL-12 released from dendritic cells (DC) and IL-12/IL-18 from macrophages strongly promote Th1-responses, with ILC1 also able to respond to these two cytokines (Barnes and Wizel 2000; Messing et al. 2020). In addition, IL-23 and IL-27, further members of the IL-12 family of cytokines, can also have a Th1-polarising activity (Brombacher et al. 2003). Lastly, NK cells depend on IL-15, amongst other factors, for their development (Annunziato et al. 2015), and is a further member of the IL-2 family of cytokines. Within teleost fish most of these cytokines are present, with the exception of TNFβ, as outlined below. However, additional complexity is apparent in terms of extra genes present, generated from tandem and WGD events, one of which has led to the discovery of homologues in other vertebrate groups that are apparently absent in primates and rodents due to gene pseudogenization (Dijkstra et al. 2014). Since TNFα molecules in teleost fish were covered above under "Pro-inflammatory Cytokines", they will not be discussed here.

10.4.1 Interferon-γ

IFN-γ was discovered in teleost fish by analysis of the fugu genome (Zou et al. 2004a). Subsequent analysis of the zebrafish genome found that in fact there are two type II IFN in teleost fish, with the second termed IFN-γ-related (IFN-γ-rel) (Igawa et al. 2006; Savan et al. 2009). These genes occur side by side in the genome, the latter likely a teleost-specific duplication of IFN-γ (Redmond et al. 2019). The zebrafish proteins differ significantly and have only 16.6% aa identity, with IFN-γ-rel lacking a complete nuclear localisation signal in the C-terminus (Igawa et al. 2006), which is required for translocation into the nucleus to effect IFN-γ bioactivity. In addition, the IFN-γ protein, as in mammals, appears to be produced as a dimer (Yoon et al. 2016; Li et al. 2019), whilst the IFN-γ-rel proteins are thought to be active as monomers (Shibasaki et al. 2014) as well as dimers (Gan et al. 2020b), with the possibility of IFN-γ/IFN-γ-rel heterodimers also postulated (Li et al. 2019). The zebrafish IFN-γ transcript is not expressed constitutively in tissues but is induced by poly I:C. In contrast IFN-γ-rel is constitutively expressed in gills and induced by LPS (Igawa et al. 2006). In zebrafish embryos, overexpression studies (injection of mRNA into 1–2 cell stage embryos) have shown that both IFN-γ and IFN-γ-rel can induce up-regulation of a panel of ISG, such as IFN-ϕ1 and lmp2, with no clear differences between them (Sieger et al. 2009). Knockdown of these genes in the embryos, using antisense morpholinos, revealed that when done individually there was no impact on survival to bacterial infection (*Escherichia coli* or *Y. ruckeri*); however, when both were targeted mortality increased. Whilst this suggests an overlap in functionality (i.e. one gene could compensate for the absence of the other), other studies have reported some differences. For example, overexpression of IFN-γ-rel increased susceptibility to viral infection in larval zebrafish whilst IFN-γ overexpression had no impact, and the overlap in ISG was only partially redundant (López-Muñoz et al. 2011). This may relate to differences in receptor chain usage and signalling for these two molecules in zebrafish (Grayfer and Belosevic 2009; Aggad et al. 2010; Ruan et al. 2017), as also seen in other teleost fish species (Li et al. 2019; Gan et al. 2020b). Curiously overexpression of the IFN-γ genes led to heightened mortality after bacterial challenge, suggesting potential deleterious effects can also occur, perhaps due to overactivation of the inflammatory response as occurs in sepsis (Sieger et al. 2009). At least one population of cells making IFN-γ has been revealed as CD4-1$^+$ T cells, using CD4-1 cells isolated from CD4-1 transgenic zebrafish (Dee et al. 2016) or using specific antisera (Yoon et al. 2015). In the latter case antigen restimulation (in vivo) was shown to specifically increase IFN-γ expression in the CD4-1$^+$ cells, which were potentially equivalent to Th1 cells.

Further studies in carp have shown that IFN-γ expression is elevated in leucocyte populations enriched for T cells (e.g. IgM$^-$) after PHA stimulation whereas IFN-γ-rel is increased in both IgM$^-$ and IgM$^+$ cells by LPS (Stolte et al. 2008), implying they are likely produced by different cell populations. Several studies in carp and goldfish have compared the bioactivity of IFN-γ and IFN-γ-rel directly. In carp only IFN-γ was able to induce iNOS expression in phagocytes, particularly in combination with LPS (Arts et al. 2010), whilst in

goldfish IFN-γ-rel induced higher phagocytosis by monocytes compared to IFN-γ but down-regulated the induction of ROS by IFN-γ treatment (Grayfer et al. 2010). In channel catfish the two genes were shown to be expressed in macrophage, T cell and NK cell lines, and a splice variant of IFN-γ was found that differs by 3 aa in the loop between α-helices A and B (Milev-Milovanovic et al. 2006). More recently a second IFN-γ and IFN-γ-rel gene was reported in ginbuna crucian carp, with two IFN-γ also present in carp and two IFN–γrel in goldfish (Araki et al. 2013; Shibasaki et al. 2014), likely due to the fourth WGD in this teleost lineage. In ginbuna carp IFN-γ1 was expressed more highly than IFN-γ2 in gills and was more inducible following allogeneic scale grafting, but no difference in antiviral activity or expression was detected between IFN-γ-rel 1 and IFN-γ-rel 2.

Two isoforms of IFN-γ have also been described in salmonids, where IFN-γ2 is more highly induced in HK cells after poly I:C stimulation or IHNV infection (Purcell et al. 2009) but follow a similar profile of induction after vaccination (Veenstra et al. 2021). Interestingly intron 1 of the rainbow trout gene contains multiple copies of a minisatellite together with an insertion of a DNA repeat that identifies at least 6 alleles (Zou et al. 2005), which in mammals can be linked to disease resistance/susceptibility (Coltman et al. 2001). This has not been seen in IFN-γ-rel genes to date (Chen et al. 2010). Extensive analysis of the trout IFNγ1 bioactivity has been undertaken (Zou et al. 2005; Martin et al. 2007a, b) and recently it was shown in both trout and Atlantic salmon that rIFN-γ can highly up-regulate one of the Mx subgroups present in these species (SMG2 genes) (Robertsen et al. 2019; Wang et al. 2019b). Whilst IFN-γ-rel has still to be reported in salmonids, multiple copies are present as expected (Wang, SFIRC, personal communication). IFN-γ/ IFN-γ-rel have also been found in other teleost groups including the gadoids (Furnes et al. 2009) and perciformes (Pereiro et al. 2016; Velazquez et al. 2017; Peng et al. 2018a; Kong et al. 2018; Li et al. 2019) where in general similar findings regarding expression and bioactivity have been found as those outlined above. However, splice variants of IFN-γ have been described in medaka, with a possible role in germ cell proliferation (Mohaptra et al. 2015).

10.4.2 Interleukin-2 and Interleukin-15

IL-2 and IL-15 are related cytokines that signal via a receptor that in mammals contains three molecules, with a private chain the difference between the two receptors (i.e. IL-2Rα or IL-15Rα, joining with IL-2Rβ and γC) (Rochman et al. 2009). Analysis of teleost fish genomes revealed the presence of an IL-2 gene (Bird et al. 2005b), as well as two forms of IL-15, termed IL-15 and IL-15L (Bei et al. 2006). The IL-2 gene in zebrafish was more difficult to find, likely due to a gap in the genome, nevertheless recent papers confirm its presence and modulation (e.g. Jiang et al. 2019). In other cyprinids its genomic location has been confirmed (Lv et al. 2020) and in common carp (4R species) two genes are present (IL-2A, IL-2B) encoding proteins with 51% aa identity (Wang et al. 2018). IL-2 is expressed at high levels in the thymus of grass carp, and at medium levels in head kidney

and gills. The grass carp rIL-2 stimulates HK leucocyte proliferation, associated with increased expression of CD4 and a variety of cytotoxicity-related genes (pfp-2, gzmbl, fasl), and up-regulation of type 1 (IFN-γ, IL-12p35, TNFα) and type 2 (IL-4/13A and IL-4/13B) cytokines (Lv et al. 2020; Wang et al. 2021). The crystal structure has also been determined in this species, where significant similarity to human IL-15 was found (Wang et al. 2021). This may relate to the fact that whilst IL-2Rβ, γC and IL-15Rα are all present in teleosts, IL-2Rα is absent. Indeed, that IL-2 can bind to IL-15Rα has been shown recently in grass carp (Wang et al. 2021).

In salmonids two IL-2 genes are also present, at two loci, generated from the salmonid (4R) WGD (Wang et al. 2018). The proteins show a lower aa identity than in carp, ranging from 39% to 43% aa identity over a range of species, with exon 2 of IL-2A being relatively small compared with IL-2B but exon 3 larger. In rainbow trout IL-2A is more highly expressed constitutively in tissues but IL-2B is more highly induced. The two trout rIL-2 proteins also up-regulate type 1 and type 2 cytokines, although in this species only IL-4/13B was up-regulated (i.e. not IL-4/13A). Importantly both rIL-2 proteins could maintain the expression levels of T-cell markers (CD4-1, CD4-2, CD8α, CD8β) that decreased in cultures of PBL in the absence of IL-2 (Wang et al. 2018). More recently it has been shown that trout rIL-2 induces STAT5 phosphorylation (used as a means to indicate cell activation/proliferation) in lymphocytes isolated from thymus and systemic immune tissues (spleen, head kidney) but not mucosal tissues (intestine, gills) (Yamaguchi et al. 2020). This includes cells that are CD4$^+$/CD8$^-$, CD4$^-$/CD8$^+$, CD4$^-$/CD8$^-$ and in thymus also CD4$^+$/CD8$^+$ cells. IL-2 can also impact trout (IgM$^+$) B cells, where in culture it increases their number, proliferation and antibody secretion (Abos et al. 2020). Interestingly in percomorphs a second IL-2 gene (IL-2L) has also been found, adjacent to the IL-2 gene, suggesting in these species a tandem gene duplication has occurred (Wang et al. 2018; Buonocore et al. 2020). These proteins appear even more divergent than those generated by WGD, ranging from 22% to 33% aa identity. Comparative studies have been carried out in sea bass, where despite many shared activities rIL-2L was more potent at inducing a range of cytokines in HK and IL-4/13 expression in spleen, whereas rIL-2 induced Th1 type cytokines in spleen. In flatfish, IL-2 has been reported in olive flounder and turbot (Tang et al. 2019a) but to date only a single gene has been found, perhaps suggesting the gene duplication happened after the divergence of the pleuronectiformes within the Acanthopterygii. The flounder IL-2 has been shown to interact with the IL-2Rβ chain, and following injection of rIL-2 an increased number of IL-2Rβ cells and CD4$^+$ cells are found in gill and spleen (Zhou et al. 2020b). This is interesting as the β and γ chains form only a low-affinity receptor for IL-2 in mammals but perhaps this is different in teleost fish that lack the IL-2Rα chain. Interestingly, binding of IL-2 to the IL-15Rα has also been reported in tetraodon and trout (Wen et al. 2011; Yamaguchi et al. 2020).

In zebrafish the two IL-15 genes (IL-15, IL-15L) are located on different chromosomes, and show only 11% aa identity (Bei et al. 2006; Gunimaladevi et al. 2007). A degree of gene synteny is retained at these loci from gar (Holostei/2R fish) through to mammals, and suggests that these genes appeared early and co-evolved with IL-2 (Bei et al. 2006; Dijkstra

et al. 2014). However, IL-15L has been lost or pseudogenised in several vertebrate groups (e.g. amphibians, birds and various mammalian lineages, incl. humans). IL-15 is composed of 6 exons/5 introns, and in teleosts generally there are no introns in the 5′UTR in contrast to mammals and birds (Wang et al. 2007). IL-15L on the other hand contains 4 exons/3 introns, similar to IL-2. The zebrafish IL-15L gene undergoes alternative splicing, giving 3 isoforms, termed IL-15L, IL-15La and IL-15Lb (Gunimaladevi et al. 2007). These transcripts have been studied comparatively; all are widely expressed but IL-15Lb is low relatively. Modulation of IL-15 expression has been studied more extensively in other cyprinids, such as rohu (Das et al. 2015) and loach (Chen et al. 2018), during ontogeny and following PAMP stimulation or infection. Similarly in grass carp IL-15 expression and bioactivity has been studied (Wang et al. 2020), with the latter revealing a role in inducing type 1 immune responses and NK cell activation but inhibiting type 2 immune genes (e.g. IL-4/13).

In salmonids IL-15 and IL-15L are also known, with two paralogues of the latter (IL-15La, IL-15Lb) present at different loci (Yamaguchi et al. 2020), likely generated by the salmonid WGD. In rainbow trout IL-15Lb has a more restricted expression profile than IL-15La and when expressed is generally lower. In addition, the IL-15La protein (as with IL-15 and IL-2) can be glycosylated unlike IL-15Lb. Trout IL-15 has several forms, with three known splice variants (SV-1, SV-2, SV-3). However, all have a premature stop codon and are expressed at lower levels than the "authentic" mRNA (Wang et al. 2007). Both IL-15 and IL-15L can bind the IL-15Rα, and co-expression with soluble IL-15Rα enhances their release from transfected HEK292T cells (Yamaguchi et al. 2020). The potential ability to form a stable heterodimer with IL-15Rα allows so-called trans-presentation, as established for IL-15 in mammals (Stonier and Schluns 2010). Subsequent functional analysis of IL-15/IL-15L bioactivity using fusion proteins coupled to IL-15Rα has shown, remarkably, that whilst trout rIL-15 increases marker genes in splenocytes for type I immunity (e.g. IFN-γ), as with grass carp, trout rIL-15L induces expression of type 2 marker genes (e.g. IL-4/13) (Yamaguchi et al. 2020). Hence a functional divergence exists between these two related cytokines that appear to have opposite roles regarding immune stimulation. This may occur at the level of innate as well as adaptive immunity, since CD4⁻/CD8⁻/IgM⁻ splenocytes (possible ILC) show these responses. Lastly, in percomorphs IL-15 and IL-15L have been described, with IL-15L again having a restricted expression pattern vs IL-15 (Bei et al. 2006; Fang et al. 2006; Bae et al. 2013).

10.4.3 Interleukin-12 Family Cytokines

IL-12 is the prototypical member of the IL-12 family of cytokines that also include IL-23, IL-27, IL-35 and IL-39. These proteins are all heterodimers, with an alpha chain and a beta chain. Chain sharing is common, as seen with the alpha chain (p35) of IL-12 and IL-35, the alpha chain (p19) of IL-23 and IL-39, the beta chain (p40) of IL-12 and IL-23, and the beta chain (EBI3) of IL-27, IL-35 and IL-39 (Lu et al. 2020). IL-27 has a unique alpha chain,

p28/IL-30. However, some functional homodimers and monomers are known, as with the p40 dimer that is anti-inflammatory and an antagonist of IL-12 signalling (Heinzel et al. 1997). All of these chains have been found in teleost fish, and in principle all of these molecules can be formed. Whilst the genes encoding p35 and p40 were first described in fugu (Yoshiura et al. 2003), p19 was found first in zebrafish (Holt et al. 2011) and p28 in salmon (Husain et al. 2014). Interestingly multiple genes for some of these molecules are found in teleosts. For example three p40 genes exist in zebrafish and carp, termed p40a, p40b and p40c (Huising et al. 2006; Nascimento et al. 2007), and two p35 genes are known (p35a, p35b) (Nascimento 2007; Wang and Husain 2014), and these duplicates are likely present in all (3R) teleost fish. Indeed, synteny analysis of the p40a/p40b and p35a/p35b loci suggest they were generated by the teleost WGD event, but the origin of p40c appears older and less clear (Nascimento 2007). This means that potentially 13 IL-12 family members could be formed from these genes. In 4R fish this number could be even larger. In salmonids p35a has been duplicated into p35a1 and p35a2, and this has also happened with p19 (p19a, p19b) and p28 (p28a, p28b), and p40b exists as p40b1 and p40b2. Since p40a has not been described to date in salmonids (lost?) this limits the number of IL-12 family members that may exist in this group to 22! (see Table 10.2). A splice variant of EBI3 has been found in grass carp, termed EBI3b, giving even more potential variants (Zhang et al. 2019).

Transcript analysis of the individual chains has been studied but it can be difficult to predict which of the family members is being made as protein. Nevertheless, some associations have been seen that suggest similar proteins are being made to those in mammals (Ribeiro et al. 2010; Wang and Husain 2014; Matsumoto et al. 2016; Wang et al. 2017). Several of the IL-12 family (IL-12, IL-23, IL-35) have been made as recombinant proteins by cloning the alpha and beta chains into a vector with a linker between (Wang et al. 2014a/IL-12; Wang et al. 2017/IL-23; Zhang et al. 2019/IL-35). These heterodimers have been shown to be active and, interestingly, the beta chain used can have subtle effects on the bioactivity seen. For example, in grass carp three IL-23 proteins were made containing p19 combined with p40a, p40b or p40c (Yin et al. 2019). All three proteins promoted Th17/type 3 responses, in terms of increasing expression of IL-17A/F1 (see "Cytokines of type 3 immunity" below) and IL-22 in HK leucocytes but IL-23a and IL-23b were more potent than IL-23c. In rainbow trout two forms of IL-12 were produced that also differed in the beta chain (p35/p40b and p35/p40c) (Wang et al. 2014a). Whilst both could induce IFN-γ and p40 (b and c) expression in HK leucocytes, only IL-12 p35/p40c induced IL-10. Since these two p40 chains have only 46% aa similarity it is possibly these different IL-12 proteins might bind to the IL-12R with different affinities or even bind to different receptors (since the receptor chains will likely be duplicated as well). Lastly, in amberjack all six possible forms of IL-12 were made (Matsumoto et al. 2016). Whilst four of them could increase IFN-γ expression and down-regulate IL-10 expression in HK cells co-stimulated with formalin killed bacteria (*Nocardia seriolae*), two forms (p35a/p40b, p35b/p40b) lacked the ability to affect IFN-γ transcript levels. Lastly,

Table 10.2 The potential number of IL-12 family members in teleost fish

	Chain	3R Teleosts	4R Teleosts (e.g. salmonids)
α-chain	p19	p19	p19a, p19b
	p28	p28	p28a, p28b
	P35	p35a, p35b	p35a1, p35a2, p35b
β-chain	p40	p40a, p40b, p40c	p40b1, p40b2, p40c
	EBI3	EBI3	EBI3
Known IL-12 family members	IL-12 (p35 + p40)	**6**	**9**
	IL-23 (p19 + p40)	**3**	**6**
	IL-27 (p28 + EBI3)	**1**	**2**
	IL-35 (p35 + EBI3)	**2**	**3**
	IL-39 (p19 + EBI3)	**1**	**2**
	Number of possible proteins	**13**	**22**

The IL-12 family proteins are formed as heterodimers consisting of an alpha and beta chain. However, due to the additional paralogues of these chains present in teleost fish multiple isoforms could exist (as outlined above), as seen with IL-12 itself where between 6 and 9 possible molecules may be formed dependent upon the species examined

monomeric p40 has been shown to have activity (pro-inflammatory) in teleost fish, with all three forms tested in grass carp (Zhang et al. 2014; Feng et al. 2019).

10.4.4 Interleukin-18

IL-18, also known as IFN-γ inducing factor, is a member of the IL-1 family of cytokines (termed IL-1F4) present in all jawed vertebrates. However, recent analysis suggests that it likely evolved in an evolutionary independent event to IL-1β (Rivers-Auty et al. 2018). The locus and gene organisation (6 exons/5 introns) are well conserved in teleost fish (Zou et al. 2004b; Huising et al. 2004; Rivers-Auty et al. 2018) and splice variants can be present, as seen in rainbow trout (Zou et al. 2004b). There is a conserved ICE cut site in the protein suggesting it is cleaved to become active. In trout, PAMP stimulation has no impact on IL-18 gene expression in HK cells and a macrophage cell line, but LPS and poly I:C increase expression of the splice variant in RTG-2 cells (Zou et al. 2004b). In fugu, an NLRP3 inflammasome inducer (Nigericin) induces IL-18 in HK leucocytes, especially in combination with LPS (Biswas et al. 2016a), as do probiotic intracellular products in rohu

macrophages (Giri et al. 2015). The protein has yet to be studied and hence its role in type 1 immunity of teleost fish remains to be verified.

From the above it can be concluded that the cytokines needed for activation and regulation of type 1 immunity exist in fish. In many cases the teleost molecules are true homologs of their equivalents in other vertebrates, although multiple paralogues may exist (e.g. IL-2, IFN-γ). The impact on the IL-12 family is particularly impressive, with the potential for many more functional proteins to be produced, able to have subtle impacts upon immune activation. The recent progress with understanding the function of novel molecules such as IL-15L is also of note, and provides an insight into the evolution of the type 1 vs type 2 dichotomy.

10.5 Cytokines of Type 2 Immunity

Type 2 responses are characterised by the recruitment of eosinophils to a site of inflammation, although basophils, mast cells and alternatively activated macrophages (M2 cells) can also be involved. Such responses typify the host response to helminth parasites and noxious environmental stimuli but also participate in wound healing after injury and can lead to a variety of immunopathologies (Gurram and Zhou 2019). In mammals these responses are driven by the type 2 cytokines IL-4, IL-5, IL-9 and IL-13, produced by ILC2, Tc2 and Th2 cells (Annunziato et al. 2015; Akdis et al. 2020). Other Th cell subsets may also have a role, in producing these cytokines, although this is still being clarified, such as Th9 cells (derived via reprogramming of Th2 cells) that secrete IL-9 and Tfh cells that can release IL-4 (IL-4^{+} Tfh) or IL-13 (IL-13^{+} Tfh) to promote B-cell class switching to IgE (Veldhoen et al. 2008; Chen et al. 2019b; Gause et al. 2020). In addition, IL-31, an IL-6 family member, can be produced by Th2 cells (Gibbs et al. 2019) and an association between type 2 cytokines and IL-20 has been seen in particular situations, as seen in asthma (Wu et al. 2014). Whilst specific antigens trigger the T-cell responses, for ILC2 cells it is well known that they are activated to secrete type 2 cytokines in response to particular alarmins released from epithelial cells after exposure to helminths, allergens or toxins, some of which are cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) (Gurram and Zhou 2019; Akdis et al. 2020). In fact both ILC2 and Th2 cells highly express the receptor for IL-33, ST2 (Gieseck III et al. 2018), whilst TSLP can also stimulate Th2 cell development, directly or via DC conditioning (Gause et al. 2020).

10.5.1 Interleukin-4/13

In mammals IL-4, IL-5 and IL-13 are clustered in the genome, on human Chr 5q31 and mouse Chr 11, together with IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). In humans IL-9 is further upstream on Chr 5, whilst in mice it is located on Chr 13. A similar situation has been reported in chicken (Chr 13) but with an additional gene

present at this locus, termed KK34, with IL-5 a pseudogene (Avery et al. 2004). IL-4 and IL-13 are gene neighbours, with RAD50 next to IL-4 and KIF3A next to IL-13. The type 2 cytokines known in teleost fish were discovered by genome analysis, with RAD50 and KIF3A helping to identify the loci (Bird and Secombes 2006; Li et al. 2007). Analysis in zebrafish (and other species) revealed that in addition to a gene next to RAD50 on Chr 9, a second gene is present on Chr 14 next to KIF3A (Ohtani et al. 2008). Since both these genes have homology to IL-4 and IL-13, they were termed IL-4/13A (linked to RAD50) and IL-4/13B (linked to KIF3A), and likely represent an ancestral gene (for both molecules) that was duplicated in fish by the teleost WGD event, and at a later point in evolution to generate IL-4 and IL-13. This is further supported by the presence of a single locus containing a single IL-4/13 gene in Holosteans (gar fish) that have not undergone the teleost WGD event (Wang and Secombes 2015). In teleosts both genes have a typical 4 exon/3 intron organisation and 4 predicted alpha helices in the translated protein, encoded in exons 1, 3 and 4 (Wang et al. 2016). A second IL-4/13B gene has been found in grass carp, termed IL-4/13B-like (IL-4/13Bl), that has a much larger intron 3 (951 vs 297 bp in IL-4/13B) (Yang et al. 2019). Whilst there are several nucleotide changes throughout the molecule that impact on the protein translation, the largest single difference is a 3 aa deletion in helix C of IL-4/13Bl.

In cyprinids the IL-4/13A and B genes are widely expressed constitutively, as seen in goldfish and grass carp (Hodgkinson et al. 2017; Jiang et al. 2020). Interestingly, in grass carp the expression level of the different isoforms has some differences. For example, in HK of *A. hydrophila* challenged fish IL-4/13A transcript levels decrease whilst IL-4/13B levels increase (Yang et al. 2016a), and after *Flavobacterium columnare* infection IL-4/13B levels increase sooner than IL-4/13A (Jiang et al. 2020). Comparing the two IL-4/13B isoforms in this species, IL-4/13B expression is higher in gills and skin whilst IL-4/13Bl is higher in kidney and brain (Yang et al. 2019). In individual cell types these differences can be even more apparent. Goldfish macrophages and monocytes express high levels of IL-4/13A, whilst in carp, a Th2-like clone developed from bulk cultured T cells up-regulates only IL-4/13B on PHA stimulation (Yamaguchi et al. 2013). These latter cells also express CD4-1/CD4-2 and GATA-3 but not CD8α/CD8β, and differ from non-clonal T cells in which PHA also up-regulates IFN-γ and IL-4/13A. Similarly in CD4-1^{+} cells isolated from gills of transgenic CD4-1 reporter zebrafish, relatively high expression levels of IL-4/13B and GATA-3 were found but not IL-4/13A (Dee et al. 2016). Lastly, in antigen re-stimulated zebrafish, spleen/kidney cells showed enhanced levels of IL-4/13B relative to fish given only a primary immunisation or fish boosted with an unrelated antigen, with no induction of IL-4/13A (Yoon et al. 2015). A similar phenomenon was seen in CD4-1[+] lymphocytes isolated from these fish using an anti-zfCD4-1 antiserum.

Bioactivity studies have also been performed with the cyprinid IL-4/13 proteins. In goldfish rIL-4/13A and rIL-4/13B both increase macrophage arginase activity and expression of arginase-2, and when used to pre-treat the cells inhibition of IFN-γ-stimulated NO production occurs in a dose-dependent fashion (Hodgkinson et al. 2017). Similarly, grass carp rIL-4/13Bl can up-regulate macrophage arginase 2 expression/arginase activity and

promote their proliferation following M-CSF pre-treatment but down-regulates NO pro-
duction and iNOS/IFN-γ expression in these cells (Yang et al. 2019). Both IL-4/13A and B
have also been shown to stimulate the proliferation of IgM⁺ B cells, as seen in zebrafish and
carp (Zhu et al. 2012; Yamaguchi et al. 2016), in line with the finding that IgM⁺ B cells
express IL-4Rα on their surface (Zhu et al. 2012). Injection of zebrafish with IL-4/13A can
also increase DC-SIGN/CD209 expression on PBL (Lin et al. 2009), whilst in grass carp
such treatment increases survival rates following challenge with *A. hydrophila* (Yang et al.
2016a). IL-4/13A and IL-4/13B single and double mutants have also been generated
recently in zebrafish (Bottiglione et al. 2020). Mutant fish have higher levels of
proinflammatory genes in whole larvae, more pronounced in the double mutants, whilst
transcript analysis (RNA-seq) in gills reveals a shift towards type 1 immunity, suggesting a
role in suppression of inflammation and maintenance of a type 2 phenotype, at least in the
gills.

In the salmonids the two loci have been duplicated, as with most of the cytokines, giving
two IL-4/13B genes but one of the duplicated IL-4/13A genes has been pseudogenised, at
least in Atlantic salmon (Wang et al. 2016). Interestingly there appears to be a degree of
(fish) lineage-specific differences in the IL-4/13 genes, in that exon 3 of the salmonid IL-4/
13 genes is relatively small whilst in IL-4/13B it is relatively large. This exon encodes both
helix B and C. As in cyprinids, the IL-4/13 genes are widely expressed constitutively,
although there is a higher expression of IL-4/13A vs IL-4/13B, and mucosal sites such as
gills and skin have the highest transcript levels and are considered Th2-skewed
environments (Takizawa et al. 2011). The IL-4/13A gene is the only paralogue expressed
in trout fibroblast cell lines, whilst in RTS-11 cells (macrophages) all three are expressed
but IL-4/13A is highest. However, on stimulation in vitro or in vivo, it is the IL-4/13B
genes that are induced. Interestingly, following *Y. ruckeri* challenge of trout the gill
expression of all three paralogues decreases, in studies focused on sampling prior to a
time when fish are expected to succumb to the infection, but in fish vaccinated prior to
challenge this does not occur (Wang et al. 2016). Comparative analysis of the bioactivity of
the IL-4/13A and IL-4/13B proteins showed overlapping but also distinct effects.
Differences included a larger impact of rIL-4/13A, relative to rIL-4/13B, on induction of
IL-13Rα2 (a and b) expression, potential decoy receptors, but a greater down-regulation of
IL-6R and MCSFR. On the basis of these results it was proposed that IL-4/13A provides a
basal level of type 2 immunity, which can be enhanced when activated by IL-4/13B.

In perciformes, some differences between species are apparent. For example, in large
yellow croaker, puffer fish (Tetraodon and Fugu) and stickleback a single IL-4/13A and
IL-4/13B gene is present as above (Li et al. 2007; Ohtani et al. 2008; Biswas et al. 2016b;
Mao et al. 2018), whilst in medaka the IL-4/13B gene appears to be absent but two IL-4/
13A genes are present side by side, as also seen in sea bass (Ohtani et al. 2008; Stocchi et al.
2017). Whether the lack of IL-4/13B is typical of Beloniforme fish remains to be deter-
mined. Pufferfish, stickleback and medaka are all in different orders of the Acanthopterygii;
however, large yellow croaker and sea bass are both in the order Perciformes, in the
families Sciaenidae and Moronidae respectively. The expression patterns and bioactivity

of these molecules have been studied in both species. In croaker, as in salmonids, IL-4/13A is highly expressed in gills constitutively, but IL-4/13B is more inducible, as demonstrated following injection with an inactivated trivalent bacterial vaccine (Mao et al. 2018). Nevertheless, the recombinant proteins both exhibit similar activities in terms of down-regulating expression of pro-inflammatory cytokines and iNOS, phagocytosis and production of oxygen and nitrogen radicals, whilst up-regulating anti-inflammatory cytokines and arginase 2. In contrast, in sea bass the three isoforms (IL-4/13A1, IL-4/13A2 and IL-4/13B) show some unique aspects to their constitutive and induced expression. All are expressed in a range of tissues, with IL-4/13B being relatively constant/low in expression level. However, both of the IL-4/13A isoforms show variation between tissues, being highest in spleen, and having an opposite expression pattern in gills and PBL; IL-4/13A1 is relatively low in gills and high in PBL, with IL-4/13A2 the reverse. IL-4/13B is the main isoform induced by in vitro stimulation with PHA and PMA, although IL-4/13A1 shows a degree of induction with PHA whilst IL-4/13A2 is induced in spleen cells by PHA but not in HK cells (Stocchi et al. 2017). Bioactivity has focussed on the expression of a number of key immune genes in stimulated HK leucocytes. Good induction of IL-4Rα (1 and 2) is seen with IL-4/13A1, a little with IL-4/13A2 (IL-4Rα2 only) and none with IL-4/13B. IL-13Rα (1 and 2), however, shows primarily down-regulation in stimulated cells, especially marked with IL-13Rα1 after IL-4/13B treatment. Other regulatory molecules such as SOCS3 and IL-10 are also differentially affected. All three proteins up-regulate SOCS3 but with different kinetics (IL-14/13A1/2 relatively late vs IL-4/13B), whilst IL-10 shows down-regulation with IL-4/13A1, up-regulation with IL-4/13A2 and is not affected by IL-4/13B. Hints that IL-4/13A and IL-4/13B may show some subtle differences in bioactivity have also been shown in Fugu (Biswas et al. 2016b). In fish injected with rIL-4/13A or rIL-4/13B phagocytosis and respiratory burst activity of HK cells was enhanced by IL-4/13A but not with IL-4/13B. In addition, IL-4/13A had more pronounced effects on up-regulation of pro-inflammatory genes (IL-6 and TNFα) but only IL-4/13B up-regulated p35, the alpha chain of IL-12 and IL-35.

10.5.2 Interleukin-20L

In relation to the other type 2 cytokine genes mentioned above, no direct homologues have been found in teleost fish to date, although a gene related to IL-20 (termed IL-20-like; IL-20L) is present at the IL-10 locus. In mammals IL-19, IL-20 and IL-24 are found next to IL-10 on Chr 1 but in the opposite orientation. IL-20L shares highest homology with IL-20 but phylogenetic analysis suggests it is likely an ancestral molecule that diverged into IL-19/20/24 later in evolution (Wang et al. 2010a). In zebrafish, this molecule is found on Chr 11 adjacent to IL-10 (although it was called IL-34 in Stein et al. 2007), and curiously is in the same orientation as IL-10 (Piazzon et al. 2017). The gene has a 5 exon/4 intron organisation, as with other IL-10 family members (Wang et al. 2010a). In common carp, a 4R species, genome analysis has found two IL-10 loci each with a copy of IL-10 and

IL-20L, with the genes in the same orientation at both loci (Piazzon et al. 2017). Similarly in pufferfish (Fugu, Tetraodon) the IL-20L gene is present adjacent to IL-10 (Lutfalla et al. 2003; Zou et al. 2004c) and again both genes are in the same orientation (Zou et al. 2004c). IL-20L has also been discovered in salmonids, and in the rainbow trout gene there is an additional intron in the 5'UTR (Wang et al. 2010a). This can occur in some of the mammalian IL-10 family members, as seen in the 5′UTR of human IL-20. Functional studies in trout have shown that IL-20L is widely expressed in vivo, and is inducible by PAMPs (poly I:C, LPS) and cytokines (IL-1β, IFN-γ) in monocytes/macrophages (RTS-11 cell line) and in spleen after infection with *Y. ruckeri* (Wang et al. 2010a). Whether two IL-20L genes exist in salmonids (4R species) is currently unknown although two IL-10 genes are present in trout (Harun et al. 2011a), indicating the duplicated loci are both retained.

From the above it seems reasonable to conclude that a functional type 2 immunity exists in teleost fish, regulated by cytokines that are not true homologs of the equivalent mammalian genes but that have a clear evolutionary relatedness. The advantages of the zebrafish model have helped functional studies and inform on the cells and pathways involved which should be examined in production species.

10.6 Cytokines of Type 3 Immunity

Type 3 immunity refers to the responses effected by ILC3, CD4^{+} Th17/Th22 cells and CD8^{+} Tc17 cells, with ILC3 considered the innate equivalent of Th17 cells. These cells are crucial for defences at epithelial barriers/mucosal surfaces against pathogens such as extracellular bacteria and fungi (Hamada et al. 2009; Annunziato et al. 2015; Silberger et al. 2017). ILC3 are categorised into a number of subsets, such as CCR6^{+} and CCR6^{−} subsets, that have overlapping but distinct functions (Klose and Artis 2016). In mice the CCR6^{+} cells include both lymphoid-tissue-inducer (LTi) cells and LTi-like cells. ILC3 release a variety of cytokines to effect responses, such as IL-17 (CCR6^{+} cells), IL-22 (both CCR6^{+} and CCR6^{−} cells), GM-CSF (CCR6^{−} cells), IFN-γ (CCR6^{−} cells) and LT-α (soluble trimer). For example, IL-22 released from ILC3 can induce AMP secretion from epithelial cells and mucus production by goblet cells, as seen in many (extracellular) disease states, and are the predominant IL-22 producing cells during the early stages of infection (Panda and Colonna 2019). In humans ILC3 can also produce IL-26 and surface expression of LT-$\alpha_1\beta_2$ heterotrimers can activate DC (Sonnenberg and Hepworth 2019). Th17 cells were the third subset of CD4^{+} T effector cells to be discovered, and predominantly secrete IL-17A, IL-17F, IL-21 and IL-22, and are key for adaptive responses against fungi and extracellular bacteria (Wei et al. 2007; Wu et al. 2018a). Human Th17 cells can also secrete IL-26, which is lacking in mice. More recently a Th22 subset has been described that can release IL-22 in the absence of IL-17 (Plank et al. 2017). They can also release granzymes and IL-13 and have a major role in a range of inflammatory diseases. Lastly, Tc17 are an important T-cell subset involved in lung defences and secrete

IL-17A and IL-17F but do not express perforin and granzyme and hence have no cytolytic activity (Hamada et al. 2009). Upon re-stimulation Tc17 can also produce IL-21, IL-22, TNF and several chemokines (Hamada et al. 2009). Of the cytokines mentioned above, teleost fish are known to possess many members of the IL-17 family, as well as IL-21, IL-22 and IL-26, and these will be discussed here. GM-CSF and homologs of lymphotoxin have not been found in teleost fish, whilst IFN-γ and IL-13 are discussed above under "Cytokines of Type 1 Immunity" and "Cytokines of Type 2 Immunity", respectively.

10.6.1 Interleukin-17 Family

The IL-17 family consists of IL-17A to IL-17F in mammals, found at 5 loci (with IL-17A and IL-17F gene neighbours). In teleost fish the situation is somewhat more complex. In zebrafish, 6× IL-17 family members have been described at 4 loci, with three genes having similarity with IL-17A and IL-17F, and so were termed IL-17A/F1–3 (Gunimaladevi et al. 2006). These genes are likely related to the ancestral gene that gave rise to IL-17A and IL-17F later in evolution, which was itself duplicated in bony fish (Wang et al. 2015). In fact, since (2R) gar fish possess IL-17A/F1 and IL-17A/F2 at a single locus, it seems that the ancestral gene underwent an initial tandem gene duplication, followed by the generation of a second locus containing IL-17A/F3 as a consequence of the teleost WGD. IL-17A/F3 is more related to IL-17A/F1 than IL-17A/F2, and so the potential duplicate of IL-17A/F2 appears to have been lost. An IL-17C and IL-17D gene were also identified in zebrafish, with clear homology to the equivalent mammalian molecules. Further analysis of the fugu genome revealed that in fact two IL-17C genes (IL-17C1 and IL-17C2) are present at different loci (one linked to the IL-17A/F1-IL-17A/F2 locus) and that a novel IL-17 family member is also present at a fifth locus, which was termed IL-17N (Korenaga et al. 2010). The origin of IL-17N is not clear but it is also present in gar and coelacanth, and therefore appeared before the emergence of the teleosts (Li et al. 2021c). Lastly, in several fish species, including carps and catfish an IL-17B gene has been found (Wang et al. 2014b; Dong et al. 2019). That IL-17E (also called IL-25) has not been found in fish has been speculated to be due to a later appearance in vertebrates, derived from an IL-17C-like gene (Wang et al. 2010b; Kono et al. 2011).

Differences in the IL-17 gene repertoire between fish groups are apparent, such as the lack of IL-17B in more advanced teleosts, and multiple paralogues in 4R salmonids and carps. For example, in common carp two copies of IL-17A/F1, IL-17A/F2, IL-17B, IL-17C (both related to IL-17C1), IL-17D and IL-17N are present (Li et al. 2016; Dong et al. 2019), together with a single copy of IL-17A/F3 (Li et al. 2021c). In salmonids two copies of IL-17A/F1, IL-17A/F2 and IL-17C (both related to IL-17C2) are present, as well as a single copy of IL-17A/F3, IL-17D and IL-17N, although a duplicated IL-17N is detectable as a pseudogene (Wang et al. 2015). Thus, currently up to 13× IL-17 family members can be present in particular species, as in carp, although 6–7 is more typical (e.g. IL-17A/F1–3, IL-17B, IL-17C, IL-17D, IL-17N). Expression and functional studies have been performed

in many teleost species (e.g. see Zou and Secombes 2016; Secombes et al. 2016; Zhang et al. 2020a; Takahashi et al. 2020; Li et al. 2021c), but fewer studies have compared all family members in a single experiment (Wang et al. 2015; Yang et al. 2016b; Tang et al. 2019b; Gonzalez-Fernandez et al. 2020). It seems likely that the IL-17A/F molecules will have value as markers of type 3 immunity, with links to specific cell types ultimately confirming whether these are part of the innate or adaptive response.

10.6.2 Interleukin-21

IL-21 is a member of the gamma-chain family of cytokines and is situated in the genome next to another member, IL-2 (see "Cytokines of Type 1 Immunity"). It has a broad range of effects on lymphoid, myeloid and epithelial cells, increasing their proliferation, survival and differentiation. In mammals it plays a role in antiviral defence by increasing the survival of CD8$^+$ cells and impacts B-cell responses to protein antigens and anti-parasite defence, as seen in IL-2 or IL-2R deficient mice (Solaymani-Mohammadi et al. 2019). The IL-21 sequence has been reported for zebrafish (Wang et al. 2011b), and more recently in grass carp (Zhang et al. 2020b) and common carp (Yu et al. 2020). Interestingly, genomic analysis revealed that the gene is composed of 6 exons/5 introns, in contrast to tetrapods where 5 exons exist, with exons 3 and 4 in zebrafish equivalent to exon 3 in tetrapods (Wang et al. 2011b). It is not known if a further paralogue may exist in carp as a consequence of the WGD but in salmonids that is the case, with the two IL-21 genes present on Chr 14 and Chr 25 in rainbow trout and Chr 5 and Chr 9 in Atlantic salmon (Wang et al. 2018). In percomorphs a single gene is present, and indeed fugu was the first teleost species in which the IL-2/IL-21 locus was identified (Bird et al. 2005b). Curiously the fugu gene (but not the spotted green pufferfish gene) has a fusion of (teleost fish) exons 5 and 6, so has a 5 exon organisation but quite different to that of tetrapods (Wang et al. 2006b).

Recently splice variants of IL-21 have been discovered in grass carp (Cui et al. 2020b). Relative to the full-length molecule (gcIL-21sv1), the sv2 variant lacks intron 5 and thus has 5 exons, whilst sv3 has a large exon 3 but lacks exons 4–6. These changes result in the last 6 aa of sv2 differing from sv1, whilst sv3 is C-truncated giving a protein of 115 aa vs 149 aa for sv1 and sv2 respectively. Modelling of sv3 predicted that it contains 2 of the usual 4 α-helices present in IL-21. Expression analysis showed that sv1 was the most highly induced variant, but that both sv1 and sv2 recombinant proteins could induce the proliferation of HK derived IgM$^+$ B cells, and increase the expression of IgM/IgT heavy chains and IgM secretion. Other studies with teleost rIL-21 have shown that it can increase IL-10, IL-21, IL-22 and IFN-γ in HK cells or PBL, and maintains the expression of CD8 and IgM over 72 h in culture where untreated cells show reduced expression (Wang et al. 2011b; Yu et al. 2020; Zhang et al. 2020b). These data suggest IL-21 is an important regulator of T and B cells in fish, with the effects driven via multiple intracellular pathways, including activation of JAK/STAT3, Akt1/2 and PI3K (Wang et al. 2011b).

10.6.3 Interleukin-22 and Interleukin-26

IL-22 and IL-26 are both members of the IL-10 cytokine family. These genes are found at two loci in tetrapods and teleost fish, with IL-22 and IL-26 both at the locus where IFN-γ (and teleost IFN-γ-rel) is found, adjacent to each other as seen in zebrafish (Igawa et al. 2006). Despite a low sequence homology, the genomic location, gene organisation and IL-10 family signature in the predicted proteins are convincing evidence of their correct assignment. The crystal structure of zebrafish IL-22 has also been determined, which has a typical class II cytokine secondary structure as expected (Siupka et al. 2014). It was immediately apparent that zebrafish IL-22 is highly expressed at mucosal sites such as intestine and gills, and is inducible (in the kidney by LPS stimulation and spleen by poly I: C stimulation). When IL-22 production was blocked in zebrafish embryos, using an IL-22 antisense oligo morpholino, higher mortality was seen after *A. hydrophila* infection, demonstrating a key role in innate immunity (Costa et al. 2013). The involvement of the PI3K pathway in controlling IL-22 expression was also shown in zebrafish, using pten$^-$/$^-$ mutants (ptena$^-$/$^-$ and ptenb$^-$/$^-$) (Costa et al. 2013). The PTEN protein is a negative regulator of the PI3K pathway and thus if it has a similar function in teleost fish knockdown would result in enhanced IL-22 expression levels, which was seen in 3 dpf embryos from both lines. IL-22 is also linked to adaptive immunity, and in sorted zebrafish CD4–1$^+$ lymphoid cells (potential Th cells) expression is increased significantly in specific antigen (HGG) boosted fish vs cells sorted from fish given HGG only or fish boosted with an unrelated antigen (OVA) (Yoon et al. 2015).

Less is known about IL-22 in other cyprinid species but it has been reported in grass carp and siluriformes, such as the yellow catfish, where it is also constitutively expressed at mucosal sites and inducible following infection (Jiang et al. 2018; Yang et al. 2020). Furthermore, grass carp rIL-22 has been shown to up-regulate a number of pro-inflammatory cytokines and STAT3 in HK leucocytes and a grass carp kidney cell line (CIK) (Yang et al. 2020). Within the Euteleostei IL-22 is relatively well studied in the salmonids. A single gene has been described so far, although two are likely present adjacent to the two known IFN-γ genes (see "Cytokines of Type 1 Immunity") that show this duplicated locus has been retained (as may also be the case in carp where two IFN-γ genes are present). An additional intron is present in the rainbow trout gene, in the 5'UTR, giving a 6 exon/5 intron gene organisation (Monte et al. 2011). As in other teleosts, it is highly expressed at mucosal sites and inducible by bacterial infection (*Y. ruckeri*), and is one of the few immune genes that increases post-infection in trout larvae that are relatively resistant to the disease (Monte et al. 2011; Chettri et al. 2012). Interestingly, in trout vaccinated before challenge there is a significantly enhanced expression of IL-22 in the gills 24 h post-infection relative to unvaccinated-challenged fish, suggesting a potential contribution to the protection seen in these fish (Harun et al. 2011b). Trout rIL-22 has been produced and shown to increase the expression of AMPs (defensins and LEAPs) in splenocytes, in a dose-dependent fashion (Monte et al. 2011), hinting at one mechanism by which IL-22 can contribute to disease resistance. More recently MoAb to trout IL-22

have been produced and used to show the location of cells in gills (filaments and ILT) able to produce this protein in naïve and challenged fish, which look like epithelial cells or lymphoid cells depending on the location (Hu et al. 2019).

In the Neoteleostei, IL-22 has been described in both the Paracanthopterygii (gadoids) and Acanthopterygii (percomorphs). In cod and haddock IL-22 is again highly expressed constitutively in gills, and can be induced by infection (*Vibrio anguillarum*) but in haddock this induction occurs much faster in gills of vaccinated fish (Corripio-Miyar et al. 2009). Curiously in isolated cod epithelial cells induction of IL-22 expression has been shown to be selective depending on the bacterial species used for stimulation (Caipang et al. 2010). Lastly, IL-22 has been characterised in several Percomorph species, ranging from flatfish (turbot) to mandarin fish, So-iny mullet and golden pompano (Costa et al. 2013; Qi et al. 2015; Peng et al. 2017; Huo et al. 2019a). A variety of functional studies with the recombinant protein have been undertaken. In turbot challenged with *A. salmonicida* together with 100 ng rIL-22 a higher survival was found relative to fish without rIL-22 (Costa et al. 2013). Similarly, So-iny mullet injected with rIL-22 (100 μg) 24 h prior to challenge had significantly enhanced survival to *Streptococcus dysgalactiae* (Qi et al. 2015). Such fish showed increased AMP expression (defensin) in kidney, liver, spleen and gut 12 h post-rIL-22 injection, which was prolonged to 24 h in gut and kidney. In mandarin fish treatment of intestinal cells with rIL-22 increases AMP expression (LEAP1 and 2) but also SOCS3 and IL-22 binding protein (IL-22BP) that may be involved in negative regulation (Huo et al. 2019a). IL-22BP is a soluble inhibitory IL-22 receptor and is the only known soluble receptor for IL-10 family cytokines (Martin et al. 2014). It is thought to prevent deleterious inflammatory effects, by keeping IL-22 activity under tight regulation, and in the intestine is produced by DC in mammals. In line with this, the rIL-22 activity for intestine cells was completely inhibited by 30 min preincubation with mandarin fish rIL-22BP prior to addition to the cells but this was not seen when two IL-22BP mutants were used (Huo et al. 2019a). So as in mammals, it is likely that IL-22 function is precisely controlled in fish.

In contrast, relatively little is known about IL-26 in fish. The first teleost gene discovered was in zebrafish, at the IFN-γ locus (Igawa et al. 2006). Unlike IL-22, IL-26 is not constitutively expressed in zebrafish but is induced in intestine by poly I:C, and in zebrafish embryonic cells (ZF4) by exposure to IPNV (Wang et al. 2011a). IL-26 expression has also been seen to be enhanced in zebrafish made transgenic for a variety of different AMPs, constitutively (whole fish) (Wu et al. 2018b) or following infection with *Vibrio vulnificus* (Peng et al. 2010; Pan et al. 2011). More recently the grass carp protein has been studied and shown to have a role in regulating the inflammatory response (Qiu et al. 2017). Thus, rIL-26, at a relatively high dose (1 μg/ml), was able to up-regulate expression of IL-1β, IL-6 and IL-8 in HK leucocytes and iNOS in monocytes/macrophages but inhibit IL-10 expression in these cells. Whilst the IL-26 gene is known to exist in other teleost species (e.g. pufferfish – Igawa et al. 2006) there is no functional data.

Overall it is clear that type 3 immunity exists in teleost fish and is likely to have an equally important role in protecting against external pathogens at mucosal surfaces as seen

in other vertebrates. Whilst much progress has been made in identifying the relevant genes and analysing factors that affect their expression, there is still much to be learnt about the bioactivity of the proteins and the cells that produce them.

10.7 Regulatory Cytokines

All cytokines can be deemed regulatory, however in this section those that are involved in controlling the effector functions of the cytokines described above will be outlined. Since the responses that cytokines elicit are destructive, it is imperative that they do not last too long or are overly strong. In such circumstances damage to the host can occur, which in extreme cases could lead to death. The main way in which these regulatory cytokines function is either as molecules that act via discrete receptors to stimulate negative feedback pathways, or as receptor antagonists that compete with the effector cytokine to block signal transduction. A good example of the latter is the IL-1 receptor antagonist (IL-1ra), considered crucial for the control of inflammation (Hallegua and Weisman 2002). Whilst there are many other ways in which cytokine function can be regulated, for example, by the expression of decoy receptors that bind the ligand but do not signal or secreted receptors/ binding proteins that block cytokine function, these molecules are not themselves cytokines and so will not be described here. The "regulatory" cytokines are commonly released by specific cell populations, the most prominent of which are Treg cells (CD4$^+$/ CD25$^+$/FoxP3$^+$) and their various subpopulations (Shevyrev and Tereshchenko 2020; Savage et al. 2020). These cells produce several inhibitory cytokines, including TGF-β1, IL-10 and IL-35, the latter a heterodimer composed of p35 and EBI3 as described above (see "Cytokines of Type 1 Immunity"). However, regulatory B cells and regulatory CD8$^+$ T cells are also known (Peng et al. 2018b; Yu et al. 2018). These cytokines are known in fish, although the IL-1ra is a functional homologue rather than a true homologue.

10.7.1 Novel Interleukin-1 Family Member

Fish possess a member of the IL-1 family, nIL-1F that appears to function as an IL-1ra (Wang et al. 2009; Yao et al. 2015) but is novel, evolved early in vertebrates and is apparently lost in tetrapods (Eggestol et al. 2020). It is present throughout teleosts but has so far only been studied in grass carp, rainbow trout and lumpfish. The molecule is thought to be made as a precursor protein, and cleaved to release the active mature peptide, as with many IL-1 family cytokines. Predicted ICE and thrombin cut sites are present upstream of a C-terminal region containing 12 beta sheets, required for formation of a beta-trefoil protein structure, typical of IL-1 family cytokines. In grass carp binding of nIL-1F (made after the thrombin cut site) to the IL-1 receptor (IL-1R1) was revealed using the recombinant proteins, with nIL-1F able to inhibit binding of IL-1β to IL-1R1 in a competitive binding assay (Yao et al. 2015). In trout the kinetics of nIL-1F expression was studied after bacterial

infection, in comparison to two of the three IL-1β isoforms present in this species, where a delayed induction was apparent (Wang et al. 2009). Expression in trout macrophages was demonstrated, where nIL-1F increased in the trout macrophage cell line RTS-11 after LPS or IL-1β treatment. Lastly, in agreement with the concept that nIL-1F is a receptor antagonist, trout rnIL-1F (also produced from the thrombin cut site) was shown to antagonise IL-1β activity. Addition of nIL-1F to RTS-11 cells, that in itself had no clear effect on pro-inflammatory gene expression, inhibited subsequent IL-1β induction of TNFα, COX2, IL-1β, IL-8 and nIL-1F in a dose-dependent manner (Wang et al. 2009). In lumpfish comparative analysis of PAMP effects on IL-1 family cytokines showed that flagellin, poly I:C and CpG were all good inducers of nIL-1F in HK cells, which also induced IL-1β1 but not IL-1β2 or IL-18 (Eggestol et al. 2020).

10.7.2   Interleukin-10

Teleost IL-10 was first discovered by analysis of the Fugu genome (Zou et al. 2003c). Since then it has been studied in many fish species, with a recent study in zebrafish exploiting the unique ability to undertake functional studies in vivo using a mutant fish line lacking IL-10 (Bottiglione et al. 2020). A striking finding was that in these fish the gills showed an inflammatory phenotype, with marked thickening of the gill arch and filaments, and were more responsive to an exogenous irritant (R848) in terms of prolonged pro-inflammatory gene up-regulation. Such results suggest that IL-10 has a major anti-inflammatory role in teleosts and is crucial for gill homeostasis. Other studies in cyprinids have explored factors affecting IL-10 expression and the protein bioactivity. For example, in Catla bacterial infection (*A. hydrophila*) and LPS injection will elevate tissue IL-10 transcript levels (Swain et al. 2012), requiring NF-κB signalling, but in goldfish monocytes and granulocytes treated with rTNFα the expression of IL-10 is down-regulated (Grayfer et al. 2011). In grass carp IL-10 is induced quickly (3 h) by rIL-6 treatment of HK cells, as is IL-6 itself (Wen et al. 2020). However, rIL-10 can reduce this IL-6 induction by rIL-6, revealing a negative feedback loop. Interestingly, at later timings (12 h, 24 h) post-rIL-6 stimulation IL-10 expression is inhibited, showing the transient nature of this interaction. In goldfish, rIL-10 was shown to inhibit pro-inflammatory gene expression and an NADPH oxidase component (linked to a reduced ROS response) in monocytes stimulated with killed bacteria, and in splenocytes IFN-γ expression was reduced (Grayfer et al. 2011). Goldfish rIL-10 induced phosphorylation and nuclear translocation of STAT3 in monocytes, with SOCS3 mRNA levels subsequently increased, as also seen in carp and grass carp rIL-10-treated HK cells (Wei et al. 2014; Piazzon et al. 2015). An interaction with TGF-β has also been demonstrated in grass carp, whereby antibody blocking of both molecules increased pro-inflammatory gene levels after LPS stimulation but not when used individually (Wei et al. 2015). Lastly, an impact on adaptive immune responses has been shown in carp, where rIL-10 promotes the in vitro survival and proliferation of T cells from immunised fish when in combination with the specific antigen (*T. borreli* lysate), with gene

expression analysis showing a concomitant increase in CD8/ZAP70 expression but down-regulation of CD4 and other Th1/Th2 markers (Piazzon et al. 2015). The positive effect was not restricted to CD8^{+} memory cells, with carp rIL-10 also able to induce the proliferation, differentiation and secretion of antibodies from IgM^{+} B cells, although only with B cells from immunised fish in the latter case. In fact, two IL-10 molecules exist in carp (Piazzon et al. 2017), as with many other cytokines in this 4R species. They exert identical bioactivity but show differences in expression level: IL-10a is higher constitutively whereas IL-10b is the main induced form following infection.

IL-10 has been characterised in most of the other teleost lineages, including the siluriformes (Xiao et al. 2019), gadoids (Seppola et al. 2008), salmonids (Harun et al. 2011a; Abos et al. 2020) and percomorphs (Pinto et al. 2007; Peng et al. 2017; Matsumoto et al. 2018; Huo et al. 2019b). Salmonids also have two IL-10 paralogues that show differences in expression (Harun et al. 2011a, b). IL-10a is higher constitutively, more inducible in trout spleen following *Y. ruckeri* infection, and peaks earlier after LPS stimulation of RTS-11 cells, whereas IL-10b is more inducible in gills after *Y. ruckeri* infection and peaks later in LPS treated RTS-11 cells. Post-transcriptional regulation may account for these differences, with IL-10a having an upstream ORF in the 5′UTR whilst IL-10b has multiple mRNA instability motifs in the 3′UTR. rIL-10 can increase survival and antibody secretion in cultured trout IgM^{+} B cells, which is enhanced in the presence of CD40L and even further with IL-2 + CD40L, but does not increase their proliferation unless in combination with CD40L (Abos et al. 2020). The effect on antibody secretion and proliferation contrasts with the carp data, in that B cells from unimmunised fish were used for the former and CD40L was required for the latter. The effects of IL-10 have also been studied in vivo in amberjack, where injection increases splenic IgM[+] B cell number (Matsumoto et al. 2018). The IL-10 protein has been modelled in sea bass (Pinto et al. 2007) and golden pompano (Peng et al. 2017), where good homology was found to human IL-10, including an intradomain hydrophobic core, and suggests it will form a homodimer.

10.7.3 Interleukin-35

Whilst the peptide chains that constitute IL-35 (p35 and EBI3) are present in teleosts, as with the other IL-12 family members it is difficult to verify that IL-35 gene expression is occurring due to the sharing of these peptide chains with other IL-12 family proteins (namely IL-12, IL-27 and IL-39). However, a single study focused on the IL-35 protein has been undertaken with grass carp, in which two forms of EBI3 are present (EBI3a and EBI3b) (Zhang et al. 2019). In co-immunoprecipitation studies binding of p35 and EBI3a was demonstrated, but not with EBI3b. Subsequently, an rIL-35 protein was produced, made as a p35/EBI3a fusion protein, and was used to stimulate HK cells in vitro. Transcript analysis of selected genes over a time course revealed that p35, EBI3, CD25 and gp130 (IL6st) were induced by rIL-35 but that CD4–1, CD4–2, IL-17A/F1, RORγ2 and IL-12Rβ2 were decreased, at least during the first 24 h post-stimulation, potentially in line with a

suppressive function of the protein. However, at a later stage (48–72 h) IL-17A/F1, RORγ2 and IL-12Rβ2 (which combines with gp130 to form the IL-35 receptor) increased. Since rIL-35 was a good stimulant of p35 and EBI3 transcript expression, immunoblot analysis of the extracellular proteins was performed and revealed dose-dependent increases in protein expression for both chains.

10.7.4 Transforming Growth Factor-β1

TGF-β's belong to the TGF-β family of proteins that play an important role in cell proliferation, differentiation and regulation. Three TGF-β proteins (TGF-β1, TGF-β2, TGF-β3) have been described in tetrapods, and also exist in fish (Laing et al. 1999, 2000). However, a fourth was first described in gilthead seabream, termed TGF-β6; TGF-β4 and TGF-β5 are in fact the bird and amphibian versions of TGF-β1 but due to low homology were initially ascribed a different number. TGF-β6 appears to be lacking in amphibians, birds and mammals but is widespread in bony fish (Funkenstein et al. 2010; Wang et al. 2019c). It is most similar to TGF-β2, and it is likely that an ancestral TGF-β gene was duplicated by the WGD events in early vertebrates, leading to the four isoforms, which have been duplicated further by the 3R and 4R WGD in teleosts. Since TGF-β1 is considered to be the main immune active isoform, it will be the focus of this section and in this context.

Two paralogues of zebrafish TGF-β1 have been found (TGF-β1a and TGF-β1b) (Funkenstein et al. 2010), likely due to the teleost 3R WGD, since gar possess a single copy. Curiously the gene organisation has been shown to vary in cyprinid TGF-β1 vs tetrapods, although in both 7 exons encode the protein (Maehr et al. 2013). Relative to tetrapods there has been a fusion of exons 2 and 3 but an intron insertion in exon 7. As in tetrapods the protein is made as a latent form, consisting of a signal peptide, the latency-associated peptide (LAP) and a C-terminal 112 aa mature peptide immediately downstream of a furin cut site (Kohli et al. 2003). The mature protein has 9 conserved Cys residues, 8 that form 4 intra-protein disulphide bonds, giving a "cysteine knot" secondary structure, and the last an inter-protein bond to form a dimer. In carp TGF-β1 is highly expressed in spleen and HK, and can be induced by stimulation of HK cells with ConA (Yin and Kwang 2000). In grass carp TGF-β1 is induced in HK cells by rIL-1β, whilst rTGF-β1 inhibits IL-1β production and bioactivity, in a classical negative feedback loop that controls IL-1β effects (Yang et al. 2014). Similarly in goldfish rTGF-β1 down-regulates the activation of macrophages by TNFα. However, in grass carp and goldfish rTGF-β1 induces the proliferation of PBL and fibroblasts (CCL71 cells) respectively (Haddad et al. 2008; Yang and Zhou 2008) and can induce pro-inflammatory cytokine and IL-10 expression in grass carp PBL (but not HK cells) (Yang et al. 2012; Wei et al. 2015). Hence TGF-β1 can have positive as well as negative effects within the teleost immune system.

In salmonids three paralogues of TGF-β1 have been described in rainbow trout (Hardie et al. 1998; Maehr et al. 2013; de Mello et al. 2014), with expression highest in lymphoid

tissues such as gills, kidney and spleen, although TGF-β1c is also relatively highly expressed in reproductive tissues. TGF-β1a and TGF-β1c are paralogues from the salmonid (4th) WGD, but a second paralogue of TGF-β1b has not been found. They also differ in that TGF-β1a and TGF-β1c have a 7 exon gene organisation as for cyprinids but in TGF-β1b exons 3 and 4 (tetrapod exons 4 and 5) have fused to give a 6 exon gene (Maehr et al. 2013). Comparative studies have shown that TGF-β1a is more highly expressed constitutively than TGF-β1b in adult tissues and during ontogeny; however, TGF-β1b is more inducible by PAMPs and pro-inflammatory cytokines and after pathogen challenge. In percomorphs only a single paralogue has been found to date, which in tilapia has a 6 exon gene organisation as for salmonid TGF-β1b (Maehr et al. 2013), but in seabream exons 1 and 2 have also fused to give a 5 exon gene (Tafalla et al. 2003). The highest levels of expression were found in seabream macrophages and in the gills, whilst in hybrid striped bass expression was highest in PBL (Harms et al. 2000).

The activities described above reveal a good number of cytokines and downstream pathways are present in teleost fish that can dampen immune responses and prevent host damage. The presence of novel cytokines (nIL-1F) and multiple paralogues again suggests a particularly complex network is operating in these fish, which requires further investigation. Association of their production to particular cell types, such as Treg cells, will allow a more clear picture to emerge as to when and where these responses are needed and whether they can be manipulated to improve fish health in aquaculture.

10.8   Concluding Remarks

It is clear that teleost fish possess a complicated cytokine network to regulate and activate their immune system, to produce appropriate protective responses to pathogens. Key cytokines associated with the functional responses outlined above are present and show that they evolved early in parallel with the emergence of (RAG-mediated) adaptive immunity. The multiple cytokine paralogues in teleosts is a novel feature, caused mainly by the additional WGD events in this lineage. This gives a high level of redundancy but also allows more subtle regulation, with potential for differences in bioactivity or when/ where the different forms are expressed/secreted. Whilst some novel genes are also present, they are related to known cytokine families and in some cases represent a more ancestral form relative to cytokines in mammals. Information on teleost cytokine bioactivity and function is increasing but the cells which secrete them are often unknown. This is an important aspect for future studies since activation of lymphocyte subpopulations able to secrete particular cytokine repertoires may be crucial for disease resistance to particular pathogens, and hence a target for modulation by vaccines. Whilst the genomic resources available to study cytokines in zebrafish have huge benefits, differences in the cytokines present between fish groups are apparent and can be marked, as with the type I IFN. Thus, from a translational perspective, knowledge about cytokine function from model fish will

always need to be transferred and expanded in target species, especially those used for aquaculture where ways to maintain fish health are paramount.

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Major Histocompatibility Complex (MHC) in Fish

11

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Abstract

The term "major histocompatibility complex" (MHC) was used originally to name a polymorphic genetic region in mammals, several loci of which were involved in determining acute allograft rejection, hence the term *histocompatibility*. "MHC molecules" commonly refers to a special class of proteins expressed at the surface of somatic cells and involved in several major immunological functions. This chapter starts with a brief history of the fish MHC research, then focuses on the particularities of the fish MHC genetic region in the context of multiple genome duplications, with the discovery of a great variability of its architecture and composition among the species. The structural and functional properties of MHC class I and class II molecules are then described. Their nomenclature and classification are explained in an evolutionary perspective. Classical and non-classical MHC molecules are examined across fish

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species in which they have been characterized. The role of MHC variation in resistance to diseases is also reviewed. Finally, the importance of MHC in mate choice is discussed.

Abbreviations

CLIP	Class II-associated invariant chain peptide
ER	endoplasmic reticulum
HLA	human leukocyte antigen, the human MHC
MHC	Major histocompatibility complex
PSMB	Proteasome subunit beta type
TAP	Transporter associated with antigen processing
TAPBP	Transporter associated with antigen processing binding protein
TCR	T-cell receptor

11.1 Introduction

The term "major histocompatibility complex" (MHC) was used originally to name a polymorphic genetic region in mammals, several loci of which were involved in determining acute allograft rejection, hence the term *histocompatibility*. Allograft rejection was an artefact that masked the real and main biological function of this region, i.e. T-cell education, which was elucidated many years later, but the name of the region persists. Nowadays the term "MHC molecules" commonly refers to a special class of proteins expressed at the surface of somatic cells and involved in several major immunological functions. In humans, the *mhc* region *sensu lato* (i.e. the extended *mhc* also known as x*mhc*) spans 4 Mb on the short arm of chromosome 6 and contains more than 200 genes (Consortium 1999). It is traditionally divided into three subregions: a **class I subregion** on the telomeric side (about 2 MB) with genes encoding MHC class I antigen-presenting molecules; a central **class III subregion** (about 1 MB) with many key genes of innate immunity including several TNFs and complement factors; a **class II subregion** on the centromeric side (about 1 MB) with genes encoding MHC class II antigen-presenting molecules and proteins involved in peptide loading, as well as proteins involved in processing and transport of peptides available for MHC class I presentation. In contrast, genes encoding the proteins of MHC class I presentation and processing are closely linked

in the genome of most vertebrates. Placental mammals are the exception. Although the organization and the size of the *mhc* region vary among tetrapods, genes encoding class I and class II molecules are regularly found within a single *mhc* region.

The prototypic class I and class II MHC molecules are conserved across jawed vertebrates. **Class I molecules** comprise (1) a heavy chain (or α chain) that is a type I integral membrane protein with three extracellular domains (α1, α2, α3), a transmembrane domain (TM), and a short intracytoplasmic domain, and (2) a light (soluble) chain named β2microglobulin (β2m) that is not encoded within the *mhc* in humans (Margulies et al. 2013). Class I molecules bind endogenously derived peptides and present them to CD8^{+} T lymphocytes. The stabilization of class I molecules and peptide loading involves a number of proteins encoded in the *mhc*, including the chaperone TAPASIN and the peptide transporters TAP. Peptides are generated from cytosolic proteins via degradation by the immunoproteasome and are loaded in a groove involving the α1 and α2 domains of the MHC class I molecules. In human class I molecules HLA-A, -B and -C, α1 and α2 domains are extremely polymorphic, allowing the binding of a large variety of peptides within human populations (https://www.ebi.ac.uk/ipd/imgt/hla/stats.html). **Class II molecules** also are heterodimers. They are made of two type I proteins, named α and β chains, both consisting of two extracellular domains, a transmembrane and a short intracytoplasmic region (Margulies et al. 2013). They present peptides derived from exogenous proteins to CD4^{+} T cells. Class II molecules are first combined to an invariant chain in the ER, which stabilizes the complex. Antigen peptides are later loaded in a groove at the top of MHC class II α1 and β1 domains by a process involving a non-classical MHC class II molecule called DM. In both MHC class I and II molecules the membrane proximal domain (Class I α3, β2m, Class II α2 and β2 domains respectively) is an immunoglobulin superfamily (IgSF) member C1 domain (Williams and Barclay 1988), belonging to the same category as that encountered in BCR and TCR, with the evolutionary implications that this may suggest (DuPasquier and Chretien 1996).

The genetic, structural and functional diversity of class I/II MHC molecules and the related proteins led to a specific terminology and nomenclature. **Classical class I or II MHC molecules** (also denoted by the letter "a" in class Ia/IIa) bind a wide diversity of peptides and present them to αβ T cells, hence their peptide-binding domain is highly polymorphic. **Non-classical MHC molecules** (called class Ib/IIb) are much less polymorphic and do not present peptides to αβ T cells expressing diverse TCR, instead they have other specific functions. For example, human HLA-E (Q_A-1 in mice) are class Ib molecules that bind a limited repertoire of peptides from leader sequences of various MHC class I molecules, making a complex that is recognized by NK cells and which inhibits the cells' cytotoxic activity. HLA-E also restricts a CD8^{+} T-cell subset that controls the development of CD4^{+} autoimmune and conventional responses, illustrating the potential diversity and the complexity of class Ib functions. Finally, **MHC Class I-like molecules (CD1, MR1)** present structural similarities with MHC class I proteins, however they display low sequence polymorphism and do not bind peptides. Some of them do not bind the β2microglobulin chain. Instead, their groove has evolved to bind other types of molecules

such as glycolipids for CD1, or microbial precursors of vitamin B2 for MR1. Bioinformatic and phylogenetic evidence has allowed researchers to classify the different *mhc* genes into "lineages" based on their common origin. Different classes of the MHC molecules belong to different lineages.

This chapter is about the MHC and MHC-like proteins of fishes, and the genomic regions in which they are encoded. A fish has been defined as: "a limbless cold-blooded vertebrate animal with gills and fins living wholly in water." In this chapter, we will focus on bony fishes (i.e. Osteichthyes), but we will also discuss MHC within the Agnathans and Chondrichthyes (sharks and rays) when it is important from an evolutionary perspective. Bony fish have experienced several extra genome duplications compared to other vertebrate taxa and one might expect that it created a special context with respect to the fate of duplicated genes. Indeed, some unique features have been discovered, as the particularities of the *mhc* region and associated MHC molecules in this group of vertebrates will show.

11.2 A Brief History of Fish MHC Research (See Refs. in Table 11.1 for this Section)

The fish research related to MHC started long after the discovery of the *mhc* locus in mice (known as H2), and even a few years after that of *mhc* in chicken (known as B locus), around the time when the term HLA was first used in humans. In the mid-1950s, with the abovementioned mindset, fish immunologists probably expected from their fish scale grafting studies a situation similar to that encountered in warm-blooded vertebrates, with the existence of a major histocompatibility locus if not even a major histocompatibility complex. When things therefore proved to be different it must have been a welcome surprise! Fish were not like the amphibians studied in parallel, where everything was similar to that in mice and men. Surprisingly, it was precisely for this reason that fish were ostracized in MHC research. Fish were odd. Period. One could hear or read: "And there are too many of them, think of it; more than 25,000 species"; "One can deal with fish MHC later"; "Too complicated". Fish, however, display genetic peculiarities such as a relatively large number of intra-class whole-genome duplications that could turn out to be useful natural tools for immunogenetics. Fish offered oddities that triggered the curiosity of many immunologists long ago, such as male parasitism in several anglerfish species; a unique example of natural allograft in vertebrates which cannot exist without interesting implications for understanding tolerance, and MHC in particular. Fish, such as zebrafish, medaka and others, are also important developmental/ecological models. And last but not least the economic importance of many fish should foster research on MHC, as a field directly relevant for the health of commercially bred species.

Indeed, the scenario of the fish MHC discoveries over the years, as one sees it unfolding in Table 11.1, would make a good movie. There are several periods, with fish MHC research ongoing in parallel with studies on fish immunity, fish lymphoid organs and fish T cells/thymus.

Table 11.1 Timeline of discoveries of the main particularities of the fish MHC which will be developed in other parts of the chapter. **On the left** MHC and milestones in immunology. References can be found in textbooks or review articles. See for example Fundamental Immunology (WE Paul, ed.) and historical articles by AM Silverstein. **On the right** stepwise acquisition of knowledge, concerning cold-blooded vertebrate MHC and more specifically fish (bold). The purpose of this table is to show the difference between the acquisition of knowledge in warm- and cold-blooded vertebrate, where it converged, where it differed, and how much time elapsed between discoveries in one or the other taxon. To keep the content of the table at a reasonable level the first contribution of a group is often the only one mentioned, even though the topic was pursued further. The references that allude to all the follow-up of the basic findings can be found in recent reviews

Year	General immunology milestones (*MHC general*)	MHC cold-blooded vertebrates (***MHC in fish***)
1890–1907	Ehrlich side chain theory	(Hammar 1905) (Ver Eecke 1899) Frog thymus: Lymphocyte production, thymectomy and lower resistance to infection.
1901–1917	*Loeb: Transplantation and lymphocyte "cytotoxicity"; second graft rejected quicker*	
1914–1916	*Little: DBA mouse strain. Genetics of transplantation in mice*	
1926	Murphy: Lymphocytes in immunity	
1936	*Gorer "antigen 2" the same as that controlling susceptibility to transplantable sarcoma.*	
1944	*Medawar: Allograft rejection is a specific response*	
1946	*Owen:* In utero *tolerance of RBC by dizygotic twins*	
1948	*Snell, Gorer: H2 Ag =major histocompatibility Ag*	
1953	*Medawar: Immunological tolerance, Gvh Hasek parabiotic chicken tolerance*	
1953	*Amos, Dausset leucoagglutinins: HLA*	
1956		**(*Hildemann* 1956) *scale allograft specific rejection; multiple loci.***
1957	Talmage, Burnet: Clonal selection *Simonsen: GVH in chicken*	
1958	*Payne, van Rood: Elucidation of HLA genetics*	**(*Kallman* 1958) *genetics of fin allograft rejection in Xiphophorus***
1960		**(*Kallman* 1960) *Histocompatibility loci* (Triplett and Barrymore 1960) *Triplett: Shiner-perch specifically reject allografts***
1961	*Miller, good: Thymus role*	
1963	Oudin: Idiotypy; Jerne: Plaque assay	

(continued)

Table 11.1 (continued)

Year	General immunology milestones (*MHC general*)	MHC cold-blooded vertebrates (***MHC in fish***)
1964		(***Kallman*** 1964) *at least 9–12 H loci in Xiphophorus*
1965	Hilschmann, Craig: V and C region identified	(Du Pasquier 1965) (Cooper and Hildemann 1965) Thymus role in amphibians.
1966	Uhr Gowans: Memory lymphocytes *Cooper MD, Good: Thymus and Bursa dependent system* Claman TB: Collaboration	
1968	Miller Mitchell: Lymphocyte subsets	
1969	*Mc Devitt: H2 controls Ab production.* Edelman: Ig structure	(***Hildemann and Thoenes*** 1969) *Specific memory of hagfish in allograft response*
1971	Sprent: Ag induced recruitment of circulating lymphocytes	
1972	*Kiessling: NK cells*	
1973	Steinman: Dendritic cells	(Du Pasquier and Miggiano 1973) amphibian MLR
1974	Koehler, Milstein: Monoclonal Abs	
1975		(Du Pasquier et al. 1975) amphibian MHC
1976	Tonegawa: Ig gene somatic rearrangement. Morgan: Il-2	
1977	*Pink: MHC antigens in chicken*	
1979	Adhesion molecules, lymphocyte trafficking	(Du Pasquier et al. 1979) major changes of MHC class I and II expression at metamorphosis in *Xenopus*
1980	Hood: VDJC segments identified	(Blomberg et al. 1980) TB collaboration in *Xenopus*
1981	Boman: Silk moth anti-bacterial peptides and innate immunity	(Bernard et al. 1981) in *Xenopus*, CML & MHC restricted T/B collaboration
1982	Paul: Il-4. *Allison, Mak, Davis: TCR genes at last!*	
1983	Butcher: L selectin	(Flajnik et al. 1985) split tolerance in MHC chimaeras *Xenopus*
1984	Weigert: Ig genes somatic mutation	(Flajnik et al. 1984) MHC Class I molecules in *Xenopus* (**Caspi and Avtalion** 1984) *MLR in Carp*
1985		(Kaufman et al. 1985) MHC class II molecules in *Xenopus*; (Miller et al. 1985) *T/B cooperation in catfish*

Table 11.1 (continued)

Year	General immunology milestones (*MHC general*)	MHC cold-blooded vertebrates (***MHC in fish***)
1986	Käre: *Missing self;* Clark: CD40 Baltimore Nfkappa B, gd T cells	**(Nakanishi** 1986**) *Thymectomy & graft rejection in fish*(Miller et al.** 1986**) *Miller NW: MLR in catfish***
1987	Mosmann: TH1 Th2; R Schwartz: T and B anergy; Holmes: Il-8; *Bjorkman: MHC structure*; Kappler & Marrack: Negative selection in the thymus Mosmann: TH1 Th2	**(Raison et al.** 1987**) *MLR in hagfish***
1988	Figueroa: MHC polymorphism predates speciation	**(Kaastrup et al.** 1988**): *MLR in trout***
1989	*Von Boehmer: CD4/ CD8 expression;* Schatz: Rag1	**(Kaastrup et al.** 1989**): *MHC In carp* (segregation studies)**
1990	*Townsend, Cresswell: Role of TAP*	**(Hashimoto et al.** 1990**) *carp Class I gene***
1991	*Germain, Rammensee: MHC peptide;* Holmes: Chemokine rec.	**(Stet et al.** 1993**) *polymorphism of carp MHC;* (Glamann et al.** 1991**) *trout Class II* (Nakanishi** 1991**) *Thymectomy in Sebasticus***
1992	Nagata: Role of Fas in apoptosis	**(Kasahara et al.** 1992**) *shark class II alpha* (Vallejo et al.** 1992**) *Ag presentation in fish***
1993	Nemazee: Receptor editing; Bluestone: CTLA inhibitory role; *Klein: MHC gene number increases/ decreases depending on the need to protect the host from ever-changing parasite pressure.*	**(Ono et al.** 1993a**) *carp MHC, intron-exon in Cichlids;*(Sültmann et al.** 1993**) *Class II α in zebrafish;* (Shum et al.** 1993) Class I gene isolated in *Xenopus*; (Sato et al. 1993) class II *Xenopus*; (Flajnik et al. 1993) Variation on MHC architecture Class Ib in *Xenopus* **(Grimholt et al.** 1993**; Hordvik et al.** 1993**)** First MHC I and II in salmonids
1994	*Creswell: Tapasin*	(Kato et al. 1994; Salter-Cid et al. 1994) Factor B and HSP70 in *Xenopus* MHC; **(Grimholt et al.** 1994**) *highly variable Class I and IIb in salmon***
1995	*Monaco: Proteasome and antigen presentation; Makino: NKT cells*	
1996	Lemaitre: Toll and drosophila immunity	**(van Erp et al.** 1996**) *Z class I lineage in carp***
1997	*Kasahara: 4 MHC paralogons in human* Nei: Birth and death model of MHC evolution	**(Persson et al.** 1999**) *unusual large number of class I genes in the cod***

Table 11.1 (continued)

Year	General immunology milestones (*MHC general*)	MHC cold-blooded vertebrates (**MHC in fish**)
1998	*Creswell: Calnexin, calreticulin, Erp57 and the class I pathway*	**(Wittbrodt et al. 1998)** *first evidence for a teleost specific WGD*
1999	*Kaufmann: Chicken minimal essential MHC*	**(Persson et al. 1999)** *many class I in cod*
	Rock: Role of immuno-proteasome	
2000	Breitfeld: Follicular T helper	**(Grimholt et al. 2000)** *Class II alpha genes cloned in salmonids;* (Ohta et al. 2000) primordial synteny of class I &II; **(Hansen and Strassburger 2000)** *trout CD8*
2001		**(Clark et al. 2001)** *Clark: fugu class I region basic architecture;* (Sammut et al. 2002) Class I and II conversion and deletion in polyploid frogs; (Shum et al. 2001) *impact of lack of class I:II linkage*
2002	Tschopp: Inflammasome *Serwold: ERAP (aminopeptidase) customizes peptides for MHC presentation. Mathis: AIRE*	(Ohta et al. 2002) proteasome and class I pathway linked in shark; **(Kruiswijk et al. 2002)** *Class I Z lineage in cyprinids*
2003		**(Grimholt et al. 2003)** *disease resistance MHC linked in salmon*
2004	*Beck: Map of the extended MHC (HLA)*	**(Magor et al. 2004)** *3 polymorphic b2M loci in trout;* **(Suetake et al. 2004)** *CD4 in fugu*
2005		**(Pilström et al. 2005)** *Class II in cod? Why is response so poor?* **(Sambrook et al. 2005)** *SMHC genes in zebrafish genome;* **(Fischer et al. 2005)** *mhc I expression in trout ontogeny;* **(Quiniou et al. 2005)** *MHC genes and spontaneous allogeneic cytotoxicity*
2006		**(Laing et al. 2006)** *2 CD4 in fish* **(van Oosterhout et al. 2006)** *Variation in class IIb number in guppies*
2007		**(Utke et al. 2008)** *cytotoxic fish leukocytes* **(Lukacs et al. 2007)** *trout salmon comparison*
2009		**(Toda et al. 2009)** *CD8 functional study of fish*
2011		**(Star et al. 2011)** *many class I, no class II in cod;* **(Ohta et al. 2011)** *b2M in shark MHC*
		(Haase et al. 2013) *Class II defect in pipefish*

(continued)

Table 11.1 (continued)

Year	General immunology milestones (*MHC general*)	MHC cold-blooded vertebrates (*MHC in fish*)
2013		(**Bannai and Nonaka** 2013) *Multiple class II lineages conserved for hundreds of million years;* (**Dijkstra et al.** 2013) *DM is disposable*
2014		(Robert and Edholm 2014) invariant T cell restricted by non-classical MHC Class I in *Xenopus* tadpole.
2016		(**Grimholt** 2016) *Evolution of fish MHC*
2017	*Denzin: Antibody responses to viral infections are linked to the non-classical MHC class II gene H2-Ob.*	(**Chen et al.** 2017) *3D structure of carp MHC Class I*
2018		(Edholm et al. 2018) non-classical class I in Xenopus antimycobacterial response
2019	*Manczinger: Pathogen diversity drives the evolution of generalist MHC-II alleles in human*	(**Dijkstra and Yamaguchi** 2019) *MHC and disease resistance in fish;* (**Dubin et al.** 2019) *No class II in anglerfish* (**Svenning et al.** 2019) *inducible non-classical salmon class I*
2020		(**Swann et al.** 2020) *lack of class I/II, rag and aicda in anglerfish with male parasitism* (**Roth et al.** 2020) *trade-off between immunological tolerance and embryo rejection in evolution of male pregnancy in Syngnathids;* (**Almeida et al.** 2020) *non-classical class I in shark*

1950-early 1980, pioneering period: rejection of allografts is studied in a couple of teleost species, nothing suggests the existence of MHC in fish. Allograft experiments in the 1950s that could have revealed a major histocompatibility locus failed to do so. Multiple minor loci instead seem to be at work.

A controversy raged in the 1960s: do Agnathans have a bona fide adaptive immune system? Apparently yes, since hagfish showed allograft recognition and specific memory, and in those years this was "the" criterion for the presence of adaptive immunity. However, no immunogenetic studies were performed, hence no analysis of co-segregations (see below) of the genes encoding acute graft rejection with those encoding various cell surface markers was possible. Therefore, no relationship with an MHC could be demonstrated.

Mid-1980s–1990: the discovery of a bona fide teleost MHC, based on co-segregation of several functional (including MLR) and serological markers. The field had to wait a long time, lagging behind discoveries in anurans which were remarkably similar to mammals.

Fish T-cell biology is explored, mixed lymphocyte reaction being demonstrated in different teleost species and perhaps also in agnathans. MHC co-segregation studies strongly suggest the existence of an MHC in teleosts.

1989–1990: a turning point in the history of fish MHC, from the demonstration of an MHC by co-segregation of functional genetic polymorphic markers to the cloning of the first elements (a class I gene), in carp.

1990–1996: Cloning, cloning and more cloning. Class I, Class II, and β2m genes are cloned in several fish species, confirming structural homology of key MHC elements with other vertebrates. Polymorphism is investigated, multiple lineages of Class I are defined. Antigen presentation starts to be studied.

1997–2005: first harvest. One still clones, the diversity of species analysed increases and genomic studies become available. When fish MHC genes are assembled in 1989 (vs 1975 for *Xenopus*), the surprises start: Class I and Class II genes are not physically linked! This quickly becomes a source of speculation on the origin of MHC but eventually shark studies bring the counter back to zero by showing that the linkage is primordial. Yet, as surprising and interesting as it is, this lack of linkage is not the first example of separation of genes in the MHC: the B locus of the chicken and non-classical class I loci in *Xenopus* were other examples of regions with intra-MHC breakages and translocations. They, however, did not affect the sacred linkage of Class I-II and were not as shocking. The abundance of WGD in fish compared to other vertebrates (except *Xenopus*) provided possible explanations and different models of organization.

Key findings during this period: the basal fundamental linkage of the Class I pathway genes (LMP, TAP, Class I molecules) has been lost in the mammalian Class I region. Fish CD4 and CD8 start to be characterized.

Functional studies on the immune response of cod are puzzling and suggest problems with Class II.

Resistance to disease is linked to fish MHC which underlines functional homologies with other species having an MHC.

The coelacanth genome reveals the composition, organization and synteny conservation of its MHC locus. Genes encoding α/β and γ/δ T-cell receptors, and CD3, CD4 and CD8 co-receptors also are characterized. Ig heavy chain variable region genes and TCR components are interspersed within the TCR α/δ locus, an organization previously reported only in tetrapods.

Agnathans turn out to have a different adaptive immune system based on leucine-rich repeats variable receptors rather than IgSF members and they seem to have no MHC at all!

2005–2020: "second growth" harvest. From 2006 to 2011, fish CD8 alpha is cloned and sequenced, followed by more discoveries and surprises. Cod inaugurates a series of natural MHC elements natural knock-outs. Profound variations on the theme of Ag presentation are seen: the class II pathway seems to be disposable in Gadidae, with Class II, CD4, DM missing or non-functional. A similar observation is reported for pipefish, then anglerfish joined the bandwagon in 2019, lacking MHC class IIa and b, CD74, and CD4. Very interestingly, these genes were lost only in anglerfish species that practice male

parasitism, not in other anglerfish species! This was spectacularly confirmed recently when it was found than several species of anglerfish practicing male parasitism had also lost, depending on the level of parasitism, Rag or AID, or the MHC Class II and even Class I pathways. In the meantime, the 3D structure of a class I molecule is resolved in grass carp in 2017. Some features are distinct from known peptide-MHC class I complexes, especially the region of the α3 domain, which in mammals forms a contact with CD8. Overall, one starts to take advantage of comparative genomics and of the multiplicity of genome duplications in teleosts.

The years 2019 and 2020 have seen stunning contributions showing that *mhc class II* genes are eliminated from the genome of different fish species in which reproduction implies natural "allografts" (i.e. male parasitism in anglerfish and male pregnancy in seahorses and pipefishes).

11.3   The MHC as a Genetic Region

The original backbone of the *mhc* region is ancient and contains genes involved in innate immunity, stress response and protein degradation (Levasseur and Pontarotti 2010; Du Pasquier 2004; Flajnik and Kasahara 2001). No gene for class I or class II has been found in agnathans nor in any non-vertebrate species, but a "proto-MHC" genetic region was identified in the lancelet, a chordate, which contains a number of homologs of canonical *mhc* markers (Abi-Rached et al. 2002). The origin of this proto-mhc region is ancient, since it could be tracked back to a basal branch of the metazoans, the placozoan *Trichoplax adhaerens*, with the proteasome genes *psma*, *psmb* and *psmd* and other genes likely involved in stress response and immunity (Fig. 11.1a) (Suurväli et al. 2014). During the early vertebrate evolution, this primordial MHC generated a tetrad of paralogous regions through two whole-genome duplication events (WGD) (Ohno 1970). In the human genome, these four paralogons are located on chromosomes 1 (1q21-q25/1p11-[32P]), 6 (6p21)22)—the bona fide MHC-, 9 (9q32-q34), and 19 (19p13.1-p13.3) (Kasahara et al. 1996).

Like the other components of the RAG-based adaptive immune system, class I and class II MHC molecules appeared in early jawed vertebrates and are now found from sharks and rays (Chondrichthyes) to mammals. *Class I* and *class II mhc* genes are genetically linked within the MHC (Ohta et al. 2000), with the remarkable exception of bony fishes (Osteichthyes). In these species, classical *class I* and *class II mhc* genes have been consistently found on different chromosomes (Bingulac-Popovic et al. 1997; Hansen et al. 1999), hence they are sometimes named *mh* genes (for "major histocompatibility"). While a third WGD event occurred at an early stage of teleost evolution, only one "core" MHC has been found consistently retained in sequenced fish genomes. This region contains the classical (polymorphic) *mhc class I* genes, coding for the presenting molecules and the proteins involved in antigen processing and loading on the MHC class I molecules (*tapbp*, *tap2*, proteasomes), as well as a number of conserved markers of the tetrapod *mhc*
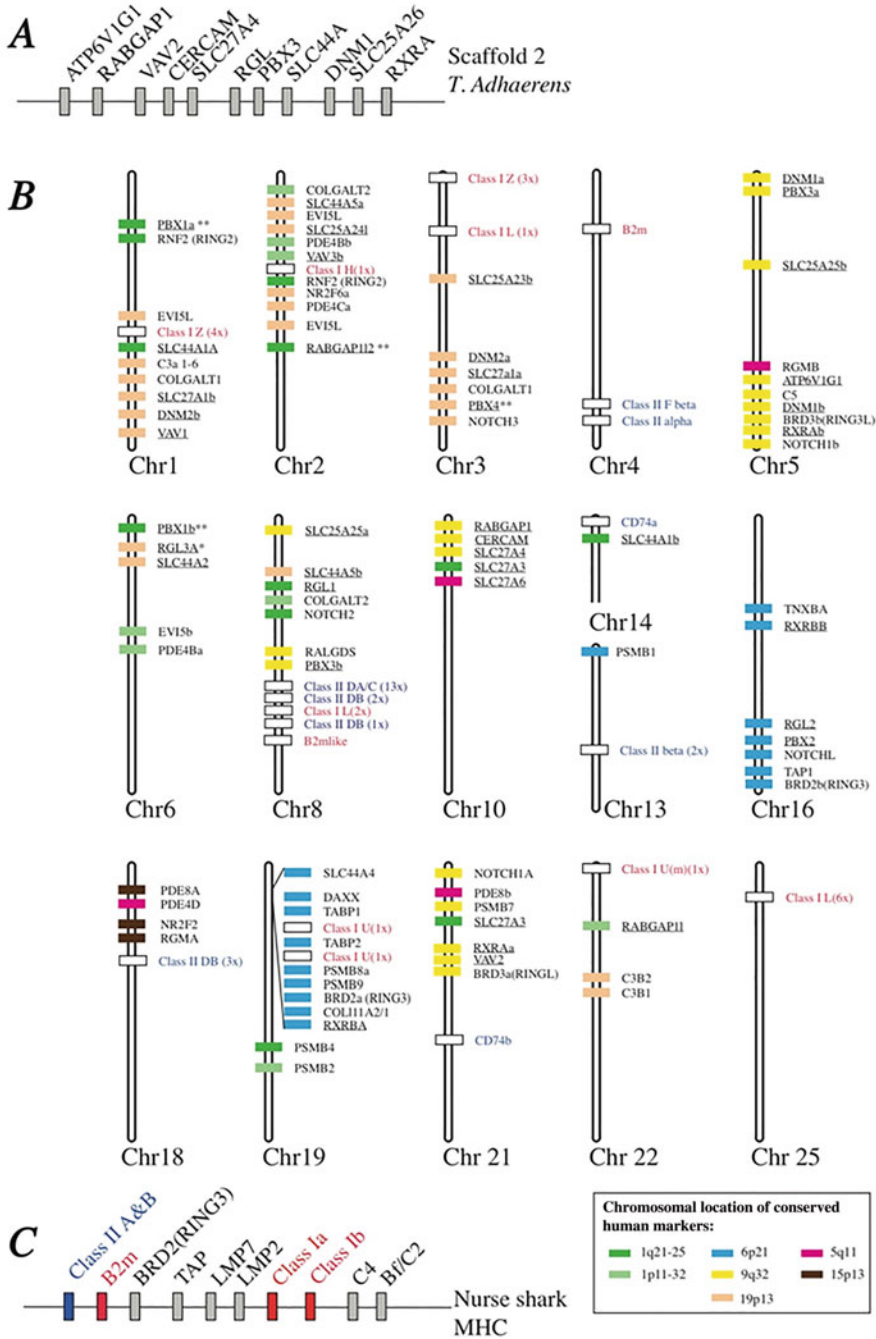
Fig. 11.1 (**a**) The *proto-mhc* in *Trichoplax adhaerens* (from Suurväli et al. 2014). (**b**) Distribution of *mhc* and markers from the *mhc* tetrad on the zebrafish genome. The colour code refers to the chromosome on which the markers are located in human. Markers present in the *Trichoplax*

(*slc44a4, daxx, rxrb*). This "core" *mhc* region is located on chromosome 19 in zebrafish (*Danio rerio*) (Fig. 11.1b; (Dirscherl et al. 2014; Sambrook et al. 2005)). In Atlantic salmon, this region has been duplicated by the salmonid (fourth) WGD event about 88 Mys ago, and two paralogons are found in LG3 and LG15 (Lukacs et al. 2010); however, only LG15 contains classical *mhc class I* genes and is considered as the core *mhc* in this species. Divergent haplotypes of classical *mhc class I* and proteasomes have been reported in zebrafish, salmon and medaka (Tsukamoto et al. 2005; Grimholt 2018; McConnell et al. 2014), suggesting that local associations of these genes into microsynteny blocks may confer a functional advantage. In contrast, the genes encoding Class Ib or Class I-like molecules are scattered throughout the genome. *mhc class II* genes are also located in multiple regions, and generally do not belong to microsynteny blocks (Dijkstra et al. 2013). For example, zebrafish *mhc class Ib* genes are located on chromosomes 1, 2, 3, 8, 22 and 25, and *class II* genes on chromosomes 4, 8 (class IIa), 13 and 18 (Fig. 11.1b).

Importantly, − *mhc* and *mhc like* genes are not randomly distributed in the genome. For example (Fig. 11.1b), in zebrafish they are generally found in the neighbourhood of the orthologs of the markers from the MHC paralogon tetrad—or share the same linkage group. Strikingly, the zebrafish chromosome 8 shows an ancient MHC linkage lost in tetrapods: that of β2m. Similar to the nurse shark (*Ginglymostoma cirratum*), *β2m* in the zebrafish is linked to a set of *mhc class II* genes plus an extra *mhc* marker RGL (RALGDS) on chromosome 8. It is also worth mentioning that, although not closely linked, a second zebrafish *β2m* gene is linked to a second set of *mhc class II* genes on chromosome 4 (see Fig. 11.1c; (Ohta et al. 2011)). Thus, the "core" region with *class Ia* genes is not the only conserved synteny block of *mhc* markers in fish genomes.

Another interesting question is how most *class I*, but also *class II*, sequences could have been linked to markers of the *mhc* tetrad. Adaptive (functional) clustering has been proposed but there might be more mechanistic reasons. One may wonder to what extent translocations have occurred in a targeted manner that would have favoured the reinsertion of chromosomal fragments into the chromosome with tetrad markers. This does not distract from the possibility that the original clustering was adaptive.

A further notable peculiarity of the bony fish MHC is the lack of a functional MHC class II pathway for Ag presentation to CD4^{+} T cells, in several phylogenetically distant species. It was discovered in the Atlantic cod (*Gadus morhua*) (Star et al. 2011) and other gadoids, in the pipefish (*Syngnathus typhle*) (Haase et al. 2013), and in an anglerfish (*Lophius piscatorius*) (Dubin et al. 2019). In these species, all *mhc* class II genes, *cd74* and *cd4* were lost or pseudogenized. In cod, other gene loss events removed important genes of the

Fig. 11.1 (continued) proto-mhc *are underlined. Unique features of a gene on chromosome 13 noted "Class II beta" here, which displays some similarity with both β2-microglobulin and MHC class II, remind a recently reported MHC suggesting that MHC class II likely preceded class I (Okamura et al. 2021). (**c**) The* mhc *of the nurse shark (from refs. (Bartl and Nonaka 2015; Flajnik and Du Pasquier 2013)).* β2m*, class I and* I-like *genes are represented in red,* class II *in blue*

immune system (Mx, Tlr5) long before the class II loss (Solbakken et al. 2016), while *mhc class I* genes expanded, maybe compensating the loss of *class II* (Star et al. 2011). Most surprisingly, in anglerfish species in which the male gets permanently attached and fused to the female, many genes involved in adaptive immunity were lost or pseudogenized including *cd8, igm, cd79, tcra and b, aicda* and even *rag1* and *rag2* (Swann et al. 2020). Evolution of pregnancy was associated with loss of several genes of the MHC II pathway in pipefish, and with loss of highly divergent invariant chain in seahorses, suggesting a trade-off between immunological tolerance and embryo rejection (Roth et al. 2020). Altogether, these observations suggest that genomic instability of fish genomes and specific selection pressures led to repeated, independent events of MHC II loss (among other immune genes), followed by loss or inactivation of the other genes of the pathway. The location of *mhc class I* and *class II* genes in distinct regions of the genome could have favoured the loss of class II genes without impact on the class I region. However, the consistent loss of both classical and non-classical *class II genes* in all three species cited above raises unresolved evolutionary issues. In contrast to CD4 or CD74, functions of MHC class IIb molecules are presumably independent from the classical class II pathway, hence the loss of the latter should not have removed protective selection pressure for the former. Also, the origin of the MHC class II loss in cod, in absence of male pregnancy or male parasitism, remains difficult to explain.

11.4   MHC Class I Molecules

Nomenclature Rules and Notations
Six lineages of fish MHC class I genes have been identified to date (MHC I-U, −Z, -S, −L, P and -H (Grimholt et al. 2015; Grimholt et al. 2019)) from phylogenetic and comparative analyses. Lineages U and Z comprise molecules that presumably bind and present peptides, while the four other lineages likely do not and therefore should be considered as non-classical MHC class I or MHC I-like molecules. We will later discuss non-classical MHC and potential MHC like molecules together.

A systematic nomenclature has been established for these MHC class I molecules and their genes (Grimholt 2016), based on the rules followed by the general MHC nomenclature (Klein et al. 1990). *mhc* gene names are constituted as follows: first, "mhc", then a four-letter abbreviation of the fish species' scientific name, followed by the lineage symbol (U, Z, S, L, P and H for fish MHC class I); the gene locus; a third symbol referring to the subclass (A – for α chain –for class I); the gene identifier if required. This is followed by an asterisk and an allelic symbol designated by 01, 02, 03, etc. For example, a rainbow trout typical class I is encoded by the gene named *mhcOnmy-UBA*. *mhcOnmy-UBA*01:01* would name a particular variant: *01 defining a group of sequences with less than five amino acid substitutions, and: 01 a particular variant within that group. (See IPD-MHC nomenclature rules at https://www.ebi.ac.uk/ipd/mhc/group/FISH/).

11.4.1 Classical Class I (From the U Lineage)

11.4.1.1 Structure of MHC Class I Molecules

Fish class I molecules are structurally similar to their mammalian counterpart, with an α chain composed of three domains, a TM, and a short intracytoplasmic region. The membrane distal α1 and α2 domains form a peptide-binding domain (PBD) with a groove. The α3 domain is an IgC1 domain. This chain is associated with the β2M comprising one IgC1 domain. The 3D structure of a grass carp Ctid-UAA/β2M/peptide complex has been recently resolved, showing the typical structure of a classical MHC class I molecule with a few particular characteristics (Chen et al. 2017), one of which was the divergent topology of the CD loop of the α3 domain, which in mammals binds CD8. This may suggest that fish classical MHC I may not interact with CD8 in a canonical manner. All domains from fish U MHC I sequences can be represented by an IMGT "collier-de-perles" at http://www.imgt. org/IMGTrepertoireMH/2D-3Dstruct/.

11.4.1.2 Polymorphism, Variation in Gene Numbers

Genes from the U lineage represent a large fraction of fish MHC class I genes. They have been extensively amplified in several species such as tilapia (Sato et al. 2006), cod (Star et al. 2011) and stickleback (Grimholt et al. 2015). The number of genes may change between haplotypes (Lukacs et al. 2007; McConnell et al. 2016). The U lineage comprises classical genes (e.g. *uba* in salmonids) with high polymorphism in α1 and α2 domains; it also encompasses non-classical genes (see below). Strikingly, the numerous U genes present in the cod genome do not harbor a typical (high) level of polymorphism, and polygenism may thus play a significant role in the diversity of presented peptides. The presence of conserved ancient versions of the α1 (and α2) domain in some haplotypes across teleosts also contributes to the diversity of U molecules (Shum et al. 2001; Hansen et al. 1996). Interestingly, an endosomal sorting motif has been identified in the intracytoplasmic region of some splicing isoforms produced by a subset of U *mhc I* genes of cod and stickleback, suggesting that it might replace the class II function, at least in cod (Grimholt et al. 2015), or have atypical functions.

11.4.1.3 Diversity of Genes Involved in MHC Class I Processing and Peptide Loading

Genes involved in the peptide loading onto classical MHC I molecules (*tap, tapbp, psmb-8,-9,-12,-13*) are closely linked to *uba* genes. Some of these genes can be polymorphic (as in zebrafish (McConnell et al. 2016)) or not (as in Atlantic salmon (Grimholt 2018)). Importantly, a feature common across teleosts is a retention of the paralog sets produced by the second vertebrate WGD, which likely leads to alternative versions of the peptide-loading complex. In salmonids, an additional WGD further increased the number of genes available, and the potential diversity of the peptide-loading molecular pathway (Grimholt 2018).

11.4.1.4 Expression, IFN Induced Expression

The expression of the MHC class I UBA was analysed during rainbow trout ontogeny by immune-histochemistry (Fischer et al. 2005). Early expression was observed in the brain including in some neurons, but not in adult fish. This MHC class I protein was expressed very early in the thymus, and this tissue still showed the highest expression levels in 1-year-old fish. Other lymphoid tissues and epithelia also expressed the UBA molecule. Interestingly, while the gut was one of the tissues where class I UBA was highly expressed in the adult, intestinal UBA was detected rather late in ontogeny (circa 40 days post hatching) compared to gills (18 days post hatching), perhaps in connection with oral tolerance. Viral infection induced rainbow trout UBA transcripts in lymphoid tissues, PBL and liver (Landis et al. 2008), as well as other genes of the type I IFN and class I pathway (Hansen and La Patra 2002). While the main regulator of classical MHC class I expression is the enhanceosome in mammals, IRF1 seems to be the master regulator in fish, at least in epithelial cells (Grimholt et al. 2020, see also below Sect. 11.5.2.3 about MHC class II).

11.4.1.5 Classical Class I and CD8+ T-Cell Responses

Several lines of evidence indicate that cytotoxic antiviral responses mediated by fish CD8^{+} T cells are restricted by class I molecules (Somamoto et al. 2013; Dijkstra et al. 2001; Utke et al. 2008). For more details, see the Chap. 4 "Cell-mediated immune responses" by Fischer and colleagues, in this book.

11.4.2 Non-classical MHC Class I (All Lineages, Including the Non-classical U)

In lieu of functional data, fish non-classical MHC class I sequences are classified into lineages based on a combination of phylogenetic clustering, lineage-specific motifs and presence or absence of canonical peptide termini binding residues (Grimholt et al. 2015; Lukacs et al. 2010; Dirscherl et al. 2014). Among the six MHC class I lineages (U, Z, S, L, P and H) only the U lineage harbours genes with both classical and non-classical characteristics (Miller et al. 2006). Comparably Z (Hashimoto et al. 1990; Kruiswijk et al. 2002), S (Shum et al. 1999; Lukacs et al. 2010), L (Dijkstra et al. 2007), P (Grimholt et al. 2015) and H (Grimholt et al. 2019) sequences are all classified as non-classical and among these only the Z lineage members are predicted to bind peptides in the same mode as classical class I (Dirscherl and Yoder 2014; Grimholt et al. 2015; Lukacs et al. 2010).

Non-classical MHC class I genes are encoded across multiple chromosomes (see Fig. 11.1 for an example). The majority of non-classical U lineage genes reside within the core MHC region with a few genes located on other chromosomes (Grimholt et al. 2015). Based on compiled genomic analysis of a variety of different fish species it has been suggested that the Z lineage genes originally resided in the extended MHC class I core region (Grimholt et al. 2015). In some species, close proximity among U and Z lineage genes has been maintained while in others the Z lineage genes have been distanced from the

MHC class I region, likely as a result of large insertions and translocations. Genetic linkage between some L lineage genes and the MHC class II loci has been described in salmonids and zebrafish (Dijkstra et al. 2007) (Dirscherl et al. 2014). However, the majority of L lineage genes are scattered throughout the genome, as are the P, S and H lineage genes.

11.4.2.1 Species-Specific Variation in Non-classical Class I Lineages and Gene Numbers

Chondrichthyans have both classical and non-classical MHC class I U lineage genes but to date no additional non-classical class I lineages have been described (Bartl et al. 1997; Ohta et al. 2000; Almeida et al. 2020; Wang et al. 2003). In contrast, sequences belonging to both the U and Z lineages are found in all bony fish species studied to date suggesting an evolutionary ancient origin and long co-existence of these two lineages (Grimholt 2016; Dirscherl et al. 2014). The actual number of U and Z lineage genes however varies dramatically among species ranging from a single Z lineage gene in species such as cod, tetraodon and stickleback to 18 in the Mexican tetra (Grimholt et al. 2015). Comparably, S, L, P and H lineage genes, which are postulated to originate from duplication events of the U and/or Z lineage genes, are unevenly distributed and found in some species but not in others. Z lineage has been found across ray-finned fish groups, but is also present in lungfish, indicating it has been lost during tetrapod evolution (Grimholt et al. 2015). This extraordinary degree of non-classical MHC class I diversity and variation, both in the presence or absence of distinct lineages, and in the high degree of flexibility within a given lineage, highlights the dynamic evolution typical of non-classical class I genes and may be indicative of species-specific functional adaptations and pathogen driven co-evolution.

11.4.2.2 Non-classical MHC Class I Lineage-specific Characteristics

Non-classical U lineage genes likely represent diversified duplicates of their classical U lineage counterparts and some have highly conserved peptide termini binding residues while others are less conserved. Typically, non-classical class I U lineage genes have limited polymorphism, and in most cases a more restricted expression pattern compared to their classical counterparts. *Z lineage genes* also exhibit many features common to classical class I including highly conserved peptide-binding groove (Grimholt et al. 2015). Low to moderate levels of polymorphism are present within individual zebrafish Z lineage genes (Dirscherl and Yoder 2014). However, Z-lineage genes encode for proteins that, unlike most other class I sequences, have variable α3 domains and highly conserved α1/α2 domains. This has prompted the supposition that many Z lineage genes bind a highly conserved or even identical ligand (Grimholt et al. 2015; Lukacs et al. 2010). A feature unique to some *L lineage genes* is loss of introns. In these genes the alpha chain, TM and cytoplasmic domain are encoded on one exon and only the leader sequence is separated by an intronic region (Dijkstra et al. 2007). Common to all L lineage sequences is a complete lack of typical peptide anchoring residues and a relatively high hydrophobicity within the peptide-binding domains. This may indicate that these molecules have a role in presenting lipids in a manner similar to the mammalian MHC class I-like CD1 family (Grimholt et al.

2015). *S lineage sequences* also lack the peptide anchoring residues typical for classical class I and, as opposed to other non-classical lineages, have a short cytoplasmic tail (Grimholt et al. 2015). *P lineage sequences* are absent or non-functional in many species and the sequences are quite divergent. None of the canonical peptide-binding residues are conserved and based on sequence analysis it is inferred that the antigen-binding groove has a unique and rigid structure (Grimholt et al. 2015). The most recent addition to the non-classical class I are the *H-lineage genes*, named so as they are present in holostei (gar and bowfins) and teleost species (Grimholt et al. 2019). Teleost H-lineage sequences have a high degree of deterioration of the extracellular domains and a unique, relatively long, tyrosine-containing cytoplasmic tail motif, suggesting a role of H-lineage genes in intracellular signalling through tyrosine phosphorylation.

11.4.2.3 Inducible Expression

Studies aimed at elucidating the functions of non-classical class I molecules in fish are scarce. However, a few studies have been conducted demonstrating altered levels of different non-classical class I gene transcripts in response to various immune stimuli and disease states, suggesting that non-classical class I are important for fish immune functions. For example, expression of four non-classical U lineage genes (UGA, UCA, UDA and UEA) was induced in virally infected rainbow trout (Landis et al. 2008). Similarly, in Atlantic salmon distinct L lineage genes are differentially induced in response to viral and bacterial infections and immunological stimuli (Svenning et al. 2019). Increased levels of the zebrafish Z-lineage gene, ZEA, were observed in the liver following immunization with an attenuated bacterial vaccine (Yang et al. 2012) while transcription levels of other Z-lineage genes decreased in zebrafish in response to silencing of a bacterial recognition molecule (Chang et al. 2009), and in zebrafish embryos injected with transformed mesenchymal stem cells (Mohseny et al. 2012).

11.5   MHC Class II Molecules

11.5.1 Nomenclature Rules and Notations

Three lineages of fish MHC class II molecules have been identified by phylogenetic studies: DA, DB and DE. Only the DA lineage comprises classical class II molecules. A nomenclature has been established for fish *mhc class II* genes, following the general recommendations from (Klein et al. 1990). Specifically, these genes are named D (for "duo" to refer to the second class of MHC molecules), followed by a letter corresponding to the "family" or "lineage", and finally by a third symbol referring to the subclass (A for α chain, or B for β chain). Classical MHC class II genes therefore belong to the DA lineage, and a rainbow trout gene encoding the α chain of a classical MHC II molecule is named *mhc Onmy-DAA*, while an allele would be designated by *mhc Onmy-DAA*01:01* as explained above for MHC class I.

11.5.2 Classical Class II (From the A Lineage)

11.5.2.1 Structure and Peptide Loading Mechanisms

Fish classical MHC class II molecules have a typical α/β heterodimeric structure, each chain containing a PBR domain, an IgC1 domain, a TM, and a short cytoplasmic region (see IPD-MHC database for a listing of trout and salmon MHCI/ MHCII- alleles; https://www.ebi.ac.uk/ipd/mhc/group/FISH/). These molecules are polymorphic and display highly conserved residues in the PBR of both chains; the β chain also has conserved residues that are critical for the interaction with CD4 in mammals (Grimholt 2016). They are synthetized in the endoplasmic reticulum where they form a complex with the ortholog of the chaperone CD74 (aka invariant chain or Ii): CLIP (the class II-associated invariant chain peptide) which interacts with the peptide-binding groove of MHC class II, increasing the stability of the heterodimer. In mammals, this complex is modified in late endosomes, where a non-classical MHC class II called "DM" competes with CD74 and facilitates the replacement of CLIP by peptides of exogenous origin, to be presented to T cells on the MHC Class II molecule. In fish, two CD74 (a and b) have been consistently found in many teleost species, with highly conserved motifs (Yoder et al. 1999; Dijkstra and Yamaguchi 2019). In contrast, genes encoding DM molecules have not been found in fish, and the residues in the MHC class II sequences that seem to be required for DM function are also lacking in these species (Dijkstra et al. 2013). Hence it appears that DM is dispensable in fish and the peptide loading on MHC class II is operated via alternative mechanisms.

11.5.2.2 Polymorphism

MHC class II polymorphism in fish was first described in zebrafish (Ono et al. 1992; Sültmann et al. 1994). However, it has been mainly studied in Atlantic salmon and medaka, where sufficient numbers of allelic variants have been sequenced. In these species, both α and β chains are highly polymorphic (Dijkstra et al. 2013; Bannai and Nonaka 2013); in medaka, the exon 2 of both genes showed high DS/DN ratios (i.e. synonymous/non-synonymous substitution ratios) and trans-population polymorphism (i.e. polymorphism shared by divergent populations). MHC class II polymorphism is also under investigation in other species like the olive flounder (Xu et al. 2010). MHC class II polymorphism has been linked to resistance to diseases in a few models, but further studies in multiple models will likely clarify why it seems difficult to identify such associations (see 6.).

11.5.2.3 Expression and Its Regulation

In the Atlantic salmon, the MHC class II β chain was found mainly expressed by lymphocytes, macrophages and dendritic-like cells (Koppang et al. 2003), suggesting a restriction to immune cells as in mammals.

In adult zebrafish, the characterization of the *mhc class2-dab*:eGFP reporter transgenic lines showed that the *mhc2dab* promoter was active in B lymphocytes, eosinophils and in a subpopulation of

myeloid cells which do not comprise neutrophils (Wittamer et al. 2011). Mechanisms of induction of MHC class II expression have been characterized in the zebrafish, showing that one of the co-orthologs of CIITA (Class II Major Histocompatibility Complex Transactivator) is induced by IRF1, and that IRF1 can be involved in two MHC class II induction pathways: via the ISRE present in the promoter of CIITAv2, and directly via an ISRE located in the promoter of the *mhc class II dab* gene (Hou et al. 2020). These data illustrate that fish systems provide an interesting—if complicated—opportunity to understand the possible roles of the multiple copies of these key factors controlling MHC class II expression. This issue has been recently addressed in an Atlantic salmon cell line (SHK-1) (Grimholt et al. 2020). A selective induction pattern was found, with all paralogs up-regulated by IFN-γ in some cases, while only one or a few were induced in others. IRF1 and STAT1 appeared as master-regulators; in contrast, the enhanceosome (CREB/RFX/NFY) and CIITA may not be involved in the regulation of MHC pathways in these cells, since ssRFX5 and ssNFYA/B are not expressed.

11.5.2.4 Biological Functions

Although direct evidence for the functions of fish MHC class II molecules are scarce, a number of findings suggest that they are largely similar to those reported in mammals. Fish class II molecules control allograft rejection (Cardwell et al. 2001), and likely present peptides to CD4^{+} T helper cells that cooperate with B cells to promote Ab responses. As suggested by the reduction of the secondary Ab response in trout that have been thymectomized one month after hatching (Tatner 1986), and by responses to hapten/carrier Ag systems such as DNP-BSA, TNP10-KLH and TNP10-HorseSA, fish B cells seem to cooperate with (CD4^{+}) T cells in a classical manner (Miller and Clem 1984; Miller et al. 1985). The importance of MHC class II molecules is strongly supported by the particular deficiencies of B cell responses against TD Ag in Atlantic cod, in which the MHC class II pathway is lacking (Star et al. 2011; Pilström et al. 2005). However, the modes of the Ag recognition by T-cell subsets expressing either CD4–1 and CD2–2 coreceptor, or only CD4–2 remain to be characterized.

The loss of the MHC class II pathway, which occurred independently at least three times during fish evolution, was a major surprise. It is discussed in Sects. 11.2 and 11.3.

11.5.3 Non-classical MHC Class II (A, B and E Lineages)

Among the three MHC class II lineages the A lineage, which is present in a variety of teleost species (Sültmann et al. 1994; Sültmann et al. 1993; Bannai and Nonaka 2013; Godwin et al. 1997, 2000; Hordvik et al. 1993; Miller and Withler 1996; Ono et al. 1993b; Sato et al. 2012, 1998), harbours both classical and a few non-classical class II sequences. However, a clear distinction among A lineage sequences into either classical or non-classical sequences has, to date, only been unequivocally achieved for salmonids and medaka (Bannai and Nonaka 2013; Dijkstra et al. 2013; Harstad et al. 2008; Sato

et al. 2012; Kuroda et al. 2002) so the definition may be subject to change. For example, among the A lineage molecules identified in medaka, two are considered non-classical as they are virtually non-polymorphic and predominantly expressed in the gills while a third gene pair is highly polymorphic and thus designated classical class II (Bannai and Nonaka 2013). Comparably, sequences within the well-defined E lineage and the somewhat more diffuse B lineage all comply with non-classical class II definitions including low levels of polymorphism and lack of the classical class II peptide termini anchoring residues and/or CD4 binding residues (reviewed in (Grimholt 2016)). In addition, expression patterns of B and E lineage genes deviates from their classical counterparts by being either considerably lower, as is the case for Atlantic salmon E genes, or with non-typical tissue-specific expression patterns as exemplified by certain Atlantic Salmon B lineage genes (Dijkstra et al. 2013). Based on phylogenetic analysis the A and E lineages form distinct, highly supported clades while the B lineage, sometimes referred to as B group, is more variable and consists of at least three sub-groups defined by sequence clustering (Dijkstra et al. 2013).

11.5.3.1 Species Specific Variation in Non-classical Class II Lineages and Gene Numbers

With the exception of Atlantic cod and other gadoids, pipefish and an anglerfish (which all lack MHC class II genes), all teleost species studied to date have at least one functional classical MHC class II A lineage gene. Comparably, the occurrence of the non-classical class II B and E lineages varies, most species analysed have B lineage representatives and a few have E lineage genes. In addition, the gene numbers within a given lineage vary dramatically from species to species. For example, tetraodon and fugu lack B lineage sequences while Atlantic salmon and tilapia possess seven and sixteen B genes, respectively (Grimholt 2016). Similarly, based on analysis of available fish genomes and EST database searches E lineage sequences are present in some teleost species while missing in others (Dijkstra et al. 2013). Furthermore, at least in salmonids, the E lineage genes have deteriorated, with a high frequency of pseudogenes and low expression levels (Grimholt and Lukacs 2020). E-lineage genes are however considered to represent the primordial MHC class II sequences as distinct E lineage sequences are present in primitive ray-finned fishes such as sturgeon, paddlefish and spotted gar (Dijkstra et al. 2013). In the spotted gar E lineage genes coexist alongside an A/B lineage-like sequence in a region containing the classical class I-like sequence (Dijkstra et al. 2013; Grimholt 2016).

11.5.3.2 Biological Function

In humans the two non-classical MHC class II molecules, HLA-DM and HLA-DO, are critically involved in the classical MHC class II peptide loading process (see above). Notably, none of the teleost non-classical class II genes encodes for molecules with the structural requirements to function as equivalents to the human non-classical class II HLA-DM and HLA-DO molecules (Dijkstra et al. 2013). Thus, combined with the lack of any functional evidence, the roles of teleost non-classical class II sequences remain

elusive. Based on the presence of an endosomal sorting motif in the cytoplasmic region of some distinct B lineage sequences in Atlantic salmon and stickleback it has been suggested that these molecules may assist in the classical class II peptide loading process in a manner analogous to that of HLA-DM (Dijkstra et al. 2013). However, this is somewhat hard to reconcile with the lack of B lineage genes in some species. E lineage sequences are distinct and differ significantly from all other fish class II sequences. Notably, teleost E lineage sequences share many features with classical and non-classical class II sequences of other jawed vertebrates including cartilaginous fish and tetrapods (Dijkstra et al. 2013). To date, there is no indication as to the functional role(s) of E lineage genes and, like the B lineage genes, the loss and deterioration of E lineage sequences in many species raises suspicions concerning their biological relevance in teleosts. It is possible that DE encodes classical MHC molecules in sturgeon, suggesting that this lineage may have been replaced by DA after the third (teleost specific) WGD.

## 11.6	MHC Variation and Resistance to Infections

The crux of proposed associations between MHC polymorphism and disease resistance is that classical MHC class I and class II molecules show extensive allelic polymorphism, especially in those residues that line the peptide-binding groove. This allows for different individuals within the same population to present different antigenic peptides, although derived from the same pathogen, resulting in variable immune responses between individuals. MHC polymorphism occurs, not within individuals but at population level. The evolutionary advantage of allelic polymorphism of the MHC therefore also lies at population level; balancing selection allows for maintaining genetic variation in immune responses between individuals and thus variation in disease resistance within the population.

The MHC region can be characterized as part of a large, intensively studied and gene-dense linkage group, which makes this region prone to bias towards why more diseases appear associated with the MHC than with any other region of the genome. Given this characteristic it makes sense to consider approaches such as those studying linkage of genome-wide quantitative trait loci (QTL) with polymorphisms present in the MHC region as the most objective. In humans, such QTL approaches have already provided evidence suggesting that, in fact, the majority of associations with the MHC are with autoimmune conditions (Kelly and Trowsdale 2017), rather than with resistance to pathogens. This means that also for fish, it could be beneficial to revisit past and often preliminary evidence for links between MHC polymorphism and disease resistance, and to advance with genome-wide association studies on the MHC and disease resistance. A significant difference between mammals and fish is the close linkage of MHCI and MHCII loci in mammals, implying that selection for specific MHCI alleles also selects for specific MHCII alleles, which may explain that susceptibility to many autoimmune diseases is linked to the human MHC.

Currently, the number of QTL studies in fish that have looked for disease resistance associations with MHC polymorphism are not only limited in number but also suffered from a lack of fine mapping, making it hard to substantiate preliminary associations found between the two. Yet, QTL mapping also did not exclude the possibility that genomic regions including classical MHC class II and non-classical MHC class I genes could be associated with disease resistance, although associations of classical MHC class I alleles with disease resistance certainly are not easily detected (reviewed in (Yamaguchi and Dijkstra 2019)). The very high allelic/haplotype polymorphism in teleost classical MHC class I genes (Grimholt et al. 2015) may explain why associations of classical MHC class I alleles with disease resistance, if any, may be particularly hard to detect in fish. Of course, since the classical MHC class I and class II genes of teleost fish are not linked, associations with disease resistance of any MHC class are not automatically correlated with the other. Of interest, high copy number variations in both MHC class I and class II genes, particularly in fish genomes, and also null-allele variation in non-classical MHC class I genes may have as yet unstudied associations with disease resistance. However, significant associations have been reported between MHC class II haplotypes and resistance/susceptibility to furunculosis (a bacterial disease caused by *Aeromonas salmonicida*) in salmon (Grimholt et al. 2003; Langefors et al. 2001; Lohm et al. 2002; Kjøglum et al. 2008) and brook charr (Croisetiere et al. 2008). Regarding class I, an association between two MHC class I alleles and resistance to ISA has been reported (Grimholt et al. 2003; Kjøglum et al. 2006). Future studies using quantitative genetics on the resistance to pathogens will likely further clarify to what extent MHC explains resistance and/or susceptibility to various infections.

11.7 Fish MHC and Mate Choice

In addition to pathogen-driven balancing selection, MHC-driven non-random mating has been considered as an explanation for its high polymorphism. Although mechanisms connecting MHC genes with mate choice remain unclear, a number of studies proposed female preference for MHC-diverse/dissimilar males in many species, including bony fishes (reviewed in (Kamiya et al. 2014)).

In 2001, Milinski and colleagues argued that partners should not only prefer mates with dissimilar MHC to avoid inbreeding, but also mates with higher diversity of MHC alleles to increase resistance to potential pathogens. They reported that the three-spined stickleback (*Gasterosteus aculeatus*) has evolved an "allele counting" strategy, gravid females favouring the odour of males with a large number of MHC class-IIb (Reusch et al. 2001). The same group later published several studies supporting the idea that mating decisions of fish females both sustain MHC polymorphism and promote resistance genes to infections (i.e. "good" genes) (Eizaguirre et al. 2009). However, several factors can apparently interfere with female choice of mate with diverse or dissimilar MHC, such as sexual conflicts in Chinook salmon (*Oncorhynchus tshawytscha*) (Garner et al. 2010) and

environmental salinity in stickleback (McCairns et al. 2011). Other studies showed that MHC similarity in males was correlated with higher number of offspring, suggesting that preference for MHC-dissimilar mates is not a general rule (Gasparini et al. 2015; Tentelier et al. 2017; Promerova et al. 2017). Thus, the maintenance of the MHC polymorphism is not fully understood, and the contribution of MHC non-random mating remains controversial in absence of direct molecular mechanisms linking MHC molecules and partner selection.

11.8   Conclusions

This short overview of fish MHC shows that it has become a great model to understand major variations in gene MHC content and MHC architecture. On an evolutionary timescale, fish offer natural experimentations equivalent to knock-out animals, as no other class of vertebrate. The recent findings in anglerfish, pipefish and seahorses are perhaps the best illustration of this aspect. In teleosts, by far the most abundant group of bony fish, the complex architecture of MHC might be linked to their specific evolutionary history, characterized by several WGD, a phenomenon rather rare in other classes of vertebrates. This offers a set of excellent systems to evaluate the fate of duplicated genes: neofunctionalization, deletion, maintenance, translocation, etc. With many genome sequences available (82 species in the Ensembl release 100, April 2020), the fish are by far the best model for comparative genomics in poikilotherms. MHC science in fish is now intermingled with projects investigating fish speciation, behaviour, reproduction, coevolution, trans-species polymorphism and more.

A textbook chapter about fish MHC written 20 years from now in 2040 might tell us more about some nagging, intellectually interesting questions, such as "Have agnathans an equivalent of MHC?" and "how can fish mount Ab responses in absence of MHC class II?". So far, the lamprey sequences annotated as TCR-like and CD4-like sequences did not stand a serious scrutiny and do not really deserve their nomenclature, so nobody knows how the repertoire of Variable lymphocyte receptors, the Ag-specific receptors of Agnathans, is selected. In addition, the possible class II-like functions of the large MHC class I repertoire of Atlantic cod are far from understood. Apart from answers to questions of fundamental interest like these, this 2040 review will certainly contain much more QTL information related to relationships between diseases and MHC and a deeper understanding of the functions of the various non-classical class I and class II genes. The variations observed in fish might even serve as a model for understanding human MHC-related pathologies, offering by their examples some ideas for alternative solutions to defects, diseases and abnormalities of many sorts. The future of MHC immunology will certainly continue to stress the value of comparative studies done in fish.

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Mucosal Immunity in Fish

12

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Abstract

Mucosal barriers are dynamic structures that allow fish to interact with the external environment while maintaining homeostasis. Mucosal barriers are very complex tissues with unique adaptations that optimize a myriad of physiological roles including nutrition, gas exchange, smell, and immunity, among others. Thus, mucosal barriers contain a local immune system, the mucosa-associated lymphoid tissue (MALT), that protects the host from pathogens and other insults. Fish MALTs have thus far been found in the skin, gills, gut, olfactory epithelium, pharyngeal epithelium, and buccal epithelium. Fish mucosal barriers draw many benefits from their symbiotic microbial communities, and therefore, the mucosal immune system of fish plays a key role in maintaining this symbiosis. This chapter gives an overview of the importance of the mucosal immune system in overall fish health. We introduce the key principles known to govern mucosal immune responses in teleosts as well as the innate and adaptive immune cells and molecules involved. Importantly, we review also the application of fundamental biological knowledge in fish mucosal immunity to aquaculture interventions and practices, including advances in the field of fish vaccinology and probiotics. With the goal to move the field forward, we identify critical knowledge areas that remain obscure and propose future work to illuminate them.

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Abbreviations

Ach	Acetylcholine
AGD	Amoebic gill disease
AMP	Antimicrobial peptides
AP	Alkaline phosphatase
APC	Antigen-presenting cell
BCR	B-cell receptor
CMIS	Common mucosal immune system
CNS	Central nervous system
CyHV-3	Cyprinid herpesvirus-3
d-MALT	Diffuse mucosa-associated lymphoid tissue
DC	Dendritic cells
ENS	Enteric nervous system
GALT	Gut-associated lymphoid tissue
GAPs	Goblet cell-associated antigen passages
GC	Goblet cell
GIALT	Gill-associated lymphoid tissue
IEL	Intraepithelial lymphocytes
IHNV	Infectious hematopoietic necrosis virus
ILT	Interbranchial lymphoid tissue
IPNV	Infectious pancreatic necrosis virus
LPL	Lamina propria leucocytes
MALT	Mucosa-associated lymphoid tissue
MBL	Mannose-binding lectin
MCs/EGCs	Mast cells/eosinophilic granule cells
mIgM	Membrane-bound IgM
MUC	Mucins
NALT	Nasopharynx-associated lymphoid tissue
NCCs	Non-specific cytotoxic cells
NK cells	Natural killer cells
NNV	Nervous necrosis virus
O-MALT	Organized mucosa-associated lymphoid tissue
OSNs	Olfactory sensory neurons
pIgR	Polymeric Ig receptor
PNS	Peripheral nervous system

RBL   Rhamnose-binding lectins
SALT   Skin-associated lymphoid tissue
SC   Secretory component
sIgs   Secretory immunoglobulins
SVCV   Spring viremia of carp
VHSV   Viral hemorrhagic septicemia virus

12.1   Introduction

Mucosal barriers cover all fish surfaces, thereby sensing environmental perturbations and constituting the first line of defense against pathogens. Fish mucosal tissues are also inhabited by complex and beneficial microbial communities known as the microbiome. Environment plays a fundamental role in fish mucosal health, affecting microbiota and host in very complex, yet, interwoven ways. Given the unique aspects of the environment in different aquaculture settings, understanding how the outside world is sensed by fish mucosal barriers is of utmost importance to understand how the fish mucosal immune system operates, and thus, how host-pathogen interactions are modulated at mucosal barrier tissues.

Animal mucosal epithelia are very heterogeneous; while epithelial cells form the main building blocks of all mucosal epithelia, many other cell types are present. Non-epithelial cells are critical for mucosal homeostasis and function and include neurons, stem cells, endothelial cells, goblet cells, neuroendocrine cells, and immune cells, among others. All these different cell types contribute in one way or another to mucosal homeostasis maintenance, communication with microbiota, immune defense, and tissue repair.

The collection of resident lymphoid cells found in animal mucosal surfaces are known as the mucosa-associated lymphoid tissue or MALT. The presence of MALT in teleosts has been known for over a century, since immune responses in the gills of teleosts to parasites were reported early in the 1900s (Reuling 1919). The first characterized MALTs in teleosts were the skin-associated (SALT), gill-associated (GIALT), and gut-associated (GALT) lymphoid tissues. Recent studies, however, have shown that MALTs exist also in the nasal, buccal, and pharyngeal areas of teleosts. As discussed in detail later, mucosal immune responses are induced in the so-called organized lymphoid tissues of endotherms (i.e., Peyer patches, mesenteric lymph nodes). Fish MALT, however, appears to be composed of only diffuse lymphoid tissue, and thus, it is not clear where adaptive mucosal immune responses are induced in these animals.

Because of the many critical roles played by mucosal barriers, the field of fish mucosal immunology has received the attention of comparative immunologists, aquaculture researchers, and fish health experts for decades. Growing evidence indicates that fish health is inextricably defined by mucosal health. As a result, understanding the basic biology of

the fish mucosal immune system has become an important goal for the field; the knowledge derived from such studies will be instrumental in refining current disease interventions, or designing novel ones to prevent disease outbreaks in fish farms. An important break-through in the last few years has been the discovery that a large number of fish species contain IgT/IgZ, an immunoglobulin specialized in mucosal immunity that represents the functional analog of bird and mammalian IgA. Many of the basic principles of the fish mucosal immune system, including some key roles of IgT/IgZ, have recently been unveiled from studies on fish parasites and bacterial pathogens, as well as mucosal vaccine trials. Overall, these studies continue to unravel not only how and where mucosal immunoglobu-lin responses are elicited upon infection or vaccination, but also new players of innate and adaptive immune responses associated with mucosal barriers of fish.

In addition to being crucial to fighting pathogens, mucosal barriers are also critical in providing a homeostatic habitat for the complex microbial communities that inhabit all fish mucosal barriers. The last decade has seen tremendous advances in understanding the composition of the fish microbiota at several mucosal sites, as well as how they shift in response to a number of perturbations, including infection. Moreover, important inroads have been made in understanding how mucosal immunoglobulins, especially IgT, contrib-ute to the control and homeostasis of the fish microbiota.

It is worth pointing out that teleosts are very diverse with over 30,000 species and only a handful of them are the target of scientific investigation, mostly farmed species with high economic value. Thus, it is likely that mucosal immunity within the teleost clade is very diverse and that different species have found divergent solutions to the problem of pathogen recognition and microbiota homeostasis at mucosal tissue barriers. The knowl-edge gathered thus far on teleost mucosal immunity may not therefore be representative of all species, and future studies need to start exploring other teleost species.

Overall, the main aim of this chapter is to provide a current view of the field of mucosal immunity in fish, from innate to adaptive immune responses, including the molecules and cells involved in these responses. In addition, we present critical knowledge gaps, and propose future directions to move the field forward with regards to basic, translational, and applied aspects of this re-emerging field.

12.2 Fish Mucus

12.2.1 General Aspects of Fish Mucus

Fish mucosal surfaces are coated by a mucus layer which separates the epithelium from the external environment. Fish mucus is produced by several types of secretory cells, the best known being the goblet cells (GCs). Fish mucus is constantly being produced and diffuses into the surrounding water. Fish mucus carries many critical physiological functions such as drag reduction, osmoregulation, chemoattraction of , and protection against toxicants

and other external aggressors [reviewed by Reverter (2018)]. Fish mucus is a complex mixture of many different molecules, among which are innate and adaptive immune components. Thus, fish mucus is a first line of defense against pathogen infection (Esteban 2012). Furthermore, fish mucus contains all the necessary nutrients and physio-chemical properties that enable microbial colonization. These microbial communities are also active contributors to the mucus composition since they can produce a large array of metabolites including bacteriocins, neurotransmitters, hormones, and short-chain fatty acids that shape other microbial populations as well as the host.

Mucus acts as a barrier to trap microbes. Thus, balancing colonization of beneficial microbes and clearance of pathogens is part of the double duty of mucosal secretions. Several cues alter mucin secretion rate, polymerization, and glycosylation patterns, infection being a main trigger. These changes are pivotal to hinder the entrance or facilitate the dislodgement of these pathogens (Pérez-Sánchez et al. 2013; Fernández-Montero et al. 2020b, 2021a). Finally, environmental conditions are likely major determinants of fish mucus composition and production. Some of the best-known examples include stress and exposure to irritants. Below we summarize the key aspects of fish mucus composition with an emphasis in immune molecules and their functions.

12.2.2 Mucins, the Main Constituents of Mucosal Secretions

Fish mucus is mainly composed by a large and highly glycosylated group of proteins called mucins (MUC) (Shephard 1994). Mucins are the main structural component of the mucus layer, conferring the viscoelastic and protective character of mucus, and are mainly produced by GCs (Ma et al. 2018b; Esteban 2012). Mucins can be subdivided into three subfamilies: (1) large, secreted, polymer-forming mucins (known as SGFM; (2) membrane-bound mucins, and (3) secreted non-gel-forming mucins (Linden et al. 2008). SGFMs are long and extensively O-glycosylated protein polymers that give mucus its gel-like consistency. In fish, several MUC genes belonging to SGFMs and membrane-bound types have been identified in different species. For instance, five MUC genes are found in zebrafish (Jevtov et al. 2014), five in crucian carp (Bai et al. 2020), seven gel-forming MUC genes in Atlantic salmon (Sveen et al. 2017), and eight in sea trout skin transcriptomes (Malachowicz et al. 2017). Thus far, no antibodies specific for teleost MUC have been developed and therefore we currently rely on gene expression and traditional histological stains to investigate mucin biology in teleosts.

Several studies have showcased how parasitic infection (Bosi et al. 2005; Dezfuli et al. 2010; Estensoro et al. 2013; Pérez-Sánchez et al. 2013; Marcos-López et al. 2018) or bacterial infection (Schroers et al. 2009) regulate MUC gene expression in teleosts. Other stimuli such as stress, presence of alarm molecules in the water, or the beginning of the reproduction period, may also trigger an increase in mucus production and regulate MUC gene expression, which may have an impact in hindering pathogen infection (Reverter et al. 2018; Vatsos et al. 2010; Fernández-Montero et al. 2020a, b). In addition, some

experimental functional diets increase fish MUC gene expression and mucus production with the goal to enhancing protection against pathogens (Torrecillas et al. 2014; Fernández-Montero et al. 2019; Jensen et al. 2015).

12.2.3 Innate Immune Molecules Found in Fish Mucus

Fish mucus contains a wide array of immunologically active compounds including innate and adaptive immune molecules. The most studied innate immune factors found in fish mucus are certain enzymes, including phosphatases, esterases, lysozymes, and proteases, which are often responsible for degrading, inactivating, and controlling external pathogens and pathobionts (Nigam et al. 2012; Esteban 2012; Charroux et al. 2018). With the advent of shot-gun proteomic approaches, the diversity of proteins that form fish mucosal secretions continues to be unveiled. Here, we highlight some of the most important innate immune factors found in fish mucus but many others with less known functions have been identified using proteomic approaches.

12.2.3.1 Alkaline Phosphatase (AP)

Alkaline phosphatase (AP) is a hydrolytic enzyme detected in the fish intestine, skin, and gills of teleosts. Several different cell types express AP including epidermal basal cells, epithelial cells, rodlet cells, fibroblasts, and others [reviewed by Lalles (2020)]. AP activity has been detected in the skin mucus of nearly 30 different teleost species (Guardiola et al. 2014a, b; Lalles 2019). AP activity at fish mucosal barriers has traditionally been linked to wound healing processes (Esteban 2012; Lallès 2020). AP also carries anti-inflammatory properties aimed at dephosphorylating microbial components (i.e., LPS, flagellin, and DNA CpG) which upon the action of AP are no longer recognized by toll-like receptors (Lallès 2019).

12.2.3.2 Lysozymes

Lysozymes are present in most studied fish species and are produced by a wide variety of tissues and cell types, including leukocytes. Lysozyme plays a key role in innate immunity at mucosal surfaces (Grinde 1989; Ellis 1990; Saurabh and Sahoo 2008) via its lytic enzymatic action that induces a direct bactericidal effect on Gram-positive (i.e., *Micrococcus luteus*) and Gram-negative bacteria (i.e., *Aeromonas hydrophila*, *Pasteurella piscicida*, *Edwardsiella tarda*) (Itami et al. 1992; Nakamura et al. 1996; Hikima et al. 2001; Saurabh and Sahoo 2008). In addition, lysozyme can enhance phagocyte activation and phagocytosis in an indirect manner by lysing bacterial cell products that would be recognized by PPRs, or in a direct way by increasing the activation of the inflammasome and increasing interleukin (IL)-1β production in phagocytes (Ragland and Criss 2017; Saurabh and Sahoo 2008). Lysozyme expression is inducible in fish mucosal tissues upon infection with parasites and other pathogens (Fast et al. 2002; Fernández-Montero et al. 2021a; Guardiola et al. 2014a; Sitjà-Bobadilla et al. 2006). In addition, lysozyme activity can be induced in

mucus through the treatment of fish with different agents, including prebiotics, probiotics, and phytobiotics (Hoseinifar et al. 2015a; Zorriehzahra et al. 2016; Serradell et al. 2020).

12.2.3.3 Proteases

Proteases can function as innate immune enzymes present in mucus and are produced by a wide range of immune and non-immune cells. Among them, the most abundant and relevant proteases with immune functions are the serine proteases and the metalloproteases. Their functions vary from wound healing and tissue reorganization to direct killing action of pathogens or degradation of their associated virulence factors. In addition, some proteases are also involved in the activation of other immune system proteins such as complement or antimicrobial peptides (AMPs) (Esteban 2012; Nonaka and Smith 2000; Cho et al. 2002). Proteases can modify the skin mucus structure to facilitate turnover and dislodge pathogens (Aranishi et al. 1998; Firth et al. 2000; Sanahuja et al. 2019). However, proteases are not only used by the host, as they are also considered important virulent factors that enable pathogens to digest the outer layers of mucus and epithelial cells with the goal to feed or penetrate inside the host. In addition, proteases produced by certain pathogens are also important immunological disruptors of several host defensive proteins, such as complement, immunoglobulins, or AMPs (Firth et al. 2000; Chen et al. 2019). To protect from pathogenic proteases, the host produces antiprotease molecules, including alpha-1 antiprotease and alpha-2 macroglobulin (Guardiola et al. 2014b; Wang et al. 2019a). Due to its wide range of beneficial functions, protease and antiprotease activities increase in fish mucus after certain functional feed supplementation regimes in species such as the greater amberjack (*Seriola dumerili*) or the gilthead seabream (*Sparus aurata*) (Fernández-Montero et al. 2021a; Salinas et al. 2005).

12.2.3.4 The Complement System

The complement system represents a critical innate immune pathway that upon activation plays a key role for pathogen opsonization and lysis, and it is also known to mediate in mammals the transport and catabolism of immune-complexes, the induction of proinflammatory cytokines, and to enhance antibody affinity, B-cell activation, and phagocytosis processes, among many other functions (Boshra et al. 2006; Samstad et al. 2014; Brinchmann 2016). This system is activated by three different pathways in vertebrates, the classical, lectin, and alternative pathways, which all converge at the cleavage of the central protein C3 into C3a and C3b fragments, being C3b and its derivatives the ones that remain bound to the pathogen, and contain opsonic properties and/or are required for the initiation of the complement lytic pathway (i.e., mediated by C5–C9 components). The teleost complement system shows some differences compared to that of mammals: (1) in fish, complement can be activated at much lower temperatures than in mammals; (2) it recognizes a higher range of microbes; (3) many of its components (e.g., C3, C4, factor B) are diversified into different isoforms, thus resulting in a functional diversification of some key complement roles; (4) the fish alternative pathway titers are much higher than those of mammals, which suggests a more potent role of this system in fish as an innate

immune mechanism (Boshra et al. 2004). Complement-like lysis activity against pathogens is detected in mucosal tissues upon bacteria and ectoparasite infection, in gill, skin, or gut mucus from a wide range of species (Dawood 2021; Buchmann 1998; Van Doan et al. 2019; Hoseinifar et al. 2015b). Moreover, complement proteins have been directly detected in the skin mucus in a number of proteomic studies (Cordero et al. 2015; Fernandez-Montero et al. 2021b). Complement activity has been monitored after supplementation with functional feeds in skin, gut, and gill mucus of a variety of fish species using transcriptomics or the natural hemolytic complement activity assay (Sunyer and Tort 1995), and while differences exist among studies, complement activity can increase upon treatment with certain prebiotics (Karimi et al. 2020), probiotics (Nikoskelainen et al. 2003), symbiotics (Hoseinifar et al. 2015b), and phytobiotics (Srichaiyo et al. 2020; Fernández-Montero et al. 2021a).

12.2.3.5 Lectins

Lectins are glycoproteins that bind to carbohydrates, proteoglycans, glycolipids, and glycoproteins, all of which are widely distributed in bacteria, fungi, and viruses (Brinchmann et al. 2018). A diversity of lectins has been reported in fish mucus of skin, gills,, and intestine, including C-type lectins, fucolectins, galectins, rhamnose-binding lectins (RBL), and mannose-binding lectin (MBL) (Bun Ng et al. 2015; Elumalai et al. 2019). Thus, lectins can act as agglutinins that recognize pathogens which leads to one or more of several innate immune processes including agglutination, opsonization and phagocytic uptake, complement activation, and direct killing. Indeed, several studies have demonstrated that fish mucosal lectins agglutinate, and in some instances inhibit growth, of a broad spectrum of bacteria, including *Vibrio anguillarum* (Kamiya et al. 1988), *Streptococcus difficile* (Tasumi et al. 2004), *V. ichthyoenteri* and *V. vulnificus* (Liu et al. 2016), *A. hydrophila* (Mu et al. 2017) and *E. tarda*, *Tenacibaculum maritimum*, and *V. harveyi* (Kugapreethan et al. 2018), among others. In addition, yeast and parasites, such as *Heterobothrium okamotoi*, *Ichthyophthirius multifiliis (Ich), Dactylogyrus* sp., or *Enteromyxum scophthalmi* can also be recognized and agglutinated by fish mucosal lectins (Buchmann 2001; Buchmann and Lindenstrøm 2002; Redondo et al. 2008; Buchmann et al. 2001). Certain additives such as phytobiotics increase the expression of some lectins that recognize pathogens, such as MBL and RBL in channel catfish (*Ictalurus punctatus*) (Peterson et al. 2015, 2018).

12.2.3.6 Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs) are small proteins or their fragments produced by a wide range of cell types and tissues, and act as potent wide-spectrum antibiotics against Gram-positive and -negative bacteria, in addition to neutralizing viruses, fungi, and parasites (Masso-Silva and Diamond 2014; Brogden 2005). AMPs are known to interact with negatively charged components of pathogens (i.e., hydrophobic regions of lipid components of cell membranes or hydrophilic regions of phospholipids). So far, teleosts have been shown to produce all of the main classes of AMPs, including defensins,

cathelicidins, hepcidins, and histone-derived peptides. Moreover, they also express AMPs unique to fish, such as piscidins (Masso-Silva and Diamond 2014; Brunner et al. 2020). AMPs are produced in fish mucosal surfaces especially by epithelial cells, and their expression levels are typically increased upon pathogen infection as demonstrated in different fish species (Casadei et al. 2009; Maier et al. 2008; Furlan et al. 2018; Milne et al. 2019). Probiotic, phytobiotic, and especially prebiotic supplementation of fish feeds results in increases in different AMP transcript levels in fish mucosal tissues (Milne et al. 2019; Campoverde et al. 2017; Ramesh et al. 2017) showing that dietary interventions can either directly or indirectly (i.e., via changes in the microbiota) shape AMP expression at mucosal barriers. Of note, dietary interventions do not only change mucosal innate immune parameters in the gut but also at other mucosal sites such as the skin.

12.2.3.7 Cytokines

Cytokines are composed of a broad range of small protein families that are involved in a myriad of cell signaling events that maintain homeostasis, development, and govern innate and adaptive immune responses (reviewed by Secombes et al. 2009; Zou and Secombes 2016). Cytokines can exert their functions in autocrine, paracrine, and endocrine ways. Cytokines are produced by a wide variety of immune and non-immune cells. Cytokine genes are expressed at all fish MALTs, however whether they are secreted to form part of the mucosal secretions of fish is less clear. Cytokine protein levels in mucosal secretions are thus far uncharacterized largely due to the lack of reagents to quantify them. Further, many proteomic studies from fish mucosal secretions have been published, yet, they have failed to detect the presence of cytokines in fish mucus. Clearly, further studies are necessary to ascertain whether cytokines play a role in mucosal secretions.

12.2.4 Adaptive Immune Molecules (Igs) in Fish Mucus

Igs or antibodies (Abs) are expressed on the surface of B cells (membrane-bound form) as B-cell receptors (BCR) or in soluble form in body fluids (i.e., plasma, mucus, peritoneal cavity fluid) (Salinas et al. 2011, 2021). To date, mucosal Igs [referred to as secretory Igs (sIgs)] have been found in every mucosal barrier investigated thus far in teleost fish including the gut (Zhang et al. 2010), skin (Xu et al. 2013), gills (Xu et al. 2016), olfactory organ (Tacchi et al. 2014), buccal and pharyngeal cavities (Yu et al. 2019) (Kong et al. 2019). A recent study on Atlantic salmon (*Salmo salar*) identified a bursa of Fabricius-like structure in the cloaca where IgM+ and IgT+ B cells could be detected, thus implying that sIgs are likely to be secreted to the mucus surrounding this structure (Loken et al. 2020). Interestingly, deep-sea anglerfish lack Ig-based adaptive immunity, which begs the question of whether these species base their immune strategies only on innate-like mechanisms or contain alternative forms of adaptive immunity (Swann et al. 2020).

12.2.4.1 Structural Aspects of Teleost Igs

Similar to most tetrapod Igs, teleost Igs are composed of two identical heavy (H) and light (L) chains. Both H and L chains contain an amino-terminal variable (V_H or V_L) domain involved in antigen recognition, and one or more carboxyl-terminal constant (C) domains (C_H or C_L) that, as part of the Fc region, mediate effector functions. Three major Ig classes have been identified thus far in teleost fish, IgM, IgD, and IgT/IgZ, which are defined by their heavy chains μ, δ, and τ/ζ, respectively (Wilson et al. 1997; Hansen et al. 2005; Danilova et al. 2005). In contrast to mammals, the teleost igh locus encodes all three Ig genes in a translocon organization, on which the Dτ-Jτ-Cτ clusters (for IgT/IgZ) are generally located between V_H gene segments and the Dμ/δ-Jμ/δ-Cμ-Cδ locus (for IgM and IgD) (Parra et al. 2016). In the absence of class switch recombination (CSR) in fish, the recombination of igh loci imposes an alternative junction of V_H to either Dτ-Jτ-Cτ or Dμ/δ-Jμ/δ-Cμ-Cδ segments (Mashoof and Criscitiello 2016). Accordingly, this rearrangement results in the development of mutually exclusive B-cell lineages expressing either surface IgT or IgM/D. Supporting the latter, IgT^{+} B cells do not express IgM and vice versa (Zhang et al. 2010).

12.2.4.2 Teleost IgM

IgM was the first discovered Ig in fish and is considered the most prevalent systemic Ig isotype (Parra et al. 2013). Most studied teleost species express the secreted form of IgM with four CH chains, while membrane-bound IgM (mIgM) consists of CH1–CH3 domains (Parra et al. 2016; Saravanan et al. 2020; Bilal et al. 2019; Bradshaw and Valenzano 2020; Piazzon et al. 2016). Exceptions of mIgM containing only 1 or 2 CH domains are also present in several teleosts (Magadán-Mompó et al. 2011; Quiniou et al. 2011; Hu et al. 2011). In contrast to the pentameric IgM in mammals in which all subunits are covalently bound through disulfide bonds, teleost IgM is tetrameric and is found in several redox forms, that is, their monomers are associated by various degrees of inter-heavy chain disulfide polymerization (Bromage et al. 2004; Kaattari et al. 1998). It is noteworthy that increased disulfide polymerization has been correlated with higher half-life of fish IgM and greater affinity to the antigen (Ye et al. 2010). Interestingly, teleost IgM lacks the joining (J) chain of higher vertebrate species which combined with the lower degree of disulfide bonding among its monomers; it may afford a higher degree of flexibility to the molecule.

IgM is the most dominant Ig in fish plasma and its concentration varies across the range of 0.6–21 mg/ml among different teleost species and it fluctuates with various factors (e.g., temperature, water quality, fish size, stress, infection, vaccination, etc.) (Parra et al. 2016; Solem and Stenvik 2006; Olesen and Jorgensen 1986). In contrast, IgM is present at a significantly lower concentration (~4.5–280 μg/ml) in mucosal secretions. After immunization or infection, antigen-specific IgM titers are increased significantly in serum and mediate systemic protective responses against pathogens (as discussed below), while such titers are found at much lower levels in mucosal surfaces, where IgT has been found to be the key sIg (Xu et al. 2013, 2016; Yu et al. 2019; Kong et al. 2019; Zhang et al. 2010). Thus far, studies performed over the past decades have revealed that fish IgM plays key roles

similar to their mammalian counterparts in a number of functions, including: (1) neutralization and agglutination of viral or bacterial pathogens respectively; (2) activation of the classical pathway of complement upon pathogen recognition (Ye et al. 2013; Magnadottir et al. 1997; Boshra et al. 2004); (3) coating of a significant portion of the microbiota (~12–50%) on different mucosal surfaces (Xu et al. 2013, 2016; Yu et al. 2019; Kong et al. 2019; Zhang et al. 2010).

It is well-accepted that the affinity maturation of the IgM response is much lower than that of mammals despite the fact that somatic hypermutation of IgM genes appears to occur at similar rates to that observed in mammals (Kaattari et al. 2002; Jiang et al. 2011; Yang et al. 2006; Magor 2015). It has been proposed that a reason for this singularity lies in the apparent lack of organized lymphoid structures (e.g., germinal centers) in teleosts. Thus, a key question that remains to be addressed is how are teleost fish able to mount potent antigen-specific IgM responses in the absence of such lymphoid structures, and where are such responses induced in their lymphoid organs.

12.2.4.3 Teleost IgD

Fish IgD was first discovered in channel catfish in 1993 (Wilson et al. 1997) and has been found in all teleost species investigated thus far (Banerjee et al. 2017; Xu et al. 2019). Unlike mammalian IgD and fish IgM, the fish IgD molecule possesses remarkably unique traits regarding its diverse structural plasticity among species (Parra et al. 2016) that include: (1) teleost ighδ genes encode 2–16 δ constant (Cδ) exons as a result of the multiple rounds of gene duplication and deletion; (2) teleost ighδ gene transcripts are chimeras that are generally composed by a Cμ1 domain of IgM and several Cδ segments. In addition, a new IgD/IgT chimera has recently been identified in three *Moronidae* species (Buonocore et al. 2020); (3) ighδ genes encoding IgD vary among species, for example, rainbow trout and zebrafish utilize a single ighδ gene to express both secreted and membrane-bound forms (Zimmerman et al. 2011; Ramirez-Gomez et al. 2012), while catfish employs two different genes for each form (Bengten et al. 2006). Moreover, fugu, medaka, and Nile tilapia only contain a membrane-bound form of ighδ transcript (Magadán-Mompó et al. 2011; Saha et al. 2004; Wang et al. 2016). The IgD protein has only been examined in two fish species so far, catfish and rainbow trout. Catfish contains two variants of the secreted form of IgD (~130 and ~180 kDa), with a total concentration of ~40 μg/ml in serum (Edholm et al. 2011). Rainbow trout has two monomeric variants of long (~370 and ~400 kDa) and short (~240 kDa) secreted forms, with a relatively higher concentration in serum (~2–80 μg/ml) than in mucus (~1.2–7 μg/ml) (Parra et al. 2016; Xu et al. 2016; Yu et al. 2019; Kong et al. 2019). Recent studies have suggested potential roles of fish IgD, such as functioning as a pattern recognition molecule, as proposed for secreted catfish IgD (an Ig lacking the VH region) (Parra et al. 2016; Edholm et al. 2010), and having a role in microbiota homeostasis as it coats a significant portion of the gill, gut, nasal, buccal, and pharyngeal microbiota in rainbow trout (Xu et al. 2016; Perdiguero et al. 2019; Yu et al. 2019; Kong et al. 2019). However, the role of IgD in host defense against pathogens remains largely unknown and thus far attempts to detect pathogen-specific IgD titers upon

infection have rendered negative results (Xu et al. 2016; Yu et al. 2019; Kong et al. 2019; Zhang et al. 2021).

12.2.4.4 Teleost IgT/Z

IgT/Z was the latest Ig to be identified in teleosts and was first discovered in zebrafish and rainbow trout in 2005 (Hansen et al. 2005; Danilova et al. 2005). While this isoform has been identified in most of the main aquacultured species (with the notable exception of catfish), a recent study revealed the absence of IgT/IgZ in 25 species out of 73 Actinopterygii species (Mirete-Bachiller et al. 2021). Both secretory and membrane-bound forms of IgT contain all four CH domains in most teleost species (Parra et al. 2016), while IgT carrying 2 or 3 CH domains are also identified in several species (Giacomelli et al. 2015; Kato et al. 2015; Gambón-Deza et al. 2010; Savan et al. 2005; Mirete-Bachiller et al. 2021). Structural analyses of IgT/IgZ protein have thus far only been performed in rainbow trout. Unlike IgM, at the protein level, trout IgT is expressed as a monomer (~180 kDa) in serum, while it is found mostly as a polymer associated by non-covalent interactions (~4–5 monomers) and small amounts of monomers in mucus (Zhang et al. 2010; Xu et al. 2013, 2016; Kong et al. 2019). The concentration of IgT is ~3.7–11 µg/ml in serum and ~0.31–9.0 µg/ml in mucus from different mucosal sites. Although the concentration of IgT is much lower compared to that of IgM, it is worth noting that the ratio of IgT/IgM in mucus of different surfaces is ~20–124 times higher than that in serum. Moreover, antigen-specific titers of IgT are overwhelmingly prevalent in mucus from all MALTs examined thus far after bacterial and parasitic infection, while rarely detected in serum (Parra et al. 2016; Ji et al. 2020). Remarkably, IgT is considered as the most prevalent Ig in coating the microbiota of several fish mucosal sites, including the gut, skin, gill, nose, buccal cavity, and pharyngeal cavity (Zhang et al. 2010; Xu et al. 2013, 2016; Tacchi et al. 2014; Yu et al. 2019; Kong et al. 2019). Supporting the dominant role of IgT in mucosal surfaces, a recent study showed that fish depleted of IgT became very susceptible to a mucosal pathogen (Ich) and their microbiota underwent severe dysbiosis (Xu et al. 2020).

12.3 Fish Mucosal Barriers: Tissues and Cells

12.3.1 Mucosa-Associated Lymphoid Tissues (MALTs) in Fish

MALT includes a complex network of lymphoid tissues associated with each body mucosal barrier. Since each of the anatomical locations has unique physiological functions, MALT often shares key principles yet they also present unique characteristics. In teleosts, MALTs include the GALT, the SALT, the GIALT which also includes the unique interbranchial lymphoid tissue (ILT), the nasopharynx-associated lymphoid tissue (NALT), the pharyngeal-associated lymphoid, and the buccal-associated lymphoid tissue. Figure 12.1 illustrates the anatomical location of each of the fish MALTs so far described,
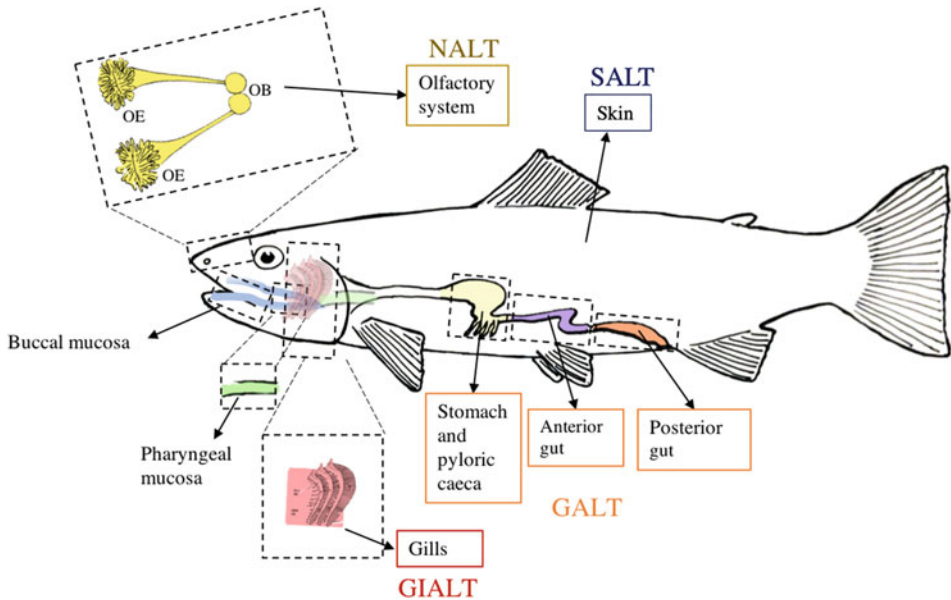
Fig. 12.1 Diagram with identified MALTs in teleost fish

while Table 12.1 depicts the main differences between fish and mammalian MALTs and sIgs.

12.3.1.1 Fish MALT Anatomy

In mammals, MALT includes diffuse MALT (d-MALT) and organized MALT (O-MALT). D-MALT is known to be present in all vertebrates (agnathans and gnathostomes) whereas *bona fide* O-MALT structures have only been described thus far in birds and mammals (Tacchi et al. 2015). d-MALT consists of scattered myeloid and lymphoid cells found intraepithelially (known as intraepithelial lymphocytes, IELs) or in the lamina propria at mucosal epithelia (known as lamina propria leucocytes or LPLs). These two populations are clearly found in bony fish MALT; their size and cellular composition may change as a result of infection or vaccination. O-MALT, in turn, consists of well-organized secondary lymphoid structures found at the mucosal barriers of endotherms. O-MALT structures such as tonsils and Peyer's patches display well-defined B- and T-cell zones and form germinal centers in response to antigen encounter. Thus, O-MALT in endotherms is critical for the affinity maturation of the mucosal adaptive Ig immune response. Whereas O-MALT appears to be absent in teleosts, this view maybe challenged by the presence of the ILT which was first discovered in the gills of Atlantic salmon (Haugarvoll et al. 2008; Dalum et al. 2015). ILT mostly consists of T cells; some B cells are also found without forming a clear zoning system like the canonical endotherm O-MALT structures. More recently, it has been shown that while GIALT is omnipresent in fish, the ILT placed at the base of the interbranchial clefts is variable across bony fishes, being absent in taxa lacking

Table 12.1 Features of MALTs and mucosal Igs (sIgs) in teleost fish and mammals

MALTs	Teleost fish	Mammals
Tissues detected	GALT (gut), SALT (skin), GIALT (gill), NALT (nose), buccal cavity, pharynx	GALT (gut), BALT (bronchus, trachea, lung), NALT (nose, pharynx), breast, conjunctiva, larynx, salivary gland, stomach, tear duct, vulva-vagina
Organization	Diffuse MALT	Organized MALT (PPs, MLNs) Diffuse MALT
SIgs Features	**Teleost fish**	**Mammals**
Key sIg	IgT	IgA
Other sIg	IgM, IgD	IgM, IgD, IgG
sIg transport to lumen mediated by	pIgR	pIgR (for IgA and IgM) FcRn (for IgG)
Role of sIg in microbiota coating	IgT++++ IgM++ IgD+	IgA++++ IgM++ IgG+ IgD+
Polymerization in mucus	Polymeric IgT (main form) Monomeric IgT (minor form) Tetrametric IgM (redox forms)	Dimeric IgA Pentameric IgM Monomeric IgG Monomeric IgD
Joining chain (J-chain)	No	Yes
Affinity maturation	Low	High
Somatic hypermutation	Yes	Yes
Class switch recombination	No	Yes

interbranchial septa (Rességuier et al. 2020). Whether the ILT plays any role in the induction and affinity maturation of sIg responses in fish, remains to be determined. For a recent review of teleost ILT refer to (Bjørgen and Koppang 2021).

One of the long-standing questions in the field of mucosal immunology is whether or not MALT forms an interconnected network of lymphoid tissues known as the common mucosal immune system (CMIS). This idea was put forward in the 1980s based on the body of work on mammalian IgA responses (Mestecky 1987). Later studies questioned its validity because only specific connections between some MALT could be documented using different mucosal routes of immunization. Thus, the current dogma in the mammalian mucosal immunology field is that there is some degree of connection among specific MALT that is supported by the presence of receptor-ligand interactions that allow homing of lymphocytes and other immune cells to specific MALTs and not others. However, a universal CMIS does not appear to be present in mammals. This intermediate view on the

level of connectivity among MALTs implies that stimulation of one MALT (i.e., with a mucosal vaccine) may or may not elicit protective responses at a distant MALT. Further, inter-connection may be also dependent on the type of antigen. In fish, the idea of a CMIS is still under debate, and the molecular basis for mucosal lymphocyte trafficking has yet to be characterized. The unique aspects of the anatomy of MALT in fish compared to mammals require careful experimental evaluation of this question in different teleost models.

12.3.2 Immune Cells in Teleost MALT

While fish MALT contains major lymphocyte populations of CD4 and CD8 T cells, γδ T cells as well as B cells, it also contains important populations of innate immune cells, including macrophages, neutrophils, mast cells/eosinophilic granule cells, natural killer (NK) cells, etc. (Smith et al. 2019; Kordon et al. 2018; Sun et al. 2014). Moreover, the presence of dendritic-like cells has also been described in fish MALTs. Below we describe the main immune cell types found in teleost MALT.

12.3.2.1 T Cells

Fish appear to contain CD4$^+$ and CD8$^+$ T cells homologous to those of tetrapod species. Two subsets of fish T cells are categorized by αβ- and γδ-TCR expressing on the cell surface. αβ T cells are more abundant and further classified into two main subpopulations, cytotoxic T cells and helper T (Th) cells, which are identified as CD8$^+$ and CD4$^+$ T cells, respectively (Nakanishi et al. 2015; Scapigliati et al. 2018). T cells are very abundant in the teleost intestine, gill, skin, and olfactory mucosa. The high abundance of T cells in those mucosal tissues suggests that fish T cells are not only important for host defense against mucosal pathogens but also potentially important for the homeostatic maintenance of the microbiota. However, studies on the specific roles of teleost T cells in mucosal areas are scarce, and whether fish T cells play a role in the induction of mucosal immune responses is a critical question that remains to be answered. Teleost mucosal T cells are usually found scattered in the epithelium and lamina propria. However, two examples of T-cell clustering have thus far been reported in teleost MALT. The first one is the ILT, a large accumulation of lymphocytes found in the interbranchial septum of several teleost taxa. According to several studies, the ILT is mainly composed of T cells (CD3$^+$ cells) and, curiously, its size appears to decrease upon antigenic stimulation (Haugarvoll et al. 2008; Koppang et al. 2010). A second example, of a smaller size, is that of the CD8$^+$ T-cell clusters found at the apical portion of the trout olfactory lamella. These clusters consist of 10–20 cells and they have a unique gene expression phenotype compared to the scattered CD8$^+$ cells found in the lateral olfactory neuroepithelium (Sepahi et al. 2016).

In contrast to tetrapods, teleost fish contain two types of CD4 genes, *cd4-1* and *cd4-2* (Takizawa et al. 2016; Ashfaq et al. 2019). Similar to mammalian CD4, CD4-1 contains four Ig domains, whereas CD4-2 contains only two. Using mAbs against CD4-1 and CD4-2, it was established that trout contains two CD4$^+$ T-cell populations, a predominant

one co-expressing surface CD4-1 and CD4-2, and a minor subset expressing only CD4-2. Both subsets produced equivalent levels of Th1, Th17, and regulatory T-cell cytokines upon bacterial infection, although the minor CD4-2 subset was less proliferative and displayed a more restricted TCRβ repertoire (Takizawa et al. 2016). Interestingly CD4-1 is also expressed by a major population of macrophages. Recently, the development of transgenic reporter zebrafish lines for CD4 and FOXP3 have revealed important aspects of T-cell biology at teleost MALT. For instance, CD4-1^{+} T cells comprise between 10 and 20% of T cells in the kidney, gills, and gut of adult zebrafish (Dee et al. 2016). In the gills, CD4-1^{+} T cells have a Th2 gene expression signature with elevated levels of *gata3* and *il4/13b* but not *il-4/13a.* In the gut, however, CD4-1^{+} cells have an increased Treg signature characterized by the expression of *foxp3a* and *il-10* (Dee et al. 2016). Further, using the f*oxp3a:EGFP* transgenic zebrafish line, Treg cells were found in the zebrafish skin. These cells are highly mobile and do not express *lck* compared to their thymus counterparts (Kasheta et al. 2017). Clearly, the use of these tools to understand the function of skin and other MALT Tregs in the context of infection would be an exciting next step. Adding to the relevance of CD4^{+} T cells in fish MALTs, a recent study in Japanese flounder has shown that upon infection with viral hemorrhagic septicemia virus (VHSV) or nervous necrosis virus (NNV), CD4-1^{+} T cells increase in numbers and proliferate in the gill and intestine (Jung et al. 2020).

CD8^{+} T cells are abundant in teleost MALT (Nakanishi et al. 2015; Rombout et al. 2011). These cells are thought to be critical for elimination of virally infected host cells. Using an anti-trout CD8α antibody, CD8^{+} T cells with cytotoxic markers have been identified in gills and intestine (Takizawa et al. 2011). Furthermore, trout skin also harbors CD8^{+} T cells and it appears that the anterior skin regions have higher proportions of CD8^{+} T cells compared to other sampled regions (Leal et al. 2016). Functional-specific cytotoxic activity assays have only been conducted in a handful of teleost species due to a lack of tools. In such studies, only systemic responses to vaccination and infection have been evaluated (Nakanishi et al. 2011) and therefore very little is known about specific CD8^{+} T-cell responses at mucosal sites. As in mammals, fish CD8^{+} T cells kill virus-infected cells by releasing perforin and granzyme (Toda et al. 2011). However, the aforementioned CD8^{+} CTLs were obtained from PBL or kidney not MALT. Transcript analysis shows that perforins are expressed in various fish mucosal tissues, including gill and intestine (Hwang et al. 2004; Athanasopoulou et al. 2009). Furthermore, a study in rainbow trout showed that sorted CD8^{+} T cells expressed higher perforin levels in the gills of VHSV-infected fish than in naïve fish (Aquilino et al. 2014). Thus, such studies suggest a potential cytotoxic role of CD8^{+} T cells in the fish mucosal surfaces against viral pathogens. Moreover, in rainbow trout, accumulations of CD8α^{+} cells around trophonts are observed in gill after Ich infection, suggesting a potential role of these cells also against parasites (Olsen et al. 2011). It is important to remember that a portion of CD8^{+} cells may not be T cells but may correspond to innate lymphocytes, an area that requires further investigation. As an example, CD8^{+} T cells are recruited to rainbow trout NALT within 15 min of intranasal delivery of live attenuated infectious hematopoietic necrosis virus (IHNV)

vaccine; such fast recruitment suggests those CD8^{+} cells behave as an innate lymphocyte subset (Sepahi et al. 2019).

CD4 and CD8 responses to infection or vaccination in teleosts are mainly focused on transcript analysis due to the lack of antibody reagents to CD4 and CD8 T cells in most teleost species. For example, transcriptome profiling of skin of orange-spotted grouper (*Epinephelus coioides*) shows an upregulation of T-cell markers, including CD4, CD9, and CD48, as well as CXCL9, 10, and 11 after infection with the parasite *Cryptocaryon irritans*, implying the presence and expansion of T-cell subsets at the local infected site (Hu et al. 2017). In zebrafish, bath vaccination with live attenuated *V. anguillarum* induces a Th17-like response in the gut, gill, and skin, as reflected by a significant upregulation of IL-17AF1, 2, and 3, IL-21, IL-22, defbl1, defbl3, and CCL20 genes. However, the Th1 and Th2-like responses were not remarkable in those tissues (Zhang et al. 2014). In rainbow trout, exposure to VHSV causes a significant decline of CD4, Tbet, and IFN-γ transcripts and an increase of IL-4 transcripts in the skin, thus suggesting a decrease of Th1-like cells in the skin mucosa. In this case, it was postulated that Th1-like cells migrated from the skin into the spleen in response to a local virus infection (Leal et al. 2016). In Japanese flounder, the CD4-1, CD4-2, and CD8α transcript levels increase in the gill after *V. anguillarum* bacterin exposure (Kato et al. 2013). In Atlantic salmon, a significant upregulation of CD4, CD8, and TCRα gene expression occurs in gills after infection with the ectoparasite *Neoparamoeba perurans* (Pennacchi et al. 2014). Overall, this body of work indicates that T-cell responses are critical in teleost mucosal immune responses, at least at the transcriptional level.

To date, γδ T cells have only been identified in zebrafish although *tcrd* and *tcrg* transcripts have been reported in the MALT of many other teleost species. In zebrafish, γδ T cells represent ~16.9–20.5% of all lymphocytes in the skin, gill, and gut tissues (Wan et al. 2017). Zebrafish γδ T cells show a surface phenotype of γ^{+}δ^{+}CD4^{−}CD8^{+} similar to the situation found in mammalian γδ T cells. Functional studies have revealed that zebrafish γδ T cells are phagocytic and potential APCs that induce antigen-specific CD4^{+} T-cell activation, B-cell proliferation, and IgM/IgZ secretion (Wan et al. 2017). Whether fish γδ T cells play a role in host defense against pathogens or in the control of the microbiota are key issues that remain to be investigated.

12.3.2.2 B Cells and Antibody-Secreting Cells

A recent review has compiled all the most current information on teleost mucosal B cells (Salinas et al. 2021). As a summary, four different B-cell subsets have been identified in teleost species: IgM^{+} B cells, IgD^{+} B cells, IgT^{+} B cells, and IgM[+]IgD^{+} B cells (Granja and Tafalla 2019; Edholm et al. 2010, 2011; Chen et al. 2009; Castro et al. 2014; Schorpp et al. 2006; Zhang et al. 2010; Xu et al. 2016). The specific proportion of the aforementioned B-cell subsets in the different fish lymphoid tissues is a subject that remains under-studied and somewhat controversial (see Salinas et al. 2021 and Chap. 14). Overall, IgT^{+} B cells are the predominant B-cell subset in all fish MALTs analyzed, while the IgM[+]IgD[+] B-cell subset is clearly predominant in systemic lymphoid organs, including the spleen, head

kidney, and blood. It has been found that both IgT$^+$ and IgM$^+$IgD$^+$ B-cell subsets are highly phagocytic and bactericidal (Li et al. 2006; Zhang et al. 2010). However, these capacities have only been studied in splenic B cells, and whether mucosal B cells have similar roles remains to be investigated. The B-cell responses to pathogens in all fish MALTs are extensively addressed below in Sect. 12.4.3. Overall, it has been shown that upon pathogenic challenge, large accumulations of IgT$^+$ B cells appear in all studied MALTs. Moreover, upon infection, many of these IgT$^+$ B cells are seen proliferating locally in most analyzed MALTs, suggesting that these mucosal responses are induced locally, or that those B cells migrate from systemic or mucosal lymphoid organs and proliferate in the infected MALTs (Zhang et al. 2010; Xu et al. 2013, 2016; Yu et al. 2019; Kong et al. 2019).

In mammals and birds, plasma cells are specialized in producing massive amounts of antibodies. In fish, the presence of antibody-secreting cells with functional features of mammalian plasma cells has been described previously (Salinas et al. 2021; Ye et al. 2013). However, little is known about the phenotypic features and specific markers that characterize plasma cells in fish. A recent study in carp has shown the presence of cells that appear to be the counterparts of mammalian plasma cells. These cells were significanlty larger than B cells, contained a large endoplasmic reticulum, and expressed high levels of soluble IgM. Moreover, recombinant carp CD40L or IL-21 alone could induce the generation of such cells as well as IgM secretion (Cui et al. 2020). Whether IgT/IgZ-plasma-like cells are phenotypically similar to these putative IgM plasma cells remains to be investigated. However, it has been shown that upon incubation of trout head kidney leukocytes with LPS and Vibrio bacterin, IgT-secreting cells contain high levels of surface IgT and are characterized by having high forward scatter (FSC) and low side scatter (SSC) properties (Zhang et al. 2010). It remains to be investigated whether these large cells are in fact plasmablast- or plasma-like cells. Nothing is known at this point with regard to the phenotypic and functional features of plasma-like cells in fish MALTs, although IgM- and IgT-secreting cells have been reported in several fish MALTs, suggesting the presence of plasma-like cells also in the fish mucosa.

12.3.2.3 Macrophages

Macrophages play an important role in host defense during pathogen infection and inflammation, as well as in maintaining tissue homeostasis. The best-demonstrated macrophages in teleost fish are comparable to the classically activated M1 macrophages in mammals (Hodgkinson et al. 2015). These cells rapidly kill pathogens through their phagocytic and microbicidal capacities, production of toxic reactive intermediates, restriction of nutrient availability, and release of inflammatory cytokines and chemokines (Hodgkinson et al. 2015; Kordon et al. 2018). Furthermore, teleost macrophages express MHC class II and B7 molecules, and thus they may play an important role as antigen-presenting cells (APCs) (Wittamer et al. 2011; Sugamata et al. 2009). Macrophages have been identified in the gill, gut, and skin in teleost fish (Mulero et al. 2008; Earley et al. 2018; Begam and Sengupta 2015). Teleost mucosal macrophages are essential in shaping

the gut microbiota and maintaining local homeostasis, similar to their mammalian counterparts (Earley et al. 2018; Gomez et al. 2013). Further detailed characterization of teleost mucosal macrophages is necessary to ascertain whether they are phenotypically different from non-mucosal macrophages.

12.3.2.4 Neutrophils

Neutrophils are the most abundant teleost granulocyte type in fish. Neutrophils are typically the first leukocytes recruited to the infected site by chemokines during acute inflammation (Havixbeck and Barreda 2015). They are also professional phagocytes and become powerful killers against pathogens through several mechanisms, including the release of neutrophil extracellular traps, the release of intracellular toxic granules, and the production of reactive oxygen species upon phagocytosis (Havixbeck and Barreda 2015; Smith et al. 2019). In the mucosal surface, neutrophils have been detected in gill (Progatzky et al. 2016; Lovy et al. 2011), skin (Jørgensen et al. 2018; Esteban 2012), and gut (Brugman 2016; Murdoch et al. 2019) of different species. Rapid recruitment of neutrophils in the gill and skin takes place after parasitic infection (von Gersdorff Jørgensen 2016; Lovy et al. 2011). Furthermore, teleost neutrophils also play a role in the establishment of gut microbiota homeostasis (Kanther et al. 2014; Murdoch et al. 2019; Milligan-Myhre et al. 2016). Transgenic zebrafish lines with fluorescently labeled neutrophils allow for live imaging of neutrophil-pathogen interactions. Most of these studies have been performed in the zebrafish larval model using both human and fish pathogens (Harvie and Huttenlocher 2015).

12.3.2.5 Mast Cells/Eosinophilic Granule Cells and Rodlet Cells

Mast cells/eosinophilic granule cells (MCs/EGCs) are a cell type that have features of both mast and eosinophil cells of mammals. They have been observed in gill, gut, and skin tissues of teleost fish (Ezeasor and Stokoe 1980; Murray et al. 2007; Dezfuli and Giari 2008; Mateus et al. 2021). Teleost MCs/EGCs have been shown to degranulate, similar to mast cells, upon stimulation (Reite and Evensen 2006), and these cells are involved in host defense against bacterial and parasitic pathogens in gills and gastrointestinal tract sites (Murray et al. 2007; Sharp et al. 1989; Fla ÑO et al. 1996; Noya and Lamas 1996; Vallejo and Ellis 1989). Local accumulation of MCs/EGCs has been observed adjacent to the infection sites (Reite 1997b; Murray et al. 2007; Sharp et al. 1989). Furthermore, degranulation of MCs/EGCs is more prevalent in infected gills and intestines than in uninfected tissues (Dezfuli and Giari 2008; Vallejo and Ellis 1989; Powell et al. 1993). Chronic inflammation in gills or intestinal tissues also causes a local increase of MCs/EGCs numbers (Reite 1997a, b). In addition, increases of the enigmatic rodlet cells are also seen under the same inflammatory conditions, and seem to localize mainly in the mucosal epithelium. Interestingly, rodlet cells have only been described in some teleost species, and are not present in other animal groups. Similar to MCs/EGCs, they release their products upon inflammatory stimuli, and may play a role in host defense against parasites (Reite and

Evensen 2006; Manera and Dezfuli 2004). These cells are easily recognizable by their club-like inclusions, which possess a crystalline inner core in their cytoplasm.

12.3.2.6 Natural Killer Cells

Two types of NK cell homologs are present in teleost fish: NK-like cells and non-specific cytotoxic cells (NCCs) (Fischer et al. 2013; Yoshida et al. 1995; Jaso-Friedmann et al. 1993). Unlike NK cells which are morphologically large and granular, NCCs are small agranular cells closely resembling monocytes in catfish (Evans et al. 1987). NCCs in seabream have highly variable morphology (Meseguer et al. 1994). Moreover, NCCs present a vimentin-like surface molecule and express the NCC receptor protein 1 (NCCRP-1) on their cell surface (Jaso-Friedmann et al. 1993, 1997). It has been shown that catfish NCCs recognize and kill a variety of human cell lines (Evans et al. 1984, 1987). Further studies revealed that NCCs express genes encoding for molecules with lytic capacity, including perforin, granulysin, and serglycin (Praveen et al. 2006). However, it appears that NCCs are not the equivalent of NK cells in fish since NK-like cells more closely related to the mammalian counterparts have been identified in teleosts. Accordingly, a few studies in catfish and rainbow trout have reported that NK-like cells kill allogeneic and virus-infected cells (Yoshida et al. 1995; Yoshinaga et al. 1994). The cell marker NK cell enhancement factor (NKEF) like gene has been identified from teleost NK-like cells. NKEF gene expression is increased in gill, skin, and other tissues after viral and bacterial infection (Huang et al. 2009, 2021; Utke et al. 2007). Interestingly, a recombinant NKEF protein has been shown to enhance the cytotoxicity of NCC from kidney in Nile tilapia (Huang et al. 2021). Whether NCCs or NK-like cells of teleosts are involved in the direct killing of pathogens remains to be investigated. Moreover, their distribution and abundance in mucosal sites is thus far unknown. Some hints come from recent zebrafish studies that used sorting of lck$^+$ cells of rag1-deficient zebrafish from the gut and performed single-cell RNA-Seq analysis. This study revealed the presence of innate lymphoid cell (ILC)-like cells in the zebrafish gut and that these ILC-like cells express *il22* and *tnfa* when exposed to inactivated bacteria or *il13* when exposed to helminth antigens (Hernandez et al. 2018).

12.3.2.7 Dendritic Cells

While the presence of dendritic cells (DC) functionally homologous to those of mammals is a matter of debate in teleost fish, teleost DC-like cells have been observed in skin, although they appear scarce within the gut and gill tissues (Wittamer et al. 2011; Kordon et al. 2016; Lauriano et al. 2018). The DC-like cells identified from zebrafish skin and channel catfish gill have been postulated to be equivalent to mammalian Langerhans cells (Lugo-Villarino et al. 2010; Kordon et al. 2016). In zebrafish, these cells express genes associated with DC function and antigen presentation such as *il12*, MHC class II invariant chain *iclp1*, and *csf1r*, and they appear to activate T cells in an antigen-dependent manner. Putative CD8$^+$ DC-like cells have been reported in trout intestine and seem to possess phagocytic activity and the ability to stimulate naïve T cells (Soleto et al. 2019).

12.3.3 Immunological Roles of Non-immune Cells at Teleost Mucosal Barriers

12.3.3.1 Epithelial Cells

Epithelial cells are the main building blocks of all mucosal epithelial barriers. These cells have numerous cell-to-cell adhesions that form a tight seal separating the host from the external environment. Epithelial barriers allow for selective diffusion of nutrients ions, solutes, and water. Epithelial cells have different morphologies at different fish epithelial tissues. For instance, the thinnest epithelial cells are those found in the respiratory epithelium of the gills, whereas gut epithelial cells have a columnar morphology and an apical brush border with microvilli. Epithelial cells are continuously being renewed from a pool of stem cells found in all mucosal barriers. Therefore, epithelial proliferation is an ongoing homeostatic process necessary for barrier maintenance.

Epithelial cells are highly susceptible to pathogen attack. Pathogens can either penetrate the epithelium between adjacent epithelial cells by breaking down tight junctions (paracellular pathway) or by direct invasion of epithelial cells (transcellular pathway). In response to infection, epithelial hyperplasia is a common tissue response in teleosts. Many examples can be found in the literature, mostly related to gill epithelial hyperplasia in response to parasitic infections (Adams and Nowak 2003; Munday et al. 2001; Parsons et al. 2001).

Fish epithelial cells respond to microbiota colonization. Most of the knowledge we currently have on this topic stems from germ-free zebrafish studies. Microbiota stimulates epithelial cell proliferation in zebrafish, a process that requires Myd88 and the Wnt signaling pathway (Cheesman et al. 2011; Rawls et al. 2004). The expression of chemokines, cytokines, and other immune mediators in zebrafish intestinal epithelial cells is regulated by microbiota colonization (reviewed by Murdoch and Rawls 2019). Furthermore, zebrafish intestinal epithelial cells recognize microbiota-derived signals and metabolites, shaping local and systemic immune responses (reviewed by Murdoch and Rawls 2019). For instance, microbiota induces expression of secreted apolipoprotein in zebrafish intestinal epithelial cells leading to neutrophil recruitment in the gut and to systemic antibacterial immune responses (Murdoch et al. 2019).

Epithelial cells are active players during the course of immune responses. Once infected, epithelial cells will mount innate immune responses by sensing pathogens via a number of pattern recognition receptors (PRRs). PRR expression in mammalian intestinal epithelial cells is known to be highly polarized in order to avoid constant pro-inflammatory responses to microbial-associated molecular patterns (reviewed by Yu and Gao 2015). Polarization of PRR expression in teleost epithelial cells remains to be investigated. During the early stages of infection, teleost epithelial cells produce cytokines such as IL-1β, tumor necrosis factor (TNFα), type I interferon (IFN), or transforming growth factor β (TGFβ), a phenomenon described in mammals (Larsen et al. 2020; Holopainen et al. 2012; Buchmann 1999). Epithelial-derived cytokines can induce several downstream responses, including hyperplasia of epithelial cells, recruitment of inflammatory cells, macrophage activation,

increase of lymphocyte activity, and increase in mucus secretion, among others (Buchmann 1999; Secombes et al. 2009). Epithelial cells also produce AMPs in all vertebrates, which effect rapid innate immunity against pathogens once released in the mucus layer. Teleost AMPs include cathelicidins, hepcidin, piscidins, histone-derived, and beta-defensins. For recent reviews on teleost AMPs please refer to (Masso-Silva and Diamond 2014; Chaturvedi et al. 2020).

Epithelial cells are also responsible for antigen uptake in teleost fish (as discussed in Sect. 12.4.2) and might play a role as non-professional antigen-presenting cells. In both mammals and teleosts, increased expression levels of MHC-II in epithelial cells happen in response to antigenic stimulation or inflammatory cues. However, definitive functional studies in teleosts remain to be conducted. The development of several teleost epithelial cell lines such as the RTgutGCs (Wang et al. 2019b) may provide in the future fundamental insights into the interactions between epithelial cells with pathogens, microbiota, and other potential external cues.

12.3.3.2 Mucus-Producing Cells

Goblet cells (GCs) are the main but not the sole producers of mucus in fish mucosal epithelia (Reverter et al. 2018). GC size, abundance, and chemical characteristics are highly variable across teleost species, tissues, and health status of the fish (Pittman et al. 2013). Fish GCs are morphologically similar to mammalian GCs. They are filled with compact mucus granules that occupy most of the cytoplasm depending on the cellular state of differentiation. GCs are continuously produced moving from basal to apical regions of the epithelium as they mature. Once they reach the apical portion of the epithelium, they develop distinct vesicles and cytoskeletal formations which enlarge the cell shape and maintain their integrity, until granule contents are released to form the mucus layer.

GCs respond to pathogens, stress, oral immunostimulation, and vaccination by either increasing in number or changing morphology in the epithelium of the infected mucosa as a first acute phase response (Buchmann 1999; Fernández-Montero et al. 2020a; Wang et al. 2019c; Arai et al. 2018; Pasetti et al. 2011). Further, glycosylation patterns of mucins also change upon infection. Aside from their immune functions associated with mucus production and mucus compounds, mammalian GCs are known to produce and secrete AMPs, chemokines, and cytokines, becoming part of the mucus defenses (Knoop and Newberry 2018). While the presence of AMPs in fish mucosal secretions has been clearly demonstrated, whether these AMPs are produced by teleost GCs requires further investigation.

Mammalian GCs play many important immune functions in the gut beyond mucin production. First, GCs create passages for antigens to reach lamina propria DCs. Following the release of mucins, goblet cell-associated antigen passages (GAPs) capture luminal antigens and deliver them to lamina propria APCs (Knoop and Newberry 2018). GCs sense microbes via Myd88 dependent mechanisms including acetylcholine (ACh) acting on muscarinic ACh receptor 4 (Knoop et al. 2015). The formation of GAPs has not been

studied in teleosts and, generally speaking, the biology of teleost GCs remains unexplored due to the absence of antibodies or genetic models to investigate them.

12.3.3.3 Neurons and Neuroendocrine Cells

Mucosal barriers are innervated by a large number of neurons that belong to the peripheral nervous system (PNS). In the case of the gastrointestinal tract, several neuronal networks have been described in mammals including the extrinsic sympathetic and parasympathetic branches as well as the intrinsic enteric nervous system (ENS). The ENS is the largest division of the PNS and regulates gut motility. The ENS has been well characterized in teleosts, especially in the zebrafish where the phox2b:GFP transgenic line marks enteric neurons (Taylor et al. 2016). Microbial colonization, infection by fish parasites, or bacterial infections induce changes in neuronal and neuroimmune mediators resulting in changes in gut motility [reviewed by Serna-Duque and Esteban (2020)]. Whether this is due to direct sensing of pathogens or pathogen-derived molecules by neuronal or neuroendocrine cells found in the fish gut remains to be elucidated. Direct sensing of pathogens and their products by peripheral nociceptive neurons is well documented in mammals (Chiu et al. 2013) and therefore these mechanisms may be in place in the teleost gut as well. The nasal epithelium is perhaps the mucosal tissue where neuroimmune communication is the most critical due to the close proximity between neurons and immune cells and the need to avoid neuroinflammation. Thus, NALT in teleosts is present among thousands of olfactory sensory neurons (OSNs) that are in direct contact with the external environment and microorganisms. In addition, OSNs provide a direct portal of entry into the brain via the olfactory nerve. As a consequence, tight regulation of immune responses in the nasal neuroepithelium is key to preserve brain integrity. In trout, a type of OSN, the crypt neuron, was shown to directly interact with IHNV (Sepahi et al. 2019). Furthermore, this interaction resulted in rapid recruitment of CD8$^+$ T lymphocytes to the nasal mucosa. These lymphocytes originate in the olfactory bulb, a part of the CNS. This example underscores the importance of neurons as pathogen sensors at mucosal sites and opens up the possibility that similar defense mechanisms occur in other teleost species and at other mucosal barrier tissues.

Neuroendocrine cells in fish are found in systemic as well as mucosal lymphoid tissues. including the gut, skin, and gills. Neuroendocrine cells have been reported as solitary cells or cell clusters (Zaccone et al. 1992). Teleost neuroendocrine cells include club cells found in the skin, gills, and olfactory organ (Pandey et al. 2021), neurochromaffin cells (Anderson and Campbell 1988), and sacciform cells (Mittal et al. 1981). However, variations among species exist. For instance, cyprinids have a large population of 5-HT positive neurons but no enterochromaffin cells in the gut mucosa (Velarde et al. 2010). Some neuroendocrine cells such as club cells appear to perform classical immune functions. For example, skin club cells of carp can phagocytose and degrade infiltrating immune cells during stress responses suggesting a role in the regulation and termination of the immune response (Iger et al. 1994).

In the gut, neuroendocrine responses regulate gut motility, a key aspect with respect to fish nutrition as well as pathogen control. Thus, it is not surprising that changes in gut neuroendocrine cells and their mediators occur during parasitic and bacterial infection in fish [reviewed by Serna-Duque and Esteban (2020)]. Neuroendocrine cells also possess oxygen receptors which mediate the cardiovascular and ventilatory response to oxygen deficiency, as reported in teleost species (Perry et al. 2009). Moreover, nitric oxide released by neuroendocrine cells contributes, through nervous stimulation, to the regulation of vascular tone and blood circulation in the gills.

12.4 Adaptive Immune Responses at Fish Mucosal Barriers

12.4.1 Antigen Uptake and Presentation at Fish Mucosal Sites

Antigen uptake at mucosal barriers involves highly specialized cellular processes. This is largely due to the high antigenic load found in the mucus, including food antigens, microbiota-derived molecules, and pathogen-derived antigens. In mammals, mucosal cells that uptake antigens include GCs, DCs, and epithelial cells. In fish, a few studies have identified cells that uptake different types of antigen (Løkka and Koppang 2016). For instance, antigens are taken up by epithelial cells in the teleost intestine. Antigen transport in the teleost hindgut occurs via endocytosis in enterocytes. Macrophages and DCs are found in the teleost gut and some reports indicate their ability to present antigen but definitive functional evidence is still missing. Further, antigen-sampling cells similar to immature mammalian M cells have been reported in the trout hindgut (Fuglem et al. 2010). In the gills, two populations of cells that can uptake antigen have been described (Kato et al. 2018). One resembles mammalian macrophage/DC cells and the second is more similar to mammalian M cells based on the expression of the lectin UEA-1 and the gene marker Anxa5.

In NALT, antigen uptake is more prominent in the apical tips of the olfactory lamella but it also occurs via the lateral neuroepithelium. Interestingly, the expression of MHC-II in the olfactory epithelium of trout increases in response to ccl19-like chemokines. In other MALTs such as SALT, the biology of antigen uptake remains largely undescribed. Overall, there are no teleost-specific antibodies to detect cells able to uptake antigen and therefore, much work remains to be done to determine how different types of antigen are taken up, processed, and presented to T cells at fish mucosal sites.

12.4.2 Inductive and Effector Sites at Fish MALT

In mammals, the mucosal immune system is traditionally separated into inductive and effector sites. Inductive sites are areas where antigen uptake takes place, and naive B and T cells are clonally selected and expanded upon antigen contact. On the other hand, effector

sites are locations where antigen-specific (effector) B and T cells are relocated upon antigen priming in inductive sites, and the sites where these activated B and T cells exert their roles. Cells activated at mucosal inductive sites migrate to effector tissues via the lymphatic system. In mammals, mucosal inductive sites include the Peyer's patches and mesenteric lymph nodes in GALT, while tonsils are found in the NALT. The exact delineation between inductive and effector sites in teleosts, however, is still blurry. This is in part due to the fact that *bona fide* O-MALTs have not been identified in teleosts, as mentioned earlier. Thus, if only d-MALT is found in teleosts, inductive and effector sites may not be anatomically separated. Alternatively, this separation may still be present but is yet to be discovered, with tools such as antibodies to several immune teleost cells potentially helping as they become more readily available. Overall, homologies between the mammalian and teleost mucosal immune system with regard to the activation of the immune response and the location of specific adaptive immune responses cannot be made at this point.

12.4.3 Adaptive Responses to Pathogens

Since most fish pathogens use the mucosa as their main route of entry into their host, onset of local mucosal adaptive immune responses is key to control and eliminate pathogens. These immune responses are probably mediated by local populations of B and T cells; whether these populations are recruited from the mucosal tissues or from systemic organs, or both, is still under investigation (Salinas et al. 2021). Below we summarize the most representative studies in which pathogen-specific Ig responses have been measured in fish MALTs, while a more comprehensive review on this subject has recently been published (Salinas et al. 2021).

12.4.3.1 Responses in the GALT

Although teleost GALT does not present organized immune structures, it plays an important role in gut homeostasis and immune defense against pathogens (Salinas and Parra 2015). As previously stated, IgT is the functional analog of IgA in these species, and it was in the rainbow trout gut where its specialized role in mucosal immunity was first described (Zhang et al. 2010). Infection with the gut endoparasite *Ceratonova shasta* leads to expansion of GALT IgT$^+$ B cells but not IgM$^+$ B cells. More critically, a compartmentalized Ig response occurs after infection, that is, parasite-specific IgT titers increase substantially only in the gut mucus while specific IgM titers are mostly observed in serum. This compartmentalized response also occurs in all other trout MALTs described herein, as well as in studies with different fish species. For example, the gilthead seabream also displays a similar compartmentalized Ig response upon infection with the myxosporidean *E. leei* (Piazzon et al. 2016). In general terms, transcriptomic studies conducted with gut parasite infection models in rainbow trout and gilthead seabream report increases of both gut IgM and IgT transcripts 60–100 days post infection, where IgT transcripts are normally higher than IgM (Estensoro et al. 2012; Picard-Sánchez et al.

2020). While a few studies on gut immune responses upon parasitic infections have been reported, evidence for the role of sIgs in teleost gut after bacterial infection is still scant. Zebrafish infected with *E. tarda* show increases in gut IgZ$^+$ B cells together with an increase in IgZ-specific titers after 3, 7, and 14 days post infection (Ji et al. 2020). With regard to gut responses to viral infection, information is even more limited. IgM$^+$ and IgT$^+$ B cells in rainbow trout gut increase after 7 days post infection with infectious pancreatic necrosis virus (IPNV), although specific sIg titers were not evaluated (Ballesteros et al. 2014).

12.4.3.2 Responses in the GIALT

Fish gills play critical immune roles supported by local immune cell populations, including B and T cells, monocytes, macrophages, neutrophils, thrombocytes, DC-like cells, NK-like cells, EGCs, rodlet cells, and melanin-containing cells, that combined form the GIALT (Aas et al. 2017; Koppang et al. 2010, 2015; Rességuier et al. 2020; Xu et al. 2016). T cells are particularly abundant in the gill ILT. Thus, the GIALT plays a key role in immune defense, since the gills are a common target of many pathogens.

Ectoparasites are one of the most common pathogen types infecting the teleost gills. Due to its economic impact in aquaculture, gill ectoparasites have been studied more in depth than other pathogens. For instance, the ciliate parasite *Ich* has often been used as a pathogen model to study mucosal adaptive responses in fish gills against parasites. *Ich* infection triggers parasite-specific IgT responses in the trout GIALT, where IgT$^+$ B-cell numbers as well as their local proliferation are significantly increased. In contrast, parasite-specific IgM titers in gill mucus are very low in comparison with those of IgT, and the numbers of IgM$^+$ B cells as well as their proliferative state remains unchanged (Xu et al. 2016). Similarly, Ich-specific IgM titers increase significantly in serum while those of IgT are very low or negligible. In addition, parasite-specific IgD titers are neither detected in the gill mucus or serum. Collectively, this information suggests the specialization of IgT in gill mucosal immune defense, similar to what has been observed in other fish MALTs. The specific IgM titers produced against certain ectoparasites (e.g., monogeneans) induce deciliation of the oncomiracidia (Matsui et al. 2020). However, some differences in Ig responses have been reported in other parasitic models such as *N. perurans*, the causal agent of amoebic gill disease (AGD). This parasite induces a reduction of the total IgM levels and a downregulation of IgM and IgT transcripts in different studies conducted in Atlantic salmon, thus suggesting an immunosuppressive effect of the parasite or an immune exhaustion of the host (Marcos-López et al. 2017; Young et al. 2008).

With regard to bacterial infections, *Flavobacterium columnare* has been used as a pathogen model to evaluate gill adaptive immune responses. Infection of rainbow trout with this pathogen induces significant bacteria-specific IgT titers in gill mucus, while IgM- and IgD-specific titers remain very low and absent, respectively (Tongsri et al. 2020; Xu et al. 2016). Bacteria-specific IgT titers detected in gill mucus correlate with an increase of both IgT$^+$ B-cell numbers and their proliferation in the GIALT. In contrast, bacteria-specific IgM-specific titers are high in the serum when compared to those of IgT, while

no specific IgD titers can be detected. In a different fish model, the zebrafish, *E. tarda* infection induces IgZ specific titers in the skin mucus, while IgZ2 titers against this pathogen are found both in the skin mucus and serum, thus suggesting a prevalent role of IgZ in mucosal immunity in this species. In addition, both IgZ subclasses carry different but complementary anti-bacterial activities, IgZ2 is implicated in bacterial coating and immobilization while IgZ is involved in C1q-mediated bacterial lysis (Ji et al. 2020). Similar to the situation in the gut, studies measuring gill Ig titers upon viral infections are lacking although a few studies have instead measured Ig transcripts, as described further below.

12.4.3.3 Responses in the SALT

Teleost skin is considered the largest physical barrier against fish pathogens and its associated lymphoid tissue, the SALT, provides a first line of defense at this mucosal barrier tissue. *Ich* has also been used as a pathogen model to characterize skin adaptive immune responses of fish against parasites. In such studies, *Ich* induces an increase of parasite-specific IgT titers in rainbow trout skin mucus, but no IgM or IgD-specific titers (Xu et al. 2013). These results correlate with a high sIgT coating of the parasites in the skin epidermis of infected fish (Fig. 12.2). IgT$^+$ B cells also increase in the infected fish epidermis in response to Ich, while neither IgM or IgM$^+$ B-cell populations coat the parasite or increase in number, respectively. Recently, very similar sIg responses were observed in trout infected with *F. columnare*. This bacterial pathogen also induces significant sIgT titers in the skin mucus, while significant IgM titers could only be detected in serum. *F. columnare* also induces local proliferation and production of IgT$^+$ B cells and specific IgT titers respectively. Collectively, these studies demonstrate that in the rainbow trout model, skin adaptive immune responses largely resemble those reported in the gills and gut.

12.4.3.4 Responses in Nasal, Buccal, and Pharyngeal MALTs

Although GALT, GIALT, and SALT are the most studied fish MALTs, the more recently discovered nasal, buccal, and pharyngeal MALTs have also been demonstrated to elicit adaptive mucosal IgT responses against pathogens. Similar to the skin studies described above, recent studies in rainbow trout NALT, buccal, and pharyngeal MALTs using the *Ich* infection model indicate that this parasite is very effective at inducing sIgT-specific titers in the mucus of all these MALTs, while IgM responses are mostly detected in the serum. As for all other studied MALTs, parasite-specific IgD responses cannot be detected in these three MALTs upon infection. Similar to what was shown in the SALT and GIALT, local IgT$^+$ B-cell proliferative responses occur in all three MALTs, while no increases in numbers or proliferative status of IgM$^+$ B cells takes place.

While all of the studies depicted above in the different MALTs have measured pathogen-specific Ig titers, a large number of studies in recent years have measured instead transcript levels of the different fish Igs (i.e., IgT, IgM, IgD) due to the lack of antibody reagents in many fish to evaluate Ig responses. Many of these studies have confirmed that IgT transcripts are the most abundantly induced in some MALTs upon pathogen infection,
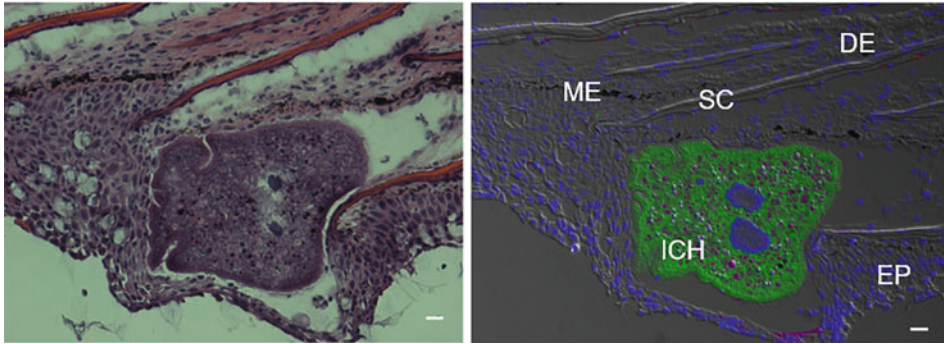
Fig. 12.2 IgT coats Ich parasites located in the skin epidermis of rainbow trout 21 days after infection. Microscope images of consecutive slides of hematoxylin & eosin staining (left) and immunofluorescence staining (right) of Ich parasites in skin cryosections from trout infected with Ich after 21 days. Ich (magenta), Dapi (blue), IgT (green), IgM (red). Scale bars, 20 μm. Skin structure is displayed with the outside part of the epidermis (EP) down and the dermis (DE) up. Scales (SC), melanophores (ME), and ICH are also indicated with white letters

while others have also shown the upregulation of IgM. Whether these increases in Ig transcripts correlate with increases in pathogen-specific Ig titers is a controversial point that remains to be addressed. In fact, some studies in rainbow trout have shown a lack of correlation between Ig transcripts and Ig-specific titers (Dixon et al. 2018; Tongsri et al. 2020), and thus, it cannot be assumed that an increase in Ig transcripts will translate into a significant increase in specific Ig titers. A recent review paper has summarized most of the studies of the last 10 years in which Ig transcripts have been used to analyze Ig responses in fish MALTs upon infection (Salinas et al. 2021).

12.4.4 Mucosal Ig Responses to Vaccines

Vaccination is considered the most effective method for disease prevention and pathogen control in fish (Rombout and Kiron 2014) and thus, it is an essential practice for the successful farming of several aquaculture species. Different types of immunization routes are currently being used for fish vaccination (Adams 2019; Bøgwald and Dalmo 2019), although it is well known that injection vaccination is the most effective at inducing highly protective and long-lasting immune responses. However, this delivery route induces for the most part potent systemic IgM responses, while the Ig responses generated in the mucosal sites appear to be less prominent. Thus, mucosal vaccines have been proposed to complement or substitute injected vaccines, where the goal is to induce the production of sIgs at mucosal barriers with the goal to protect fish from pathogen infections (Wang et al. 2020; Rombout and Kiron 2014; Munang'andu et al. 2015; Wilson et al. 2020). Thus far, the main delivery routes that are thought to promote mucosal immune responses are achieved through bath and oral vaccines. These vaccination strategies allow the simultaneous

vaccination of thousands of fish at the same time, they are much less labor-intensive than injected vaccines, and they are less stressful to fish, in part because they inflict less damage and lesions into the peritoneum when compared to injectable vaccines. Thus, bath and oral vaccines are considered ideal for mass vaccination of fish in aquaculture settings; however, their efficacy in eliciting protection is far lower than that of injected vaccines. Importantly, while bath vaccination provides in some cases a significant degree of protection, oral vaccination appears to be much less effective in that role, and only recently a few studies have shown a certain degree of efficacy in eliciting either immune and/or protective responses. Therefore, most commercially available fish vaccines are injectable, while only a handful are bath or oral so far. It is worthwhile noting that most reports on the types of sIg responses elicited by oral and bath vaccination strategies have only assessed antigen- or pathogen-specific IgM titers, thus probably missing the potential induction of specific IgT, the key mucosal sIg in most aquacultured species. Thus, there is an enormous need to improve both the detection of all sIg classes upon mucosal vaccination, as well as the efficacy of both bath and oral vaccine delivery strategies. While injectable vaccines induce specific IgM at mucosal tissues (see reviews by Salinas et al. (2011) and Sommerset et al. (2005)), below we report the most representative studies involving non-injectable vaccination strategies that stimulate mucosal responses at fish MALTs. Moreover, we only describe studies in which pathogen- or antigen-specific sIg responses have been reported, and not studies in which sIg transcripts are analyzed, since as stated above, sIg transcripts are not a reflection of antibody specificity for a given pathogen. For more extensive reviews on the topic of fish mucosal vaccines, and fish vaccines in general see (Bøgwald and Dalmo 2019; Das and Salinas 2020; Munang'andu et al. 2015; Rombout and Kiron 2014).

12.4.4.1 Oral Vaccination

Oral vaccines are probably considered the most promising strategy of fish vaccination, due to the possibility of including the vaccine as part of the fish diet. However, one of the most challenging issues of these vaccines is the protection of the antigen from proteolytic degradation once it reaches the digestive tract, which is thought to prevent the induction of efficacious immune responses against the antigen. To circumvent the problem of antigen degradation, several types of antigen carriers have been developed, mostly in the form of micro- and nanoparticles (Vinay et al. 2018; Mutoloki et al. 2015; Plant and LaPatra 2011; Moges et al. 2020). Thus, oral vaccines encapsulated with proprietary biological matrixes, alginate microparticles, sodium alginate and chitosan nanoparticles, among others, have been used to vaccinate fish against a number of pathogens, for example, *Streptococcus parauberis* in olive flounder, IPNV in rainbow trout, NNV in Senegalese sole (*Solea senegalensis*) and grouper (*Ephinephelus coioides*) (Kim et al. 2020; Ballesteros et al. 2012, 2013; Ahmadivand et al. 2017; Kai et al. 2014; Thwaite et al. 2020). In these studies, increases of serum IgM-specific titers (Kim et al. 2020; Ahmadivand et al. 2017; Thwaite et al. 2020), as well as IgM and IgT transcripts in different tissues (Ahmadivand et al. 2017; Kai et al. 2014; Thwaite et al. 2020) and increases in survival rates up to 70% (compared with control fish) were achieved (Ahmadivand et al. 2017; Kim et al. 2020). Moreover,

whenever a comparison between encapsulation and non-encapsulation was conducted in those studies, encapsulation always induced higher protective responses. Interestingly, one study with encapsulated IPNV DNA vaccine in rainbow trout showed IgT[+] and IgM[+] B-cell recruitment in the pyloric caeca after 10 days post vaccination, being higher for the IgT[+] B-cell numbers compared to those of IgM^{+} B cells. Other novel strategies recently reported for oral vaccination include the use of probiotic-based vaccines, where probiotic microorganisms are modified in order to express a target antigen of the pathogen. For example, this strategy has been used to successfully immunize common carp (*Cyprinus carpio*) against spring viremia of carp (SVCV) and cyprinid herpesvirus-3 (CyHV-3) with a reduction in mortality of 30–80% (Jia et al. 2020; Ma et al. 2020). Other strategies for oral vaccination that have been used are the combination of formalin-inactivated bacterin together with recombinant TNF as adjuvant. Upon oral delivery, this strategy induces in European seabass a protective effect, reducing mortality rates after *V. anguillarum* challenge. The protective effect correlates with increases of serum-specific IgM titers, gut IgT transcripts, and IEL infiltrates (Galindo-Villegas et al. 2013). Overall, while oral delivery represents one of the most promising vaccination methods for the aquaculture industry, further research is warranted in order to optimize antigen carriers and delivery methods to induce more efficacious protective immune responses. For more extensive reviews on oral vaccines please see (Embregts and Forlenza 2016; Mutoloki et al. 2015).

12.4.4.2 Bath Vaccination

Bath, or immersion, immunization is by far the most common strategy used for mucosal immune stimulation in fish. This is due to the easily applied and stress-free methodology used to deliver the vaccine, which can simultaneously stimulate all external MALTs (i.e., NALT, SALT, GIALT). In addition, since injectable vaccines for many farmed species are not currently cost-effective, the main strategy to vaccinate these fish is by bath vaccines. However, one of the main drawbacks of this method is that it requires a high amount of vaccine or antigen (Makesh et al. 2015). A number of studies have been conducted with immersion vaccines using inactivated or live attenuated *Yersinia ruckeri* (Raida et al. 2011) and *F. psychrophilum* (Lorenzen et al. 2010; Ma et al. 2018a) in rainbow trout, which induce an increase of serum IgM specific titers (Ma et al. 2018a; Raida et al. 2011) and reduce mortalities up to 40% upon pathogen challenge (Ma et al. 2018a). Bath vaccination has been used for some important commercial species in the USA, such as channel catfish and hybrid catfish (*I. punctatus* x *I. furcatus*). For instance, bath vaccination with an inactivated *F. columnare* vaccine (Shoemaker et al. 2011) or *A. hydrophila* bacterin (Shoemaker et al. 2018) induces a relative percentage of survival (RPS) of 57–94% after *F. columnare* challenge and 90% RPS after *A. hydrophila* challenge, respectively. Other bath vaccines, typically DNA vaccines, have been tested in common carp against koi herpesvirus and SVCV, where 30 days post vaccination an RPS of 63.3% was achieved after koi herpesvirus challenge (Aonullah et al. 2017), while 46.3% RPS was obtained upon SVCV challenge (Zhang et al. 2019). In Nile tilapia (*Oreochromis niloticus*) a polyvalent inactivated vaccine, containing *S. agalactiae*, *S. iniae*, *Lactococcus garviae*, and

Enterococcus faecalis, induces protection 3 months after vaccination against all five pathogens with RPSs ranging between 60 and 70% depending on the pathogen. This vaccination strategy also increases IgM-specific titers in serum (Abu-Elala et al. 2019).

The vast majority of immersion vaccination studies in fish deliver antigen/bacterin without a carrier. However, the use of nanoparticles as carriers to encapsulate the antigen may improve vaccine efficacy. For example, a study in olive flounder (*Paralichthys olivaceus*) reported the use a formalin-inactivated vaccine against VHSV encapsulated with PLGA nanoparticles, and squalene which served as adjuvant. The vaccine was delivered twice by bath or once by bath followed by an oral booster, and flounder challenged 30 days later with live virus. While the immersion/immersion vaccine regime provided 60% protection, the immersion/oral immunization strategy resulted in 73.3% protection, with 0% survival in the non-vaccinated group (Kole et al. 2019).

In conclusion, bath vaccination can stimulate multiple MALTs at the same time, and can induce significant protective effects. However, protection levels rarely surpass 60–70% of the vaccinated animals, and the specific contributions of the different sIg classes in providing protection upon vaccination are not yet known. Further, how T-cell immunity contributes to protection and how it can be optimized remain a black box in the field. Moreover, the role of each MALT in the induction of immune and protective responses upon immersion vaccination remains poorly understood.

12.4.4.3 Alternative Delivery Routes Stimulating Fish MALTs

While immersion and oral vaccination are the best-known mucosal delivery routes in fish, there are a number of alternative mucosal routes of antigen delivery that are currently being explored. Among them, anal vaccination induces adaptive immune responses in the gastrointestinal tract. However, the limited number of reported studies has focused on IgM responses, where the most common read-out is an increase of serum-specific IgM titers (Martín-Martín et al. 2020) together with an increase in the survival rate (Villumsen et al. 2014). Further, this delivery route is very labor-intensive and not amenable to automated implementation since the damage of the intestinal wall during delivery will result in microbiota leakage into the peritoneal cavity and systemic infection.

Another mucosal route that has recently been reported as a potential vaccine delivery method is nasal vaccination. This strategy has been shown to be highly effective in higher vertebrates (i.e., birds and mammals), and requires a lower dosage of antigen when compared with other delivery routes (Bernocchi et al. 2017). So far, studies conducted in rainbow trout have shown nasal vaccination to be efficacious in reducing fish mortalities and stimulating the recruitment of CD8^{+} T cells and increasing the gene expression of a number of chemokines, interleukins, AMPs, and sIgs (Salinas et al. 2015; LaPatra et al. 2015; Dong et al. 2020). While sIgT has been shown to be the main sIg induced in the NALT upon pathogen infection (Dong et al. 2020; Yu et al. 2018), it is unclear at this point whether nasal vaccination induces antigen-specific IgM, IgT, or IgD responses in the nasal mucosa. Clearly more studies are warranted to examine the usefulness of alternative mucosal vaccine delivery methods, including delivery to the buccal and pharyngeal

areas, that can stimulate fish MALTs with the goal to induce both immune and protective responses.

12.5 Interactions Between Fish Mucosal Immune System and Microbiota

Microbiota and mucosal immune systems have co-evolved in all metazoans. This symbiosis is known to benefit many aspects of host physiology and metabolism. In teleosts, every mucosal epithelium harbors a unique microbial community. Thus far, bacterial microbiomes are the best-studied type of symbiosis (Tarnecki et al. 2017), but we know that viromes and mycobiomes as well as archaea symbionts are all present at animal mucosal barriers (Norman et al. 2014; Vemuri et al. 2020). Comparisons between wild-caught and laboratory zebrafish microbiomes show that the microbiome of lab animals can model that of their wild counterparts (Stagaman et al. 2020). For example, the gut microbiome of wild Atlantic salmon has been shown to contain a high abundance of *Mycoplasmataceae* (Llewellyn et al. 2016), similarly to those reported in farmed and laboratory-reared salmonids (Zarkasi et al. 2014; Lowrey et al. 2015).

The first question regarding microbial symbiosis at mucosal sites is how the symbiosis becomes established at birth. In the case of fish, the microbiota is transferred from mother to offspring (Sylvain and Derome 2017; Senderovich and Halpern 2013; Beemelmanns et al. 2019). Further, once embryos hatch and become exposed to the external environment, a succession of microbial assemblies will start to form as the animal develops. Evidently in teleosts, the history of microbial assemblies during the life of an organism may be very divergent depending on the species and the habitat they occupy. Dissecting the step-wise interactions between the fish host and the microbiome during development is possible and the ability to derive germ-free zebrafish has greatly expanded our knowledge on how bacterial colonization shapes mucosal immune responses. For further reading on fish microbiomes refer to the following reviews (Stagaman et al. 2020; Llewellyn et al. 2014; Kelly and Salinas 2017).

Microorganisms that colonize fish mucosal secretions need to be able to anchor to the mucus. This is achieved by a series of molecular interactions between microbes and the different molecules that make up the mucus, largely mucins. In that regard, it has been shown that sIgA coating the microbiota in mammals plays a key role in microbiota colonization, while also preventing its epithelial translocation into the surrounding tissues. Interestingly, fish microbiota is also coated by secretory Igs. In teleosts, all three Ig isotypes can coat microbiota in all analyzed fish mucosal surfaces but sIgT is the main isotype responsible for this function. To be able to ascertain which microbiota species are coated by sIgs, a specific technique, IgA-Seq was recently developed in mammals. This technique consists of sorting microbiota coated by sIgA and then performing 16S rDNA sequencing to determine the specific composition and proportions of the sIgA-coated bacterial community. These studies show that sIgA coats specific subsets of microbes that can be both

beneficial but also pathogenic (Bunker et al. 2015; Palm et al. 2014). Recently this technique was applied to the study of fish gill microbiota to evaluate which bacterial species were coated by sIgT (Xu et al. 2020). Through sIgT-Seq analyses it was revealed that sIgT in rainbow trout coats a broad, albeit well-defined range of bacteria, similar to that shown for sIgA. The IgT-coated bacteria represent around 25% of the operational taxonomic unit (OTU) identified in the gill. Importantly, the trout gill IgT-coated microbiota is enriched in Actinobacteria and Firmicutes, which for the most part belong to anaerobic and facultative anaerobic taxa. Interestingly, IgT targets potentially beneficial taxonomic groups such as Propionibacteriales and Clostridiales, which in mammals are known to produce beneficial short-chain fatty acids (reviewed by Parada Venegas et al. 2019). On the other hand, IgT also coats potentially pathogenic bacteria including members of the orders Enterobacteriales and Pseudomonadales, as well as members of disease-driving bacteria (e.g., *Candidatus Branchiomonas*, *Acinetobacter* sp., *Streptococcus* sp.). The requirement of sIgT in the maintenance of microbiota homeostasis was recently elucidated with the use of a rainbow trout model in which sIgT was transiently depleted for over a period of several weeks. IgT depletion results in a marked dysbiosis in the gill mucosa that lead to a ~10-fold increase in the ratio of Bacteroidetes-to-Firmicutes when compared to control fish. Critically, the absence of sIgT leads to losses and expansions of beneficial and pathogenic taxa respectively. All these changes in microbiota composition are accompanied by translocation of bacteria across the gill epithelium into the systemic circulation, which indicates that IgT coating is required to keep the microbiota within the external mucosal layer. Moreover, gill tissue damage and inflammation is found in the IgT-depleted animals, probably as a consequence of the translocated microbiota. Restoration of IgT levels in the transiently IgT-depleted fish led to the recovery of tissue and microbiota homeostasis (Xu et al. 2020). Overall, the above-reported functions of sIgT coating have also been observed for sIgA in mammals (Bunker et al. 2015; Palm et al. 2014), strongly suggesting that similar evolutionary pressures have shaped mucosal Igs for millions of years to drive microbiome homeostasis. Whether mucosal sIgM and sIgD may contain similar or complementary roles in microbiota homeostasis remains unknown at this point. In that regard, it is worth pointing that unlike mice sIgM, human sIgM much like fish IgM coats a large percentage of the gut microbiota and thus, it is likely to play a role in its homeostasis.

As in mammals, fish sIgs are transported into the lumen via the interaction with the polymeric Ig receptor (pIgR) via transcytosis. In the lumen, however, it has been shown that cleaved pIgR known as the secretory component (SC) is primarily found in its free form, where it can directly bind pathogens and microbiota in rainbow trout (Kelly et al. 2017). Control and homeostasis of microbiota is also dependent on AMPs, highly potent microbiocidal molecules produced by the host that shape the localization and abundance of symbionts, and while fish contain a wide array of mucosal AMPs (Masso-Silva and Diamond 2014; Brogden 2005), their specific roles in controlling their microbiota is thus far unknown. Collectively, the current body of work indicates that forming a successful symbiosis with microbes at mucosal tissues involves complex check points by the fish immune system.

Microbial homeostasis at mucosal sites can be broken down by many stressors. Dysregulated microbial taxa are potentially damaging to the fish host and represent a source of opportunistic pathogens than can pose health problems. For instance, smoltification, transport stress, antibiotic treatment, infection, etc. all result in perturbations of the fish microbiome (Llewellyn et al. 2014; Kelly and Salinas 2017; Dehler et al. 2017). However, disentangling how the fish mucosal immune system responds to dysregulated microbiota is not straightforward due to masking effects by the original stressor. For instance, transport stress results in cortisol production, downregulation of AMP expression in the skin of rainbow trout as well as increased bacterial loads associated with the skin mucus. Finally, understanding the biological roles of the microbiota and their metabolic products in the fish mucosal immune system should be a priority because this knowledge can be harnessed to increase the productivity and sustainability of the fish farming industry.

12.6 Fish Probiotics

Probiotics are live microorganisms that confer benefits to host health (Banerjee and Ray 2017; Choudhury and Kamilya 2019). Over the last decades, the utilization of probiotics has switched from the conventional purpose of growth and nutrition in aquaculture to new objectives, such as enhancing the immune response and preventing/controlling infectious disease (Chuphal et al. 2021). In mammals, the probiotic species that are specifically selected to enhance health through activation of the mucosal immune system are called immunobiotics. However, fish immunobiotics have not been characterized yet, although a number of probiotic species appear to promote some immune parameters and enhance protective responses upon infection. Several bacteria and yeast species show promise as probiotics for the fish farming industry, for detailed studies on specific probiotic species please see these review papers (Banerjee et al. 2017; Pinpimai et al. 2015; Caruffo et al. 2015; Ibrahem 2015). Fish probiotics are mostly delivered in the feed or by direct bath/immersion treatment (Gomez et al. 2013). Although probiotics have been proven effective both in larvae and adult stages of fish, in general it is desirable to apply probiotics to fish larvae, the stage when teleosts are most vulnerable to infectious diseases as their adaptive immune systems are not fully functional or totally lacking. It has been proven that several probiotic candidates increase the fish survival rate after infection by viral and bacterial pathogens; however, the effects of probiotics in conferring resistance to parasitic and fungal diseases have rarely been assessed (Banerjee and Ray 2017). The efficacy of probiotics in the control of infectious diseases have been generally associated with the following parameters: (1) antagonistic activity against bacterial pathogens which results in growth inhibition or killing of the pathogen; (2) competition for colonization on the mucosal surface through blocking the attachment sites for pathogens; (3) immunostimulatory boosting of the cellular and humoral components of both systemic and mucosal immune compartments (Chuphal et al. 2021). However, in fish it remains largely unclear how probiotics promote innate and adaptive mucosal immune responses, a

knowledge that is required for the rational discovery and selection of probiotic-based immunotherapies (Gomez et al. 2013).

12.7   Conclusions and Outlook for the Future

The field of fish mucosal immunity continues to attract a large number of scientists from many different disciplines as well as the attention of industry partners. Exciting new breakthroughs in the field have highlighted the complexity of the teleost mucosal immune system. Three new MALTs have been described in the last 7 years in salmonids and therefore it is possible that others are yet to be described. Despite this anatomical complexity, the teleost mucosal immune system, similar to its mammalian counterpart, is governed by two main principles: the control of pathogens and maintenance of microbiota homeostasis.

With regard to the interactions between the fish mucosal immune system and pathogens, for the fish species that contain IgT, which thus far represent most of the dominant aquacultured species, it appears that IgT is the key Ig class specialized in host defenses against pathogens. However, this notion needs to be studied in a broader number of species, while the specific contributions of IgD and IgM in clearing mucosal pathogens require further work. On that line, several studies demonstrate the absence of pathogen-specific IgD responses upon infection, and thus, the role of this Ig in mucosal immunity may be restricted to the control of a portion of the microbiota. While fish can induce protective sIg responses in mucosal surfaces, we do not yet understand the specific effector functions of these sIgs (e.g., complement activation, agglutination, neutralization) involved in pathogen clearance, and thus, this is an area that clearly requires our focus. An important hurdle in the evaluation of sIg is the lack of reagents to evaluate IgT, IgM, and IgD responses in many fish species. Because of this, a very large number of studies evaluate changes in Ig transcript levels as an approximation for antigen- or pathogen-specific antibody responses. Clearly, Ig transcripts may not correlate with increases in antigen/pathogen-specific Ig titers as demonstrated by a number of recent studies (Dixon et al. 2018; Tongsri et al. 2020), and thus, efforts must be made to evaluate instead such titers with the use of newly generated antibody reagents.

While our knowledge on the roles of sIg has advanced in the last few years, we know very little regarding the types of cell-mediated immune responses that are elicited in the fish mucosa upon infection or vaccination. To gain insights into this area, critical knowledge needs to be generated on the poorly understood roles of CD4+ (i.e., Th1, Th2, Th17 helper T cells) and CD8$^+$ T cells, NK cells, and DCs in mucosal immune responses. This knowledge will be instrumental in discovering the lymphoid sites where adaptive mucosal immune responses are induced in fish. These studies will be able to address the question of whether fish contain a CMIS, a key question that will be critical for the field of fish vaccination. The presence of a CMIS would imply that stimulation of a specific fish MALT would result in the stimulation of all MALTs. Thus, finding a potential MALT through

which a CMIS maybe induced would have profound implications for the generation of effective mucosal fish vaccines in fish. On that note, very little is known with regard to vaccine strategies and immunization routes that can effectively stimulate and induce protective mucosal responses. This is due in part to the virtual lack of mucosal adjuvants that induce protective (e.g., IgT) mucosal responses, and the current inability to deliver intact antigen to the fish MALTs, although significant inroads have recently been achieved in a number of antigen encapsulation strategies that protect the antigen from degradation at mucosal sites. Clearly the future of mass vaccination for many fish aquaculture species relies on the ability to develop effective strategies of oral delivery, as vaccination by injection for many species is not cost-effective and introduces significant stress for the fish. Alternatively, bath vaccination offers also a cost-effective and less stressful strategy for many aquaculture settings, although the efficacy of most bath vaccines is significantly lower than that of injectable ones, and thus new developments are urgently needed also for improving the protective responses induced by current bath vaccination strategies.

With regard to the interactions between the fish mucosal immune system and the microbiome, such studies are in their infancy in the field. While it appears clear that sIgT is critical for the maintenance of microbiota homeostasis, a significant portion of the microbiota are also coated by sIgD or sIgM, or a combination of all three fish Ig isotypes, and thus, the roles of IgD and IgM in microbiota homeostasis remain to be elucidated, as well the significance of microbiota coating by more than one sIg class. Moreover, it is unlikely the sIgs are the only molecules involved in homeostatic control of the microbiota, and thus, future work is required to shed light into additional mechanisms and molecules by which the mucosal immune system controls the microbiota. Very little is known also with regard to how environmental and microbial (i.e., pathogens) perturbations affect the microbiota composition, and how in turn the dysbiosis induced may impact the fish mucosal immune system. In order to mitigate or to reverse the effects of such perturbations on the microbiome composition it will be critical to develop therapeutic interventions (e.g., dietary formulations, pre- and probiotics, immunobiotics) that are based on basic knowledge obtained in future studies regarding the bidirectional relationship of microbiome-mucosal immune system interactions.

Finally, given the taxonomic diversity of teleosts, the mechanisms by which fish protect their mucosal surfaces while maintaining microbiome homeostasis may be very different across teleost species and new studies may reveal alternative solutions to the ones so far identified. For example, a number of fish species do not contain IgT, and thus, it will be of interest to understand which immunoglobulin class is the one specialized in protecting their mucosal surfaces. Other fish species lack immunoglobulins altogether, and that poses the fascinating question of how these species defend themselves and control their microbiota in the absence of Igs. Fish mucosal immune responses to pathogens, immunostimulants, functional feed formulations, and vaccines continue to be documented by the use of "omic" approaches, especially transcriptomics and proteomics. Although these approaches generate large datasets, in-depth functional and mechanistic studies are still scant. Further,

the cooperation of non-immune cells such as epithelial cells, endothelial cells, and neurons to the overall mucosal immune response of fish deserves careful investigation. The use of zebrafish and the growing repertoire of transgenic lines available may fill some of the knowledge gaps that we currently have. However, translation of such findings with aquacultured species may need to be done cautiously. On that note, the diversity of teleost fish species is unparalleled in the vertebrate clade, and thus, it provides the opportunity of unearthing unique immunological innovations to the riddle of microbiota homeostasis and pathogen control. In summary, the field of teleost mucosal immunity continues to provide fundamental knowledge into the biology of vertebrate mucosal immune systems while promoting the discovery of new fish therapeutics that will, in turn, impact the development of more sustainable aquaculture practices.

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Immune Response Against *Piscine orthoreovirus* (PRV) in Salmonids

13

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Abstract

Piscine orthoreovirus (PRV) infection is extremely common in farmed salmonids. Three genotypes of PRV can infect and cause disease in different salmonid species. Red blood cells are the main target cells for all genotypes of PRV, but the outcome of the infection differs between host species and PRV genotypes. The PRV-1 genotype is known to cause heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon (*Salmo salar*), a disease that can cause up to 20% mortality. PRV-1 infection induces a strong innate antiviral response in infected red blood cells, and an adaptive immune response involving PRV-specific and poly-unspecific antibodies, and a cytotoxic lymphocyte response leading to massive immune cell infiltration in heart tissue, the hallmark of HSMI. Despite strong immune responses, PRV-1 infection is not cleared in Atlantic salmon and gives a life-long viral persistence. For other PRV genotypes, the disease course is different. PRV-2 infection in farmed coho salmon primarily leads to acute anemia, and PRV-3 infection in farmed rainbow trout can lead to both anemia and heart inflammation. Here, we discuss the antiviral immune activity against PRV and elaborate on why immune protection often is insufficient and instead can become destructive.

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Abbreviations

CTL	Cytotoxic T lymphocytes
EIBS	Erythrocytic inclusion body syndrome
HSMI	Heart and skeletal muscle inflammation
IFN	Interferon
PRV	*Piscine orthoreovirus*
RBC	Red blood cells

13.1 Introduction

A disease in Atlantic salmon (*Salmo salar*) characterized by pathological lesions in heart and skeletal muscles appeared in Norwegian aquaculture in the late 1990s. The disease was named heart and skeletal muscle inflammation (HSMI) (Kongtorp et al. 2004a), and in the following years HSMI became a common disease in Norwegian Atlantic salmon farming (Hjeltnes et al. 2016). The disease usually occurred 5–9 months after the transfer of the fish to seawater, and mortality could reach 20% of the fish in a sea cage, with most individuals in the cage showing the typical histopathological lesions (Kongtorp et al. 2006): Epi-, endo- and myocarditis, myocardial necrosis, myositis and necrosis of the red skeletal muscle (Kongtorp et al. 2004b).

The etiologic cause of HSMI is *Piscine orthoreovirus* (PRV), an ubiquitous virus in farmed salmonids (Palacios et al. 2010; Di Cicco et al. 2017). Testing of archival material revealed that PRV was present in farmed fish many years before the disease HSMI was recognized, which indicated that the virulence of the virus has evolved with time (Dhamotharan et al. 2019). PRV is a member of the *Reoviridae* family, genus *Orthoreovirus* and was the first aquatic reovirus recognized in genus *Orthoreovirus,* in contrast to other fish reoviruses that group to genus *Aquareovirus* (Palacios et al. 2010). The main model and template for understanding the PRV genome and predicting viral functions has been the comprehensively studied mammalian orthoreovirus (MRV) (Nibert and Duncan 2013). Based on these initial predictions, PRV infection mechanisms have been studied for a decade through fish trials and cellular experiments, including the study of pathogenesis, immune responses, and vaccination effects. During this period, the understanding of the PRV host interaction has gradually evolved, although many questions

remain. Here we summarize some of the main findings related to immune responses triggered by PRV, and some of the remaining knowledge gaps that keep us from fully understanding why some PRV-infected salmonids develop the pathological immune responses in the heart known as HSMI, and how protective versus pathogenic immune responses may occur.

13.2 PRV Variants, Diseases and Distribution

The PRV particle is a double-shelled icosahedral capsid with a diameter of 70 nm, which pack ten double-stranded RNA genome segments (Palacios et al. 2010; Wessel et al. 2017). The viral proteins encoded by the genomic segments are listed in Table 13.1. The functions of these proteins can be classified into three groups: i) proteins that transcribe and replicate the viral genome; ii) proteins that form integral parts of the viral particles; iii) proteins present only in the infected cells, i.e., non-structural, multifunctional proteins with important roles in the viral factories. There are functional overlaps between these groups.

Based on sequence similarity, PRV has so far been categorized into three genotypes, PRV-1-3. The different salmonid species and diseases associated with the PRV genotypes are summarized in Table 13.2. The main host species of PRV-1 is Atlantic salmon (*Salmo salar*), in which the virus may cause HSMI (Wessel et al. 2017). PRV-1 has also been detected in rainbow trout (*Oncorhynchus mykiss*), coho (*O. kisutch*), Chinook (*O. tshawytscha*), and pink salmon (*O. gorbuscha*) (Purcell et al. 2018). The only known host of PRV-2 is coho salmon, and this genotype has so far only been found in Japanese salmon aquaculture, where it is the cause of erythrocytic inclusion body syndrome (EIBS),

Table 13.1 PRV-encoded proteins and their main known and predicted functions

Segment	Protein	Function	Location in virion
L1	λ3	RNA-dependent RNA polymerase	Inner capsid
L2	λ2	Capping enzyme	Inner capsid
L3	λ1	Helicase, NTPase, RNA triphosphatase	Inner capsid
M1	μ2	NTPase, RNA triphosphatase, RNA binding	Inner capsid
M2	μ1	Part of a trimeric heteromer outer capsid protein together with σ3 (Dhamotharan et al. 2019). Penetration of endosomal membrane.	Outer capsid
M3	μNS	Organizer of viral factories (Haatveit et al. 2017)	Non-structural
S1	σ3, p13	σ3: dsRNA binding (Wessel et al. 2015b). p13: cytotoxic, nonfusogenic integral membrane protein (Markussen et al. 2013; Key et al. 2013).	Outer capsid
S2	σ2	σ2 RNA binding	Inner capsid
S3	σNS	Involved in virus inclusion formation.	Non-structural
S4	σ1	Cell attachment protein	Outer capsid

Table 13.2 Diseases associated with PRV genotypes in different salmonid species

	PRV-1	PRV-2	PRV-3
Target species	Disease	Disease	Disease
Atlantic salmon S. salar	HSMI (Wessel et al. 2017) Association with melanized foci (Bjorgen et al. 2019)	–	–
Brown trout S. trutta	–	–	?
Chinook salmon O. tshawytscha	Not confirmed	–	Not confirmed
Coho salmon O. kisutch	Association with heart inflammation and jaundice syndrome (Cartagena et al. 2020)	EIBS	Associated with heart inflammation and jaundice syndrome (Cartagena et al. 2020)
Rainbow trout O. mykiss	–	–	Heart inflammation (Olsen et al. 2015)

a disease characterized by acute anemia (Takano et al. 2016). PRV-2 can replicate in Atlantic salmon (Malik et al 2021a). PRV-3 genotype was first identified as a disease-causing agent in farmed rainbow trout (Olsen et al. 2015), and later also found to infect farmed Atlantic and coho salmon (Cartagena et al. 2020; Garseth et al. 2019; Hauge et al 2017). PRV-3 is the cause of heart inflammation in rainbow trout (Vendramin et al. 2019b) and associated with heart inflammation and jaundice syndrome in coho (Cartagena et al. 2020). PRV-3 has not been detected in wild rainbow trout or coho at the northern Pacific coast where these fish are native wild species, but is common in wild brown trout (*S. trutta*), which may indicate that this is the original host species for PRV-3.

PRV-1 is ubiquitous in areas where marine farming of Atlantic salmon occurs and found to infect most fish a few months after transfer to the sea (Bjorgen et al. 2019). The virus is also found in wild salmon populations relatively far from farm sites, but at a much lower prevalence (Garseth et al. 2013; Marty et al. 2015; Purcell et al. 2018). PRV-3 is found to be widespread in brown trout populations and in sea running *S. trutta* (Adamek et al. 2019). Heart inflammation has not been described in infected wild salmonids.

The main target cell for all PRV genotypes is the nucleated erythrocyte. The nucleated erythrocytes in fish are transcriptionally and translationally active, but such activity decreases with cell age (Gotting and Nikinmaa 2017) Infection of erythrocytes may cause hemolytic anemia, and this is the hallmark of erythrocytic inclusion body syndrome (EIBS) (Takahashi et al. 1992), as well as an important factor in the development of the jaundice syndrome in coho (Smith et al. 2006). The lysis of erythrocytes in these diseases occurs in the acute phase of the infection, shortly after the highest viral RNA load can be measured in blood (Takano et al. 2016), and consequently assumed to be a direct conse-quence of virus replication in erythrocytes. On the other hand, a modest transient anemia is also described in PRV-1 infection of Atlantic salmon, and somewhat more pronounced, anemia is described in PRV-3 infection of rainbow trout (Olsen et al. 2015).

Atlantic salmon does not eliminate PRV-1 infection. After experimental infection of Atlantic salmon, viral RNA has been detected in blood for more than a year after exposure; however, the virus was not transmitted to sentinel fish from persistently infected fish at this time (Garver et al. 2016). PRV-1 induces an innate antiviral immune response in erythrocytes (Dahle et al. 2015), while infection with PRV-1 strains from BC, Canada associated with lack of or mild clinical signs only induce a low antiviral response in the host (Polinski et al. 2019). When similar genotypes of PRV-1 were used to infect Atlantic salmon in the same trial, no significant differences in innate immune responses were detected (Wessel et al. 2020), indicating that non-viral factors may be responsible for the observed differences.

The erythrocytes represent the most numerous cell population in the body, circulating in all tissues, and at the peak of infection more than 50% of the cells may produce PRV (Finstad et al. 2014). Following the peak of infection in blood, PRV can be detected in several other cell types, including cardiomyocytes, macrophages, and hepatocytes (Dhamotharan et al. 2020). PRV infection of the heart causes influx of immune cells into the infected heart, which is the hallmark of HSMI in Atlantic salmon and in the heart lesions observed in PRV-3 infected rainbow trout (Mikalsen et al. 2012; Olsen et al. 2015). PRV-1 causes a persistent infection in Atlantic salmon, and in the persistent phase viral RNA is found in erythrocytes, erythroid progenitor cells, and macrophage-like cells (Malik et al. 2019, 2021b). In contrast to PRV-1 infection in Atlantic salmon, PRV-3 infection in rainbow trout does not always lead to persistence, and PRV-3 may be completely cleared from the fish (Hauge et al. 2017).

In Atlantic salmon, the persistent PRV-1 seems to play a role in the development of granulomatous melanized changes, "black spots", in the white skeletal muscle. The melanized changes originate from red focal changes (Bjorgen et al. 2019), which etiological cause is still unknown. PRV-1 is assumed to be a driver for development of advanced black spots from the original red spots. The PRV-1-containing spots are characterized by granulomatous inflammation with myocyte necrosis and recruitment of melano-macrophages, but also by initial regeneration (Bjorgen et al. 2019). In the melanized focal changes, macrophages and melano-macrophages are the main PRV-1-infected cells (Malik et al. 2019, 2021b).

Genetically different strains of PRV-1 can differ in virulence, and can cause more or less severe HSMI (Dhamotharan et al. 2019; Wessel et al. 2020). The main genetic differences linked to virulence have been mapped to structural proteins present in the outer capsid of PRV-1.

13.3   The PRV Infection Route, Pathogenesis, and Persistence

PRV primarily spreads by horizontal transmission, which has been demonstrated both under field and experimental conditions. The virus can enter the bloodstream via the gastrointestinal tract (Hauge et al. 2016), but gills and skin are other potential sites of entry that have not yet been investigated.

A week or so after infection, PRV replication is found in erythrocytes. How the transition from port of entry to infection of the erythrocytes occurs is not known. The virus will have to pass through the intestinal mucosa and epithelial cell layer to the bloodstream. MRV uses an epithelial junction protein, junction adhesion molecule—A (JAM-A) as a receptor (Campbell et al. 2005), but the receptor for PRV is unknown. It is not known if PRV replicates at the site of entry, or just passes through. The viral entry of the cell occurs through endosomal uptake and exit through the interaction with the endosomal membrane and outer viral capsid proteins. The infection of erythrocytes has been relatively thoroughly characterized, especially for PRV-1 in Atlantic salmon (Fig. 13.1). The first phase is characterized by a substantial increase in viral load in the infected cell as well as in the blood compartment, with high loads of viral RNA and viral protein within the cells (Haatveit et al. 2017; Wessel et al. 2017). The virus forms globular cytoplasmic inclusions called viral factories, which are compartmentalized sites of virus replication organized by the non-structural protein MuNS (Haatveit et al. 2016, 2017; Wessel et al. 2017). The viral factories are visible in phase-contrast microscopy and resemble inclusion characterized as EIBS in coho salmon. The viral factories shield the virus replication from the innate immune response, i.e., viral dsRNA must be isolated from dsRNA-recognizing receptors (Zuniga et al. 2015). Genomic orthoreoviral dsRNA may not be exposed in the cytoplasm during viral replication, since the viral mRNA transcription occurs within core particles and the progeny-positive ssRNA strands are encapsidated before they template the synthesis of negative strands to form the dsRNA genome (Lemay 2018).

The peak of PRV-1 infection in blood cells, i.e., mainly erythrocytes, is associated with a high plasma viremia which has a relatively short duration. The level of the plasma viremia is higher in infection with high virulent variants compared to low virulent variants (Wessel et al. 2020).

The pathogenesis is highly dependent on the PRV genotype and the infected salmonid species, showing both general traits across the genotypes as well as fish species and virus genotype differences important for disease development.
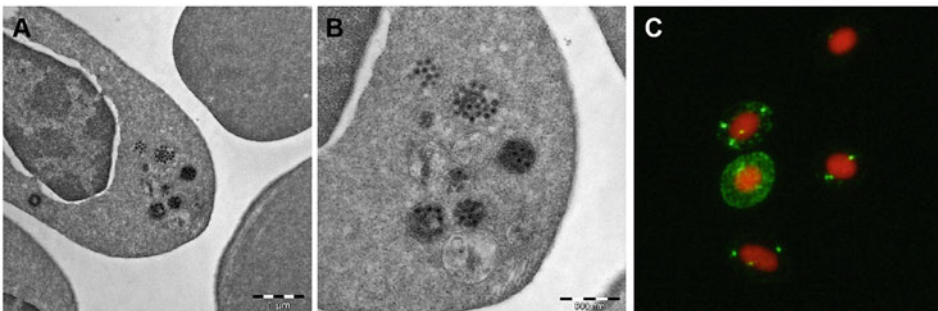

Fig. 13.1 PRV in red blood cells. (**a, b**) Electron micrographs of PRV within cytoplasmic inclusions inside a red blood cell. (**c**) Fluorescent labeling of the PRV σ1-protein (green) in erythrocytes

PRV infection of erythrocytes is linked to two disease conditions: 1) Acute hemolysis, which occurs around peak of virus infection in experimental infection, and seen as clinical anemia (EIBS), hyperbilirubinemia, and accumulation of bilirubin in the liver (jaundice syndrome) and suddenly increase in mortality (Takano et al. 2016). 2) Inflammation and necrosis of cardiomyocytes and myocytes of red skeletal muscle (HSMI) observed 2–3 weeks after peak of virus infection and clinically observed as circulatory failure and a moderate increase in mortality (Wenske et al. 1985; Kongtorp et al. 2004b).

During PRV-1 infection in Atlantic salmon the peak of infection is not associated with severe anemia. In contrast, anemia is evident after infection with PRV-2 in coho saloon, and in some field outbreaks of PRV-3 infections in rainbow trout (Olsen et al. 2015; Takano et al. 2016). Thus, different genotypes of PRV may employ different effects on erythrocytes, possibly through different mechanisms of release from the infected cell. Following the peak plasma viremia, the level of viral protein drops substantially in the erythrocytes. Although the levels of viral RNA are continually high in the cells, as reflected by RT-qPCR, the production of viral protein and thus replication of viral particles appears to be low (Wessel et al. 2017).

Cardiomyocytes are important secondary target cells of PRV-1 in Atlantic salmon. Following the peak of the plasma viremia, high loads of virus are detected in the cardiomyocytes, followed by the development of severe cardiac inflammation (Fig. 13.2). Low virulent PRV variants also infect cardiomyocytes, but the viral load in the cells is low and the cardiac lesions are mild. Heart lesions are also observed following PRV-3 infection in rainbow trout.

In the outbreaks of jaundice syndrome in seawater-cultured coho which is associated with PRV infection, however, the etiology is not proven, hemosiderosis found in the kidney suggests that hemolysis occurs in the intravasal compartment and that the yellow color is caused by large quantities of bilirubin due to rapid destruction of red blood cells (Smith et al. 2006). In Atlantic salmon it has been shown that hepatocytes are susceptible,
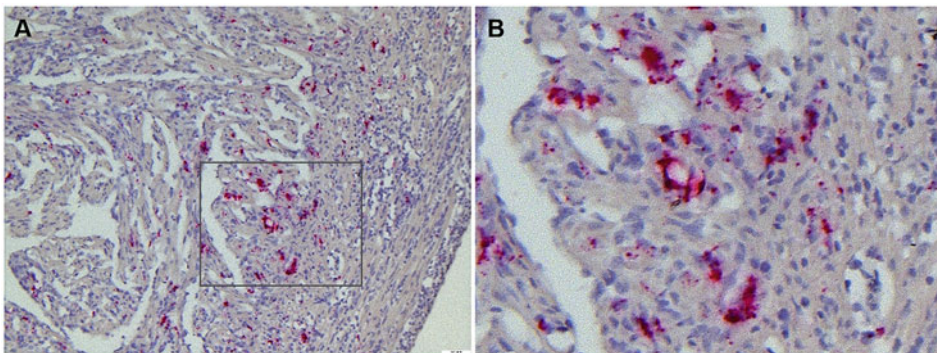
Fig. 13.2 PRV-1 in heart. (**a**) PRV-1 detected by in-situ hybridization (red) in cardiomyocytes during the development of heart and skeletal muscle inflammation in Atlantic salmon. Rectangle depicts area enlarged in (**b**) highlighting virus-infected cardiomyocytes within inflamed tissue

but not very permissive to PRV-1 infection (Dhamotharan et al. 2020). The lack of infiltration of inflammatory cells in the liver, in contrast to observations in the heart, could mirror different permissiveness for PRV-1 replication.

13.4 The PRV-Infected Cell: Innate Antiviral Response and Viral Counteractivity

From research on the MRV replication cycle, PRV has been predicted to bind to a cell surface receptor and to sialic acids via the outer capsid protein σ1 (Markussen et al. 2013), and to be taken up through receptor-mediated endocytosis. The orthoreovirus' outer viral capsid proteins µ1/σ3 partly disassemble in the endosome and the virus core particle is transferred to the cell cytoplasm through the membrane-penetrating properties of a proteolyzed µ1/σ3 complex (Nibert and Fields 1992). The conserved protease target sites in µ1 indicate that this mechanism has been conserved in PRV (Markussen et al. 2013). The transcription occurs within the core particles, ensuring that exposure of the genomic dsRNA to the cytoplasmic dsRNA recognizing receptors does not occur (Lemay 2018). Such receptors initiate signaling cascades that activate interferons and other innate immune responses (Poynter et al. 2015). The innate antiviral response to PRV is primarily studied at the transcriptional level in erythrocytes and whole organs from infected salmonids (Dahle et al. 2015; Wessel et al. 2015a). Studies on the erythrocyte transcriptome response to PRV infection have revealed a strong upregulation of innate antiviral effector genes, showing that the erythrocytes clearly sense and respond to PRV (Dahle et al. 2015; Haatveit et al. 2017). The ability of salmonid erythrocytes to mobilize an innate antiviral immune response is shown also for interaction with other viruses (Morera and MacKenzie 2011; Puente-Marin et al. 2018). Erythrocytes express genes like Toll-like receptors (TLRs) 3 and 22, retinoic acid-inducible gene I (RIG-I), and dsRNA-activated protein kinase (PKR) that have roles in the sensing of dsRNA (Dahle et al. 2015). These genes are significantly upregulated in erythrocytes after PRV-1 infection (Dahle et al. 2015; Johansen et al. 2016). In line with this, the PRV-regulated response, as indicated through transcriptional activation, includes genes associated with antiviral immune responses characteristic for RNA viruses, including interferon-regulating factors (IRF3/IRF7), interferons (IFNa1), interferon signaling mediators (STAT-1, JAK2), and interferon-regulated antiviral effector genes (Mx, ISG15, Viperin) (Wessel et al. 2018b). Most genes are induced in correlation with the loads of PRV RNA detected in the cells (Dahle et al. 2015, Haatveit et al. 2017). ISG15, Viperin, and PKR are antiviral effectors directly shown to control MRV in mammals (Smith et al. 2005). MRV can counteract the antiviral response by binding and inactivating IRF3 and thereby blocking IFN production and ISG expression (Tian et al. 2015). The long-lasting transcription of IFN-regulated genes in PRV-infected Atlantic salmon does not indicate that IFN signaling is blocked by PRV-1 (Dahle et al. 2015; Haatveit et al. 2017; Røsæg et al. 2018). Despite this the PRV sigma 3 protein binds dsRNA and has been predicted to be involved in regulation of dsRNA sensing (Wessel

et al. 2015b). PRV replication cycle and innate antiviral response is illustrated in Fig. 13.3. For PRV-1 infection in Atlantic salmon erythrocytes, the peak of antiviral gene expression coincides with a decrease in PRV protein level and decrease in plasma PRV RNA (Haatveit et al. 2017; Malik et al. 2019; Røsæg et al. 2018; Wessel et al. 2017). At the same time, the high level of PRV RNA in blood cells persists, indicating that the antiviral response may have induced a blockage on PRV protein production and viral release. Lower hemoglobin level is also observed in PRV-infected erythrocytes (Lund et al. 2017; Wessel et al. 2020), which could be an additional result of a general translational block induced by the antiviral response. The mammalian counterpart, MRV, is reported to block host translation through interaction with PKR and inhibition of stress granule formation (Schmechel et al. 1997). However, MRV is shown to bypass the host translational block and continue its own virus protein production (Schmechel et al. 1997). This does not appear to be the case for PRV-1 in erythrocytes.
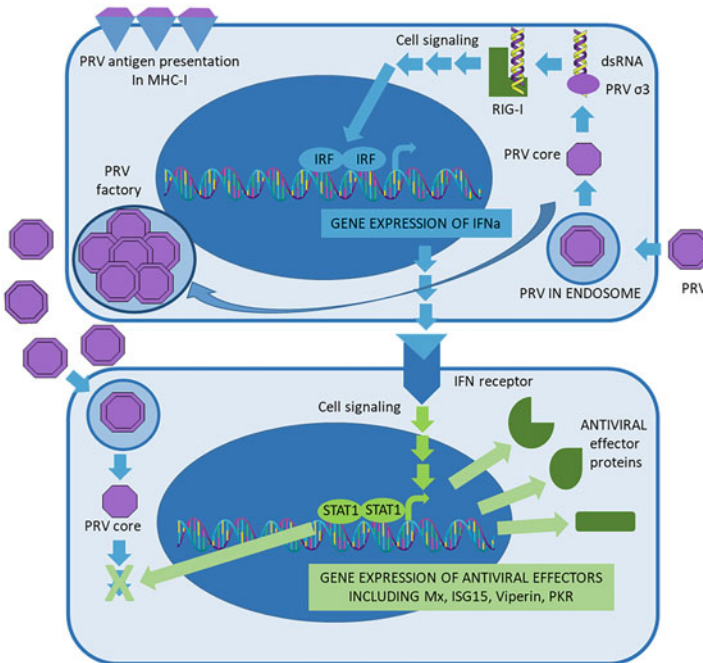
Fig. 13.3 PRV replication and antiviral response. PRV infects via receptor-driven endocytosis, and the viral core is transferred to the cell cytoplasm. The PRV dsRNA genome, if exposed, can bind to cytoplasmic pattern recognition receptors for dsRNA like retinoic acid inducible gene—I (RIG-I). The PRV σ3 protein is shown to bind dsRNA, but not to reduce the interferon response. RIG-I induces a signaling pathway resulting in activation of interferon response factors (IRFs), which are transcription factors able to induce expression from the IFNa gene. IFNa is secreted and can interact with IFN receptors on neighboring cells. These receptors activate a JAK/STAT signaling pathway that turns on a multitude of interferon-regulated genes (ISGs) that function, among others, as different antiviral effectors that can block further viral production and release

Differences in the innate antiviral response have been reported for PRV-1 infection in Atlantic salmon. Norwegian PRV-1 isolates associated with HSMI have been reported to trigger a strong innate antiviral response in blood in multiple challenge trials and during ex vivo infection of erythrocytes (Dahle et al. 2015; Haatveit et al. 2017; Wessel et al. 2015a, 2020). In contrast, infection with Canadian PRV-1 isolates in coho, Sockeye, and Atlantic salmon has been reported to be associated with little or no immune response (Garver et al. 2016; Polinski et al. 2016, 2019). Similarly, Atlantic salmon respond very little to infection and replication of PRV-3 compared to rainbow trout (Hauge et al. 2017). This could be due to the ability of some PRV variants to block interferon signaling or hide its dsRNA genome from sensing in some hosts species. The PRV infections which are not associated with innate antiviral responses have not been associated with pathological outcomes, which have suggested a link between strong innate immune responses and pathological outcomes. However, when comparing different genetic variants of PRV-1 with different disease outcomes in the same trial, no significant differences in innate antiviral responses were found in blood cells (Wessel et al. 2020).

The long-lasting innate immune response induced by PRV-1 in infected erythrocytes is suggested to explain the observed cross-protective effect of PRV against unrelated secondary viral infections (SAV and IHNV) (Lund et al. 2016; Vendramin et al. 2018). Interestingly, studies from Canada infecting Sockeye salmon with a slightly different genetic variant of PRV-1 led to a very low innate antiviral response and no ability to cross-protect (Polinski et al. 2016).

Fish erythrocytes were recently reported to go into a shape-shifted phase where they express both MHCI and MHCII, indicating that they may present both intra- and extracellular antigens on their surface (Chico et al. 2018). This shape-shifted phase may drive accumulation of infected erythrocytes in the spleen following PRV infection, which could be a reason for peak phase anemia. Professional antigen-presenting cells like macrophages can also be PRV-infected (Bjorgen et al. 2015; Malik et al. 2019). Macrophages may also present PRV antigens after engulfing PRV-infected erythrocytes (hemophagocytosis). In this way PRV-infected cells may initiate an adaptive immune response (Fig. 13.3).

13.5 The Adaptive Immune Response and Long-Term Protection

Adaptive immunity to PRV is a two-edged sword, associated both with protection and pathological effects. Next to nothing is known about the initial activation of the B-cell and the T-cell arm of adaptive immunity following a PRV infection, and mainly the timr course and end effects have been studied (Fig.13.4). After PRV infection, there is an increase in soluble and membrane-bound IgM transcripts in the head kidney, while CD8 and granzyme mRNAs are primarily induced in the spleen (Johansen et al. 2016).

The antibody response has been studied using bead-based multiplex immunoassays, detecting PRV-specific IgM targeting both the PRV outer capsid proteins σ1 and μ1c and the intracellular PRV factory protein μNS in plasma. Specific antibody production is detected at the same time as decreased viral loads and regeneration of the infected heart
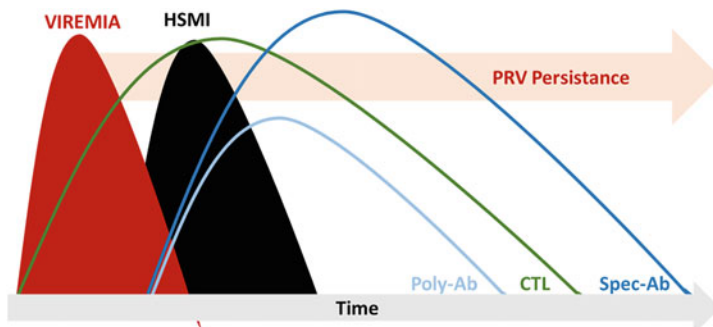
Fig. 13.4 The time course of the adaptive immune response to PRV-1 in Atlantic salmon. The time course of the PRV-1-specific antibody response (Spec-Ab), polyreactive unspecific antibody response (Poly-Ab), and cytotoxic T-cell (CTL) response relative to PRV-1 viremia and HSMI pathology

start (Teige et al. 2017, 2019). In PRV-1 infection trials, a specific antibody response against PRV-1 outer capsid proteins develops a couple of weeks after the peak in erythrocyte infection, at the time when HSMI is in early development (Teige et al. 2017, 2019). A similar time course for the antibody response is observed for PRV-3 in rainbow trout (Vendramin et al. 2019a). The anti-PRV-1 and anti-PRV-3 antibodies cross-react (Dhamotharan et al. 2018; Malik et al. 2021a).

In addition to the antigen-specific antibody response, PRV-1 infection in Atlantic salmon also induces production of antibodies that bind to negative control antigen in the assay, and thus are polyspecific (Teige et al. 2019; Malik et al. 2021a) This appears to be a phenomenon typical for PRV-1 infection in Atlantic salmon, as the phenomenon is not observed in PRV-3-infected rainbow trout (Vendramin et al. 2019a; Malik et al. 2021a), nor in Atlantic salmon infected with PRV-2, PRV-3 or salmonid alphavirus (SAV) (Teige et al. 2020; Malik et al. 2021a).

As judged from gene expression studies targeting the T-cell costimulatory molecules CD4 and CD8, a T-cell-mediated response also plays a role during PRV infection. Influx of CD8+ T cells is characteristic for the histopathological changes in the heart (Johansen et al. 2015; Mikalsen et al. 2012), indicating that HSMI represents an immunopathological disease, in contrast to other common viral infections in the salmonid hearts that rather cause tissue necrosis as a result of viral replication and cell lysis (Garseth et al. 2018; McLoughlin and Graham 2007).

Transcriptomic data from PRV-infected Atlantic salmon hearts indicate that CD8+ T cells, CD4+ T cells, macrophages, and B cells all are recruited after disease onset. However, CD8+ T cells appear to be particularly involved, and associated with elevated granzyme A mRNA levels, which suggests a cytotoxic T-cell attack on PRV-infected heart cells (Johansen et al. 2015, Mikalsen et al. 2012). Following infiltration of immune cells in the heart, as typical for HSMI, virus levels decrease (Dhamotharan et al. 2020; Finstad et al. 2012).

Field observations from both Norway and Chile indicate that fish suffering from HSMI may be sensitive to stress, and there is a general awareness that infected fish should be handled with care. However, not many studies have directly addressed this topic. An experimental study showed that PRV-infected fish were more sensitive to acute hypoxic stress compared to uninfected controls (Lund et al. 2017). A Canadian study performed with the Canadian PRV-1 genotype did not find the same effects (Zhang et al. 2019).

13.6 Solutions, Vaccination, and Future Outlook

Conclusion/Outlook

PRV infections in salmonid fish are common in both farmed and wild populations (Adamek et al. 2019; Garseth et al. 2019; Lovoll et al. 2012).

PRV-mediated diseases have only been found in farmed and captivated salmon and may have emerged through aquaculture of salmonids. Viruses coevolve with their host, and the viral fitness in wild fish is adjusted to enable it to coexist with the host. However, PRV infections are commonly subclinical in farmed fish and often not associated with disease outbreaks.

PRV has resisted cultivation in cell cultures and this has been a hurdle in the vaccine development against HSMI. However, vaccine prototypes against HSMI based on either purified virus particles obtained from infected fish (Wessel et al. 2018a), or DNA vaccines (Haatveit et al. 2018), have been shown to induce moderate to good but not full protection. Live vaccination with a non-pathogenic PRV variant is another possibility based on the ability of PRV-3 to completely block PRV-1 infection and HSMI i Atlantic salmon (Malik et al. 2021a). In general, there is a drive to develop vaccines if the disease causes large economic loss or complicates the export of products.

The complexity of PRV infections, caused by different PRV genotypes infecting different salmonid species and leading to a variety of outcomes, call for systematic species-specific studies. A result from one combination of host species and PRV subtype may not mirror all possible combinations regarding pathogenesis and immune responses versus PRV. Continuous spread of PRV will contribute to further evolution and generate new virus variants.

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Antiviral Innate Immune Responses: Infectious Pancreatic Necrosis Virus and Salmonid Alphavirus

14

Cheng Xu, Amr A. A. Gamil, Koestan Gadan, Aase B. Mikalsen, and Øystein Evensen

Abstract

Infectious pancreatic necrosis virus (IPNV) and salmonid alphavirus (SAV) are important viral infections of salmonid fish. The impact and losses due to IPNV infection have been significantly reduced in Atlantic salmon through genetic selection for resistance, but infection with SAV in salmonid aquaculture causes major loss to the industry. Vaccination has been developed to reduce the impact of both diseases but with limited success so far. The innate immune responses are the first line of defense against infection and play crucial roles in directing adaptive immune responses. This chapter addresses the innate immune responses to IPNV and SAV infections in vitro and in vivo and summarizes the current knowledge. Understanding the detailed aspects of the innate host-virus interaction is crucial for developing prophylactic interventions.

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Abbreviations

AGK	Asian grouper strain K cells
Aim2	Absent in melanoma 2
ALRs	Aim2-like receptors
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
CARD	Caspase activation and recruitment domain
CPE	Cytopathogenic effect
DAI	DNA-dependent activator of IRFs
DIP	Defective interfering particles
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
DVG	Defective viral genomes
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
FADD	Fas/Apo-1-associated death domain protein
GCN2	General control non-depressible 2
HRI	Heme-regulated inhibitor
IFN	Interferon
IkB	Inhibitor of NFkB
IKK	IkB kinase
IL-1	Interleukin-1
IPN	infectious pancreatic necrosis
IPS-1	IFNB-promoter stimulator 1
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
JNK	JUN N-terminal kinase
LGP2	Laboratory of genetics and physiology 2
LRR	Leucine-rich repeats
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MDA5	Melanoma differentiation-associated gene 5
MOI	Multiplicity of infection
MyD88	Myeloid differentiation factor 88
Mx	Myxovirus resistance protein
NFkB	Nuclear factor-κB
NLR	Nod-like receptor, nucleotide-binding domain LRR-containing family
NOD	Nucleotide-binding oligomerization domain
OAS	Oligoadenylate synthase

ORF	Open reading frame
PACT	PKR activating protein
PAMP	Pathogen-associated molecular pattern
PERK	PKR-like endoplasmic reticulum kinase
PKR	dsRNA-activated protein kinase R
PI	Propidium iodide
Poly I:C	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
PS	Phosphatidylserines
RD	Repressor domain
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor-interacting protein
RLR	RIG-1-like receptor
RNA	Ribonucleic acid
RTG	Rainbow trout gonads cells
TAB1/TAB2	Tak-binding proteins
TAK1	TGF-b-activated kinase
TANK	TRAF-family-member-associated NFkB activator
TBK	TANK-binding kinase
TCID	Tissue culture infective dose
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TIRAP	TIR domain-containing adaptor protein, a.k.a. MAL (MyD88 adaptor like)
TNF	Tumor necrosis factor
TRAF	TNF-receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFNβ
uORF	Upstream ORF
UPR	Unfolded protein response
VP	Virus protein
ZBP	Z-DNA-binding protein

14.1   Introduction

Infectious pancreatic necrosis virus (IPNV) and salmonid alphavirus (SAV) are among the most important viral infections in salmonid fish. IPN virus is a naked, double-strand RNA virus while SAV is a positive strand, enveloped virus. IPNV infection occurs in freshwater and after seawater transfer in Atlantic salmon, while pancreas disease, caused by SAV infection, is a seawater disease, not seen in freshwater in Atlantic salmon. Salmonid alphavirus is divided into six different subtypes where subtypes 1, 2, and 3 are the most important in Atlantic salmon farming.

IPN is a disease of salmonid fish and an economically important disease caused by aquatic birnaviruses. Disease is seen in all age groups, with high losses at start-feeding and post sea transfer.

Alphaviruses are typically spread by hematophagous vectors but not so for salmonid alphaviruses. Infected fish play an important role for spread but the marine source of SAV is not known. E1 and E2 are major spike proteins and are important determinants of virulence for alphaviruses infecting higher vertebrates, but little is known for the piscine alphaviruses. Amino acid variants in E2 have been linked to differences in virulence for salmonid alphavirus but details are not known.

Losses due to IPNV infection in Atlantic salmon have been significantly reduced due to development of genetically resistant fish, likely also combined with the use of inactivated vaccines against IPN. The impact of SAV on salmonid aquaculture is still significant and vaccines have been used in salmon farming over the last decade but the impact on disease prevalence is questionable.

The innate immune responses are key in early detection of infection and play important roles for the adaptive immune responses. In this chapter, we will present the current understanding of innate immune responses to IPNV and SAV in salmonids and view this in light of prophylactic interventions.

14.2   The Innate Immune System of Fish

The innate immune system is independent of any previous exposure to a particular antigen and employs germline-encoded pattern recognition receptors (PRR) to identify the infectious agents; in contrast to the receptors involved in adaptive immunity, which are generated by rearrangements and ultimately hypermutations of the receptor-encoding genes (Akira et al. 2006). PRR recognize unique and conserved microbial molecular signatures known as pathogen-associated molecular patterns (PAMPs), including dsRNA, ssRNA, unmethylated CpG DNA, lipopolysaccharides (LPS), peptidoglycans, mannan-binding lectin, 1,3-beta-glucan, and sugar mannose plus others, which are shared by groups of related microbes but absent in eukaryotic cells (Ellis 2001). Sensing of PAMPs initiates PRRs downstream signaling pathways that activate the transcription of pro-inflammatory and immunomodulatory genes that play important roles in both innate and adaptive immunity (Takeda and Akira 2005).

Cytokines are a large family of more than 100 small proteins that are involved in different cellular functions such as proliferation, pro- and anti-inflammatory, antiviral responses and immune activation, regulation of immune responses, and linking the innate and adaptive immunity (Lunney 1998). Interferons (IFN) are one of the important cytokines and were identified based on their capacity to induce cellular resistance to viral infections (Lindenmann et al. 1957). The mechanism by which IFNs exert their effect includes signaling through specific receptors, called IFN receptor, that eventually triggers signaling cascade through the JAK/STAT pathway, leading to the induction and/or

increase of the transcription of several antiviral genes and thereby establishing an antiviral state (Schoggins and Rice 2011). The cytokine repertoire of teleost fish seems to have resemblance to those of mammals. To date, many cytokine homologs have been cloned and characterized in different fish species (Whyte 2007).

The best characterized PRRs are retinoic-acid-inducible gene I (RIG-I)-like receptors (RLR) and Toll-like receptors (TLR) which mediate the induction of type I interferon and pro-inflammatory cytokines. RIG-I, melanoma-differentiation-associated gene 5 (MDA5), and Laboratory of Genetics and Physiology 2 (LGP2) are located in the cytoplasm of most cells and preferentially bind 5′-triphosphate ssRNA and dsRNA, respectively (Kato et al. 2006). These receptors are considered the main cytosolic sensors for induction of type I IFN in non-immune cells after virus infection (Fig. 14.1). Different TLRs are predominantly expressed in immune cells, located in endosome or as extracellular receptors (Kawai and Akira 2007). TLR3 recognizes dsRNA, TLR7 and 8 sense ssRNA, and TLR9 senses CpG-rich DNA. These TLRs are located on endosomal membranes. In mammals, the cell surface TLR4 can mediate IFN induction in response to a non-nucleic acid ligand, namely the bacterial lipopolysaccharide (LPS) (Kawai and Akira 2007). In contrast to the ubiquitous RIG-I/MDA5, the expression of TLRs is cell-type-dependent. TLR3 and TLR4 are expressed in many cell types including epithelial cells, fibroblasts, and monocytes, while TLR7, 8, and 9 are mainly expressed in myeloid DC (mDCs) and plasmacytoid DC (pDC) (Hornung et al. 2002) in higher vertebrates.

A total of 11 TLRs (TLR 1, 2, 3, 5, 7, 8, 9, 14, 21, 22, and 23) have been identified so far in teleost fish and some have similar functions as the mammalian orthologs (Jault et al. 2004; Roach et al. 2005). Orthologs of MDA5 and RIG-I have also been cloned and characterized at a functional level in fish (Chang et al. 2011; Biacchesi et al. 2009; Sarkar et al. 2008). Furthermore, the adaptor protein mitochondrial antiviral signaling protein (MAVS) for RIG-I-like receptors has been identified in salmon at a functional level (Biacchesi et al. 2009) Fig. 14.2). The presence of TLR and RLR orthologs in fish suggests that teleost fish possess the same pathogen recognition mechanism as mammals. Further, leukocytes of teleost fish respond to several TLR ligands that induce cytokine expression with similar patterns to those seen in mammals, suggesting the conservation of signaling cascades through vertebrate evolution. TLR signaling molecules identified in teleost fish include myeloid differentiation factor 88 (MYD88), TIR-domain containing adaptor inducing INF-β (TRIF), TIR domain-containing adapter protein (TIRAP), TRIF-related adaptor protein (TRAM), and interferon regulatory factor 3 and 7 (IRF3 and 7) (Purcell et al. 2006).

Type I IFN genes have been cloned from several fish species including Atlantic salmon, pufferfish, channel catfish, and zebrafish (Altmann et al. 2003; Robertsen et al. 2003; Long et al. 2006). Cloning of fish IFN genes made it possible to produce recombinant IFN, providing a valuable tool to investigate its antiviral activity, as well as its involvement in the host-pathogen interactions. The fish type I IFN family contains at least the four subtypes IFNa, IFNb, IFNc, and IFNd (Sun et al. 2009; Chang et al. 2009). Two IFN-α/β-like proteins (SasaIFN-α1 and SasaIFN-α2) were first cloned and characterized
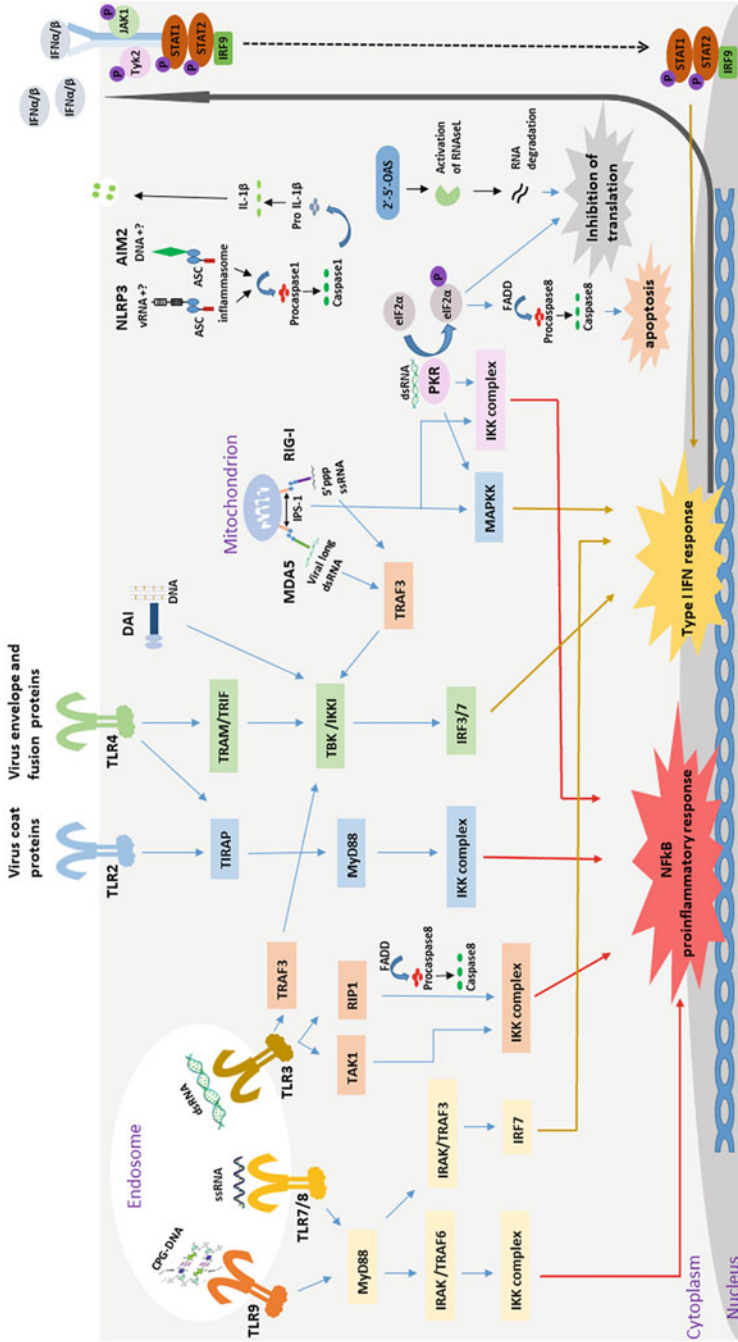
Fig. 14.1 Sensors of virus infection; modified from (Laz and Fitzgeraldi 2008). TLRs localized at the cell surface (TLR2 and TLR4) sense surface virus structures such as coat proteins while those localized in the endosome (TLR 3, 8/7, and 9) sense virus genome. Virus nucleic acid present in the cytoplasm is sensed by different cytoplasmic sensors including RLRs, NLRs, ALRs, PKR, DAI, and 2′-5′OAS. Signaling from TLRs signal through the adaptor proteins MyD88 (TLR2, TLR4, TLR7/8, and TLR9) and TRIF (TLR3 and TLR4) while IPS-1 mediates the signaling through Rig-I and MDA5. Responses generated by the different signaling cascades include type I IFN responses, NFκB-mediated proinflammatory responses, inhibition of protein synthesis and apoptosis. The produced IFN in turn binds its receptor on the cell surface and induces signaling cascade through JAK/STAT pathway leading to the transcription of several antiviral genes
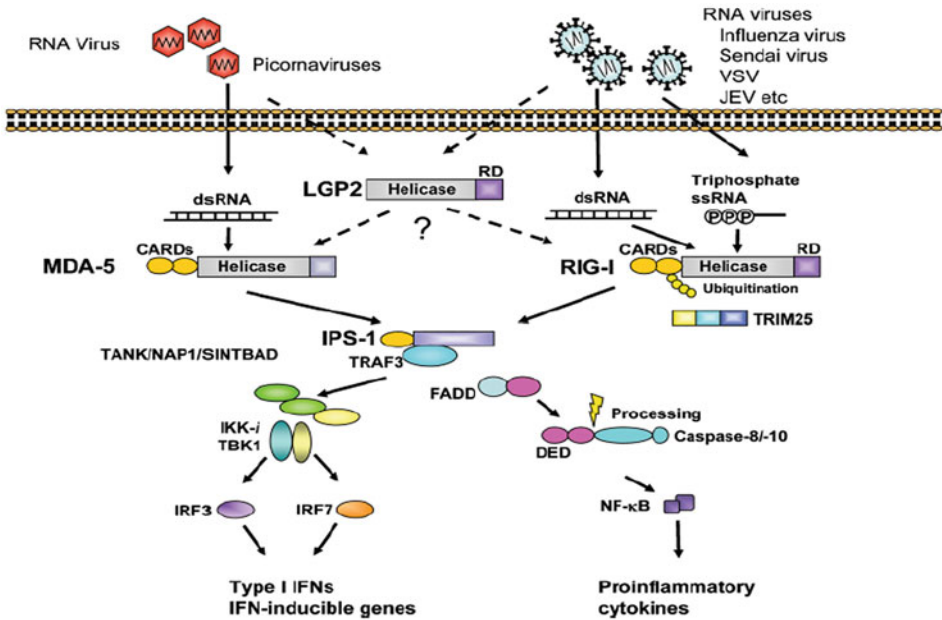
Fig. 14.2 RIG-I-like helicases mediated induction of type I IFN by virus (Takeuchi and Akira 2007)

from Atlantic salmon, encoded by distinct genes, but share around 95% amino acid sequence identity (Robertsen et al. 2003). An additional cluster of 11 IFN genes was identified in the genome of Atlantic salmon that include 2 IFNa genes, 4 IFNb genes, and 5 IFNc genes (Sun et al. 2009), with IFNd encoded by single genes outside of this cluster (Chang et al. 2009). IFNa genes (SasaIFN-α1 and SasaIFN-α2) contain NF-κB motif and are upregulated by poly I:C, likely induced through RIG-I/MDA5 or TLR3 pathway similar to the mammalian IFN-β gene. IFNb genes lack NF-κB motif and are upregulated by S-27609, likely through TLR7 pathway as mammalian IFN-α gene. IFNc genes have expression patterns different from IFNa and IFNb. Functional studies have revealed that IFNa and IFNc have similar antiviral activities and ability to induce antiviral genes, while IFNb is less active, and IFNd with no activity (Svingerud et al. 2012). There is a possibility that specialized high IFN-producing cells exist in Atlantic salmon (Svingerud et al. 2012).

14.3 Type I IFN Induction and Signaling Pathways in Fish

As already mentioned, orthologs of TLR3, TLR7/8, and TLR9 have been identified in teleost fish, suggesting that teleost fish also possess pathogen recognition mechanism comparable to those in mammals (Jault et al. 2004; Roach et al. 2005). Furthermore, functional identification of orthologous RIG-I, MDA5 and LGP2, and MAVS suggests that RLR pathway plays a key role in IFN induction and antiviral immunity in fish

(Biacchesi et al. 2009; Chang et al. 2011). Many of the important kinases (TBK1, IKKs, IRAKs), adaptors (MyD88, NEMO, TRIF), and transcription factors (IRF3, IRF7) have been characterized in different fish species (Stein et al. 2007; Skjaeveland et al. 2009). There are limited data showing the presence of type I IFN receptor in fish, but studies have shown possible receptor genes in the pufferfish and zebrafish (Stein et al. 2007; Lutfalla et al. 2003) genomes. Orthologs of most components in the JAK-STAT pathway have also been identified in fish, including STAT1, STAT2, Tyk2, Jak1, and IRF9 (Stein et al. 2007; Leu et al. 1998, 2000; Zhang and Gui 2004; Collet et al. 2009). Functional studies have been performed with STAT1 homologs from crucian carp, zebrafish, and Atlantic salmon. Salmon STAT1 has been shown to be able to translocate to the nucleus following treatment with IFN-γ (Skjesol et al. 2010).

Type I IFNs upregulate the expression of several hundred IFN-stimulated genes (ISGs) but only a few possess antiviral activity (Der et al. 1998; de Veer et al. 2001). Of the known antiviral ISGs, some of the best characterized are Mx proteins (Haller et al. 2007), protein kinase R (PKR) (Barber 2005), and the oligoadenylate synthetase (OAS) (Silverman 1994). Type I IFN also induces a form of nitric oxide synthase (iNOS2) and the MHC class I, all of which play important roles in immune responses to infections (Samuel 2001). Mx proteins are GTPases that belong to the superfamily of dynamin-like GTPases (Staeheli et al. 1993) and its antiviral activity have been demonstrated against several fish viruses including nodavirus (Chen et al. 2008), IPNV (Larsen et al. 2004) and SAV (Lester et al. 2012).

PKR activation constitutes one of the first lines of antiviral defense acting at the early stage that precedes the secretion of type I IFN (Garcia et al. 2007). PKR is constitutively expressed at basal levels in most of the mammalian tissues, but is upregulated through type I IFN signaling after virus infection. Activation of PKR however is triggered by binding to dsRNA which leads to dimerization, autophosphorylation, and phosphorylation of its substrate alpha subunit of eukaryotic initiation factor 2 (eIF2α). Phosphorylated eIF2α sequesters the guanine nucleotide exchange factor eIF2B, making it unable to recycle eIF2-GDP into the active eIF2-GTP complex (Hershey 1989). As a result, global cellular translation and virus protein synthesis is inhibited in the infected cell (Lee et al. 1996; Stojdl et al. 2000).

14.4   Salmonid Alphavirus

Pancreas disease (PD) caused by *salmon pancreas disease virus* also named salmonid alphavirus (SAV) causes infection and great economic losses in farmed Atlantic salmon, *Salmo salar* L. and rainbow trout *Oncorhynchus mykiss* in Europe. There are currently six subtypes of SAV (SAV1–6) (Fringuelli et al. 2008), with only SAV2 causing sleeping disease in trout (Villoing et al. 2000). All subtypes have been detected in Atlantic salmon with pancreas disease. Partial sequenceing of nsP3 and E2 have shown that subtypes causing PD in Atlantic salmon are subtype 1 in Scotland, subtype 2 in Scotland and

Norway, subtype 3 in Norway only (also infecting rainbow trout), subtype 4 in Ireland and Scotland, subtype 5 in Scotland, and subtype 6 in Ireland (Fringuelli et al. 2008).

Salmonid alphaviruses belong to the genus Alphavirus, a primarily arthropod-borne group of viruses within the family *Togaviridae*. SAVs are the only members that infect fish. Alphaviruses are classified into New World and Old World alphaviruses, depending on the geographical location where they were originally isolated (Strauss and Strauss 1994). Old World alphaviruses are arthritogenic viruses (Suhrbier et al. 2012) and include Semliki Forest virus (SFV), Sindbis virus (SINV), Chikungunya virus (CHIKV), O'Nyong-Nyong virus (ONNV), and Ross River virus (RRV) which typically cause fever, rash, and arthralgia. New World alphaviruses typically cause encephalitis and include Venezuelan Equine Encephalitis virus (VEEV), Eastern Equine Encephalitis virus (EEEV), and Western Equine Encephalitis virus (WEEV) (Ryman and Klimstra 2008).

The alphavirus RNA genome is about 11–12 kb, single-stranded, positive-sense, 5′ capped, and 3′ polyadenylated. The genome is divided into two open reading frames (ORFs) that encode the non-structural (nsPs) and structural proteins, respectively, separated by the promoter for the 26 s subgenomic mRNA. The 5′ two-thirds of the genome encode nsP1, nsP2, nsP3, and nsP4 that are synthesized as a precursor polyprotein which undergo proteolytic cleavage. The 3′ third of the genome, corresponding with viral 26S sub-genomic mRNA, encodes a structural polyprotein which ultimately produces five structural proteins, capsid (C), E3, E2, 6K, and E1 (Simmons and Strauss 1972; Strauss et al. 1984), Fig. 14.3).

Structurally alphaviruses contain an icosahedral nucleocapsid enclosed in a tight-fitting envelope where the glycoproteins E1 and E2 are arranged on an icosahedral lattice. E1 and E2 associate as heterodimers and are further assembled into trimers to form spike protrusions. There are 240 heterodimers on the surface of the virus, assembled into 80 spikes (Vogel et al. 1986; Rice and Strauss 1982; Wahlberg et al. 1989). The C-terminal cytoplasmic region of E1 and E2 transmembrane proteins is thought to interact with the nucleocapsid (Owen and Kuhn 1997; Zhao et al. 1994). Alphaviruses replicate in the cytoplasm and buds from the plasma membrane of infected cells (McLoughlin and Graham 2007). The nucleotide and amino acid sequences of SAV subtypes are very conserved, and the amino acid identity between SAV1 and SAV2 reference strain is 95% and 93.6% over their non-structural and structural proteins, respectively, and shows low homology (30 and 40%) with other alphaviruses. SAV3 has a nucleotide sequence homology with SAV1 and SAV2 of about 91.6% and 92.9%, respectively (Hodneland et al. 2005). SAVs differ structurally from other alphaviruses by having larger individual structural and non-structural proteins, non-glycosylated E3 glycoprotein, and short 5′ and 3′ untranslated regions. Phylogenetic comparisons indicate the segregation of SAV from the remainder of the genus, forming a cluster distinct from previously recognized geno-complexes (Weston et al. 2002; Powers et al. 2001).

Histopathological changes induced by variants of SAV include different degrees of lesions in exocrine pancreas, heart, and skeletal muscle. The classical pancreatic lesions include acute necrosis of the exocrine pancreas followed by chronic fibroplasia or
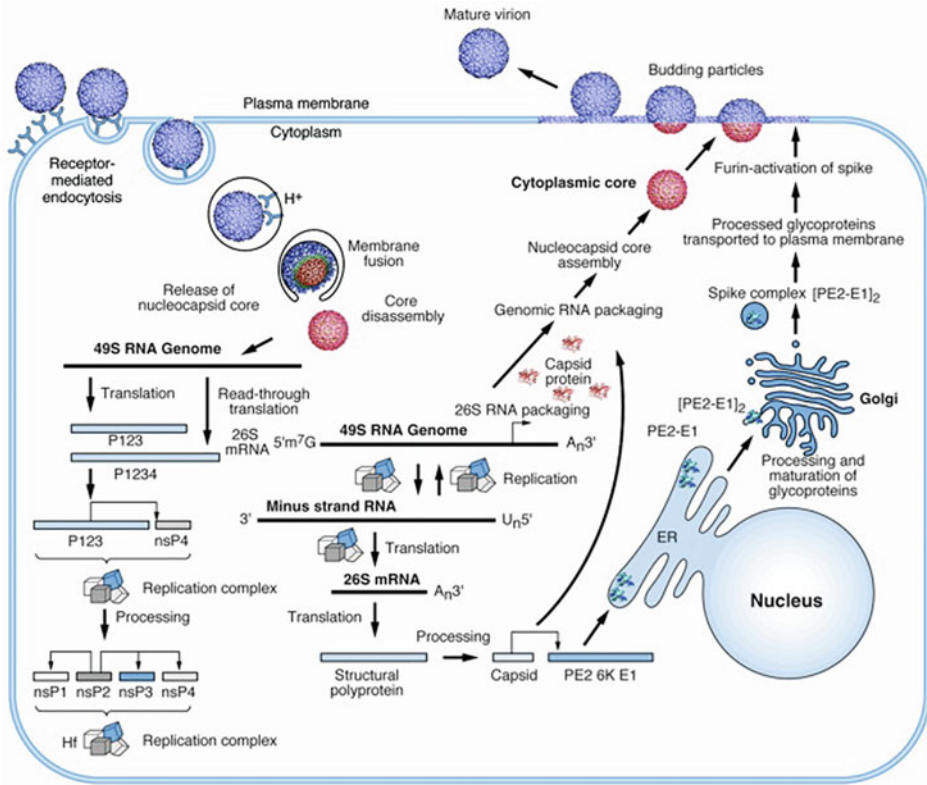
Fig. 14.3 Alphavirus life cycle (Fields Virology, ed. 6, Chapter 22, Togaviridae, reproduced with permission)

regeneration in recovering fish (Murphy et al. 1992; McLoughlin et al. 2002). Heart lesions are observed concurrently with or developing after acute pancreatic lesions (Xu et al. 2012), which include focal to diffuse cardiomyocytic degeneration and necrosis in spongy and compact layers of the ventricle, in addition to inflammatory changes (McLoughlin et al. 2002; Ferguson et al. 1986). Skeletal muscle lesions usually appear 3–4 weeks after the onset of pancreatic and heart lesions, and are characterized by hyaline degeneration, a variable degree of inflammation and fibrosis in both red and white skeletal muscles (Ferguson et al. 1986; Murphy et al. 1992; McLoughlin et al. 2002).

14.4.1 Pathogenesis of Alphavirus-Induced Diseases

Alphaviruses are spread by hematophagous arthropod vectors, typically a mosquito. For salmonid alphaviruses, sea lice may act as a reservoir or vector as there is some evidence that they can carry the virus (Petterson et al. 2009) but sea lice likely play little role in viral

spread. However, the sealice immunemodulation of host responses may facilitate establishment of infection and virus replication (Gamil et al. 2020). In mammals, the alphavirus-induced pathogenic sequence begins with deposition of virus in the subcutaneous tissues, replication at the site of infection, and transport to the draining lymph nodes (DLN) before migration and systemic dissemination. Dendritic cells (DCs) and macrophages in the DLN are targeted by alphaviruses initially after introduction, their resistance or susceptibility to infection play important roles in the pathogenesis and severity of alphavirus-induced diseases (Ryman and Klimstra 2008). The importance of interferon I in protection against infection is seen from studies in IFN-α/β-receptor-deficient mice where the frequency of Sindbis virus-positive cells in the DLN increases dramatically, suggesting that type I IFN-induced antiviral pathways are important determinants for permissibility of macrophages and/or DC upon their first encounter with the virus (Ryman et al. 2002). In the absence of IFN-α/β signaling, mice succumb rapidly to fatal Sindbis virus infection, as a result of systemic infection of the sentinel cells of the immune system, macrophages, and DC (Ryman et al. 2002). The ability to infect target organs is dependent on viral invasiveness and duration and height of viremia (Sherman and Griffin 1990; Griffin 1989). Skin is the target for alphaviruses that cause rash, the joints for those that cause arthritis (arthritogenic), and the nervous system for those that cause encephalitis (neurotropic) (Tesh 1982; de la Monte et al. 1985). SAV may infect fish via gill or intestine, virus can be detected in different organs including gill, brain, pancreas, heart, kidney, and skeletal muscle after infection (McLoughlin and Graham 2007). However, the exact transmission route and target cells for the virus are not yet known.

E1 and E2 glycoproteins have been identified as important determinants of virulence (Davis et al. 1986; Polo et al. 1988) for alphaviruses infecting higher vertebrates, but little is known for the piscine alphaviruses. E2 is the major attachment protein and is presumed to start interaction with target cells. This presumption is supported by the fact that amino acid changes in E2 can influence virulence (Tucker and Griffin 1991). Four amino acids in E1 have been identified to attribute to differing virulence of Sindbis virus (SV) strains (Lustig et al. 1988; Polo and Johnston 1990), but the mechanism related to virulence is yet to be illustrated. Amino acid variants in E2 have been linked to differences in virulence for salmonid alphavirus as well (Merour et al. 2013) but the underlying mechanisms are not known.

14.4.2 Innate Immune Response to Mammalian Alphavirus Infection

The type I (α/β) IFN antiviral pathway is the primary innate immune response protecting animals from fatal alphavirus infection (Ryman et al. 2000; Muller et al. 1994; Grieder and Vogel 1999). After alphavirus infection, viral RNA is recognized by endosomal TLRs 3, 7, and 8 as well as the cytoplasmic RNA sensors MDA-5, and/or RIG-I, which activates IRF-3/7-dependent signaling pathways to induce type I IFN production (Knudsen et al. 2013; Pichlmair et al. 2009b). The primary source of type I IFN after peripheral infection is likely to be macrophages or DCs in the lymphatic tissue (Ryman et al. 2002; Hidmark et al.

2005; Pichlmair et al. 2009a). In vitro, pre-treatment of cells with type I IFN strongly inhibits alphavirus replication although with differing sensitivity depending on virus species studied. Timing of interferon induction or stimulation at early times post infections is important for the outcome of the virus infection (Frolov et al. 2012). Nevertheless, the exact mechanism by which the IFN-induced host responses control viral replication is not well understood. However, the IFN-induced proteins shown to influence alphavirus replication are Mx and PKR.

In vitro, PKR exerts an early antiviral effect on the alphavirus replication, the presence of PKR promotes IFN-α/β induction, delays viral protein synthesis, and reduces the yield of infectious virus. PKR suppressed virus replication approximately tenfold in DCs before the initiation of IFN responses (Ryman et al. 2002; Barry et al. 2009). However, at a later infection stage, SV virion production was reduced in the absence of PKR by a type I IFN receptor-dependent mechanism (Ryman et al. 2002). Furthermore, IFN-α/β priming protected PKR knockout DC as potently as in wild-type cultures (Ryman et al. 2002, 2005). In vivo, Sindbis virus infection of mice deficient in PKR developed only subclinical disease whereas infection of mice deficient in IFNAR1 was rapidly fatal. Taken together, these results indicate that the IFN-α/β-mediated antiviral responses are essentially intact in the absence of PKR; and systemic dissemination of virus is restricted by an alternative IFNAR-dependent mechanism (Ryman et al. 2002).

Pre-treatment of bone marrow-derived DCs with IFN-α/β exerted a strong antiviral effect at very early stages of Sindbis virus replication and most likely inhibited the initial translation of infecting genomes. This mechanism effectively suppresses the production of nsPs and blocks the initial replication of the genome (Ryman et al. 2005). This translation inhibiting activity specifically targets cap-dependent translation initiation and is independent of PKR and eIF2-α phosphorylation. These PKR-independent antiviral pathway mediators are transcriptionally regulated via IFN-α/β signal transduction, as inhibition of host gene transcription by actinomycin D, which prevents ISGs induction, restores the virus replication and progeny virions production, indicating ISGs other than PKR are involved in IFN-induced early antiviral response to alphavirus infection.

14.4.3 Alphavirus counteracts Type I IFN Response

One of the main mechanisms exploited by alphavirus to counteract type I IFN response is suppression of host macromolecular synthesis, thus limiting induction of type I IFN and ISG (Ryman and Klimstra 2008). We have shown that salmon type I IFN has a strong inhibitory effect on SAV3 replication in vitro (Xu et al. 2010) (Fig. 14.4), in conformity with what has been shown previously for other alphaviruses in mammalian studies (Zhang et al. 2007; Grieder and Vogel 1999; Aguilar et al. 2005). In addition, we have also demonstrated inhibition of protein synthesis following SAV3 infection (Xu et al. 2010) similar to mammalian alphaviruses (Vernon and Griffin 2005). While non-structural proteins have been implicated in the protein shut down for mammalian alphaviruses, it has not been decided which viral proteins of SAV3 play a key role for downregulation of
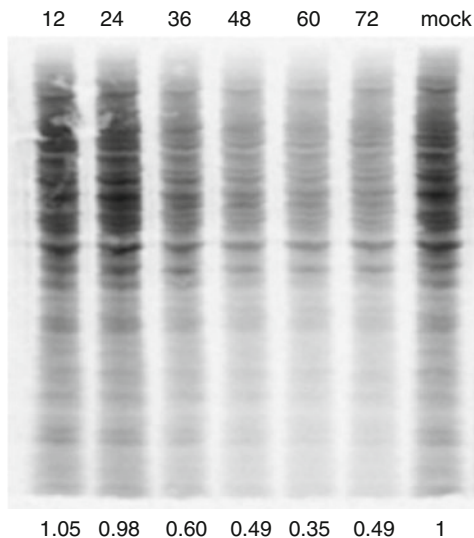
Fig. 14.4 Residual protein synthesis in TO cells infected with SAV3 (Xu et al. 2010). Confluent TO cells were grown in a six-well plate and infected as described in Materials and Methods. The membrane was exposed in a PhosphorImager cassette and then scanned by using a Typhoon imager. Numbers above the figure represent hours post infection. The protein amount was quantified by densitometry with ImageJ software. The results show 40, 51, and 65% reduction by 36, 48, and 60 h post infection, respectively. Mock infection is shown at the far right. Copyright© American Society for Microbiology, Journal of Virology, 84, 2010, 8903–12, https://doi.org/10.1128/JVI.00851-10 (Xu et al. 2010)

protein synthesis. Moreover, we have found that recombinant IFN-γ (from salmon) has marginal inhibitory effect on SAV3 replication, in contrast to others (Sun et al. 2011). The observed difference could be that we used IFN-γ of salmon origin while the study by Sun et al. (Sun et al. 2011) included IFN-γ from rainbow trout. The difference between the two sequences is 10% and such difference might affect biological functions.

We have also studied the induction of antiviral responses by interferon treatment and virus infection. We found that pre-treatment with rIFN-α before infection results in strong ISG induction (Xu et al. 2010). We have also shown that Mx, ISG-15, Jak1, STAT1 and 2 as well as IRFs 1, 2, 3, and 7B are upregulated in infected cells and also in rIFN-α-treated cells (Xu et al. 2010, 2015) (Fig. 14.5). Similar findings were also reported in vivo where infected fish exhibited upregulation of Mx and ISG-15 following experimental infection with SAV3 (Xu et al. 2012). Upregulation of some important sensors of virus infection including RIG-I, NLR-5, MAD-5, and LGP-2 was observed in rIFN-α treated as well as SAV3-infected cells (Xu et al. 2015). The latter finding points toward important roles for these sensors during SAV3 infection.

Pretreatment of permissive cells gave an antiviral state that protected against CPE but was dependent on time of treatment relative to infection, and a minimum of 4 h was
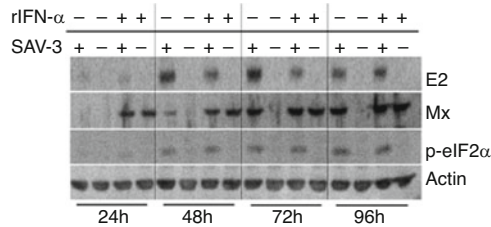
Fig. 14.5 Protein analysis of E2, Mx, and p-eIF2α in virus-infected cells. TO cells were infected with SAV3 (MOI of 1) or left uninfected, and at 24, 48, 72, and 96 h post infection, uninfected (−) and infected (+) cells were treated with 0.47 μg/ml of rIFN-α for 16 h or left untreated. Cells were lysed by using CelLytic M reagent (Sigma) and scraped from the dish. The expression of the E2, Mx, p-eIF2-α, and actin proteins was detected by Western blotting. Copyright© American Society for Microbiology, Journal of Virology, 84, 2010, 8903–12, https://doi.org/10.1128/JVI.00851-10 (Xu et al. 2010)

required for the cells to be protected against CPE (Fig. 14.6). Treatment after 2 h prior to infection gave no protection against CPE, although there was a reduction in viral copy numbers. These findings strongly indicate SAV-3 has developed mechanisms to overcome/circumvent the type I IFN response. These results also suggest that the first few hours of alphavirus replication play a key role in determining the outcome of the infection as observed for some mammalian alphaviruses (Frolov et al. 2012). It is not unlikely that alphavirus use both non-specific shutoff of host macromolecular synthesis and specific mechanisms, such as inhibition of STAT1 phosphorylation, to counteract type I IFN mediated responses. One interesting observation was that translation of the E2 protein occurred even when eIF2α was strongly phosphorylated (Xu et al. 2010) suggesting that SAV-3 is able to counteract the antiviral effect of PKR by translational resistance of 26 s sub-genomic mRNA to eIF2α phosphorylation. Put together, it seems that protein translation from genomic viral RNA (incoming positive strand) is sensitive to IFN-α-induced responses. At the same time, it seems that the synthesis of spike mRNA driven by the 26S promoter is resistant to IFN-α-induced cellular responses. It has been proposed that presence of a prominent RNA structure referred to as a downstream loop (DLP) located in viral 26S transcripts allows an eIF2-independent translation initiation of the mRNA, and while such DLPs have been found in several alphavirus species they have not been shown in SAV variants (Ventoso 2012) or Chikungunya virus (CHIKV), Western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV) (Ventoso 2012). There are at least two possible explanations for these findings. Firstly, SAV, CHIKV, WEEV, and VEEV might have an alternative mechanism to counteract PKR activation in infected cells. Secondly, a DLP structure could involve two nonadjacent RNA sequences which then fold to form a functional DLP that folding programs are (currently) unable to detect (Ventoso 2012). Thus, further studies are needed to elucidate the mechanisms used by SAV-3 (and other alphaviruses) to circumvent PKR/eIF2 phosphorylation.
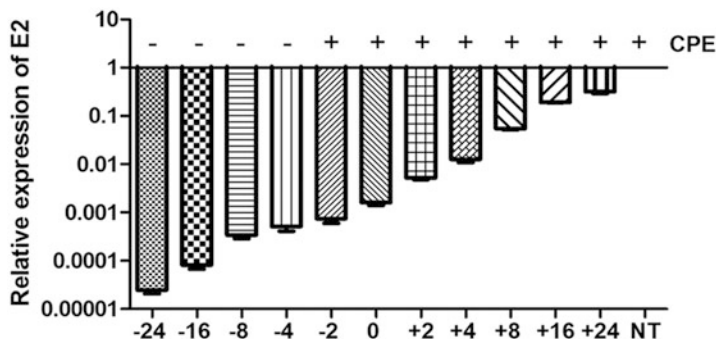
Fig. 14.6 Timing of IFN-α treatment of SAV3-infected cells (Xu et al. 2010). Treatment 4–24 h prior to infection results in a marked reduction in virus replication (2000- to 40,000-fold reduction) as measured by real-time PCR and protection against CPE. rIFN-α treatment at the time of infection or up to 24 h postinfection gives a reduction in virus replication but does not give protection against CPE. TO cells were infected with an MOI of 1, and collection was done at 4 days post infection. Copyright© American Society for Microbiology, Journal of Virology, 84, 2010, 8903–12, https://doi.org/10.1128/JVI.00851-10 (Xu et al. 2010)

Studies have shown that alphavirus replication occurs in the cytoplasm and results in the production of RNA intermediates that are recognized by and engage TLRs including TLR3 (Schulz et al. 2005), TLR7 (Neighbours et al. 2012), and TLR8 (Pichlmair and Sousa 2007). MDA5 and PKR but not RIG-I are involved in recognizing alphavirus RNA for induction of type I IFN (Burke et al. 2009). In higher vertebrates, it appears that normal cells rely mainly on recognition of viral dsRNA or 5′-triphosphate ssRNA through RIG-I/MDA5 for induction of type I IFN, and myeloid cells utilize TLR3 for sensing viral dsRNA, and/or TLR7/8 sensing viral ssRNA to trigger the response (Melchjorsen et al. 2005). If the same mechanisms are found in salmonids is not known, but we have shown that TLR3, TLR8, and RLRs (MDA5, RIG-I, and LGP2) were significantly upregulated after SAV-3 infection in a salmon cell line (TO), in addition to PKR and NLR5 (Xu et al. 2015) in line with what others have found (Nerbovik et al. 2017). Therefore, different PRRs are involved in sensing SAV infection and induction of type I IFN and other proinflammatory cytokines in Atlantic salmon macrophages.

14.4.4 Mechanisms of Type I IFN-Mediated Protection Against SAV Infection

Antiviral effectors are essential for protection against viral infection. Mx proteins interfere with virus replication by inhibiting the activity or trafficking of virus polymerases (Stranden et al. 1993), thereby impairing the replication of a wide range of RNA viruses at the level of virus transcription and at other levels of the virus life cycle. PKR inhibits mRNA translation resulting in preventing viral protein synthesis through phosphorylation

of eukaryotic initiation factor 2 alpha (Garcia et al. 2007). ISG15 exerts its antiviral activity by conjugating to target viral proteins and thus affect their function (Skaug and Chen 2010). Viperin interferes with budding and release of virus particles by disrupting the endoplasmic reticulum transport system that translocates virus particles to the plasma membrane (Wang et al. 2007). We have found that antiviral effectors like PKR, Mx, ISG15, and Viperin were all significantly upregulated after SAV-3 infection and type I IFN treatment, indicating that different antiviral mechanisms with combinatorial antiviral effect play a role in protection against SAV-3 infection at different virus replication stages. Further, we have seen that TRIM 16, 21, 25, and 39 were upregulated after SAV-3 infection in TO cells and it is not unlikely that they play a role in the antiviral effects in infected cells (Xu et al. 2015), including participating in the induction of IFN synthesis. For example, it has been shown that TRIM25 is involved in the synthesis of IFN-β through the RIG-I pathway (Gack et al. 2007), and it is possible that it plays a similar role in fish cells. However, further studies are needed to elucidate the mechanisms exploited by TRIMs in restricting SAV-3 replication in fish cells.

The IFIT family genes (interferon-induced proteins with tetratricopeptide repeats) are induced by type I IFN treatment and virus infection, and specifically recognize viral RNA bearing 5′-triphosphate (5′-ppp) groups, thereby acting as a sensor for viral ssRNAs (Zhou et al. 2013; Bela-Ong et al. 2020). We have found that both IFIT1 and IFIT5 were strongly upregulated after SAV-3 infection and type I IFN treatment (Xu et al. 2015), and it is not unlikely that they bind to the 5′-ppp-RNA of SAV-3 to exert direct inactivation of the virus RNA as well as exert a nonspecific antiviral effect through translational regulation mediated by eIF3.

Recently, there are studies on miRNAs that potentially play a role in SAV-3 infection in salmon (Andreassen et al. 2017), in response to SAV infection. It has been discussed if these miRNAs are involved in prohibiting harmful inflammatory responses and/or promote early immune responses. Additional studies are needed to understand underlying mechanisms.

14.4.5 SAV Circumvents Type I IFN System

In vivo studies have shown increasing severity of pathology in target organs coinciding with high viral loads despite significant induction of IFN-α and ISGs after SAV-3 infection in Atlantic salmon (Xu et al. 2012). In concert with these observations, we found that when TO cells treated with rIFN-α 2 h prior to infection (or later), virus replication is not efficiently inhibited and cells were not protected against virus-induced CPE (Xu et al. 2010). These findings indicate that SAV has developed mechanisms to circumvent the type I IFN-induced antiviral response. One of the main mechanisms exploited by alphavirus to counteract type I IFN response is by suppression of host macromolecular synthesis, thus limiting the production of type I IFN and ISGs (Ryman and Klimstra 2008). In vivo studies in salmon experimentally infected with SAV-1 showed that several cellular transcription

and translation associated genes were differentially expressed after experimental infection. This finding suggests that inducing transcriptional shut-off maybe one of the mechanisms employed to counteract the antiviral responses as seen for mammalian alphavirus infection (Herath et al. 2012). In our studies, we could not document protein shutoff in SAV-3-infected cells, but rather a downregulation (shutdown) (Xu et al. 2010), which likely partially attenuates the effect of IFN responses and contribute to IFN resistance. This is more in line with New World alphaviruses that result in a more moderate downregulation of protein synthesis (Garmashova et al. 2007). It is noteworthy that the similarity and differences in the interplay between the different SAV subtypes have not been addressed and whether different strategies are employed to counteract interferon responses is therefore not known.

14.4.6 Presence of Defective Viral Genomes of SAV Triggers Type I IFN Responses

SAV3 has recently, through both in vitro and in vivo experiments, been shown to be able to recombine the viral genome (Guo et al. 2014; Petterson et al. 2016). Such recombination might affect the evolution of the virus as new variants with increased or reduced virulence might emerge. It has been shown, however, that such recombination is imprecise and might result in presence of SAV-specific defective RNA with various deleted regions when compared to a full-length genome (Petterson et al. 2013; Gallagher et al. 2020). Virus-specific RNA with presence of deletions of various sizes, mutations, or other changes that render the RNA non-replicative is a well-known phenomenon of nearly every virus family and is often referred to as defective viral genomes (DVG) or if confirmed as interfering with replication, defective interfering particles (DIP) (Manzoni and López 2018). This is based on confirmed or expected characteristics of the defect RNA, interfering with the viral replication as it propagates and accumulates when it infects the cells at high concentrations and attenuates the virus and/or its replication.

Several studies on, e.g., virus in plants and fungus and also mammals indicate that the presence of DVGs could act as a virulence factor and a major determinant of pathogenesis and disease outcome as reviewed by Rezelj and coworkers (Rezelj et al. 2018). The mortality and pathogenesis of PD caused by SAV3 varies over the whole range from low to severe. For SAV2-caused PD it is in general low, although high mortality has been reported. As described above, no convincing virulence motifs have been found in the SAV genomes and it is still unknown why there are such diverse outcomes between separate SAV3 infections or between infections with SAV3 versus SAV2.

Studies on other viruses have shown that DVGs or DIPs can act as primary triggers of innate immunity and increase the effect of the host antiviral response, e.g., as pathogen-associated molecular patterns (PAMPs) are preserved in the defective RNA, which then is also recognized by the pattern recognition receptors (PRRs) (reviewed in (Rezelj et al. 2018). Such DVGs may then act as strong inducers of IFN, as previously shown for

alphaviruses (Fuller and Marcus 1980). The presence of DVGs shown for SAV may relate to the specific importance of IFNs shown against SAV infection. Viral strategies where DVGs or DIPs are acting as immune decoys to subvert the host surveillance mechanisms have not been explored so far. In general, underlying mechanisms of the possibility of DVGs or DIPs to exacerbate symptoms of the disease at infection remains poorly understood.

14.5   Infectious Pancreatic Necrosis Virus

14.5.1 Structure and Genome Organization

The aquatic birnaviruses are a member of the family *Birnaviridae* classified under the genus Aquabirnavirus. IPNV is a non-enveloped, double-stranded, icosahedral RNA virus, ≈60 nm in diameter (Villanueva et al. 2004). Viral proteins are encoded for by two genomic segments A and B (Dobos 1995), where segment A comprises two open reading frames (ORFs), a long one encoding a 107KDa polyprotein that is post-translationally cleaved to give structural virus proteins VP2 and VP3 as well as the non-structural protein VP4 (Duncan and Dobos 1986; Duncan et al. 1987; Macdonald and Dobos 1981; Magyar and Dobos 1994), Fig. 14.7). The other ORF, which is short and overlaps the first ORF, encodes a non-structural protein, VP5, only detected in infected cells/cell lysates but not in purified viruses, i.e., it is not part of the virus particle (Heppell et al. 1995; Magyar and Dobos 1994). Segment B consists of a single ORF that encodes the virus polymerase, VP1 (Duncan et al. 1991).
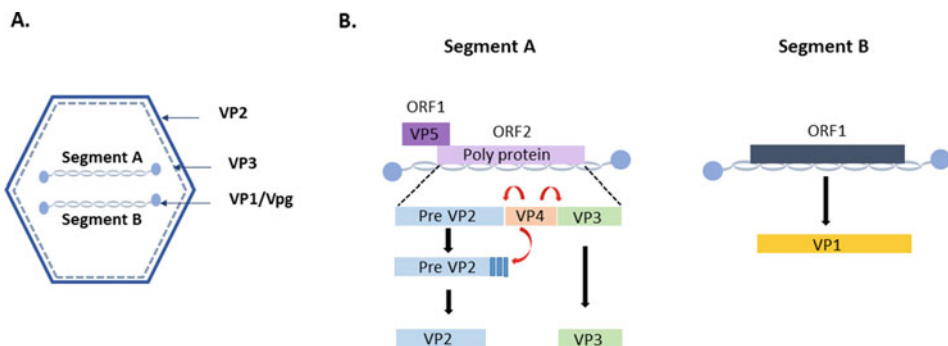
Fig. 14.7 IPNV structure and genome organization; modified from (Delmas et al. 2012). A. Schematic presentation of the virion structure. B. The genome organization of segment A and B, their encoded proteins and the cleavage sites in segment A are illustrated. Illustration Amr Gamil

14.5.2 Disease Caused, Host Range, and Classification

Aquatic birnaviruses have been isolated from many aquatic vertebrate and invertebrate species inhabiting fresh, brackish, and seawater (Reno 1999). The type species is infectious pancreatic necrosis virus (IPNV), the causative agent of infectious pancreatic necrosis (IPN) in salmonid fish (Delmas et al. 2012; Reno 1999). IPN has been known since the 1920s as "whirling sickness" that gave clinical disease in trout fingerlings, while the first proper publication dates back to 1941 and the name suggested then was "acute catarrhal enteritis" (M'Gonigle 1941). The virus was isolated and first described in 1960 (Wolf et al. 1960).

IPN is a disease of salmonid fish and is the most economically significant disease caused by aquatic birnaviruses, it was suggested that isolates causing overt IPN disease in salmonids are called or referred to as IPNV while the rest of the isolates are to be called aquabirnaviruses (Rimstad 2003; Reno 1999). The disease affects all age groups, but susceptibility varies (Frantsi and Savan 1971; Wolf and Quimby 1971). Marine aquabirnaviruses (MABV) have been used to describe viruses isolated from non-salmonid aquatic vertebrate and invertebrate species, particularly in Asia (Hosono et al. 1994; Kusuda et al. 1993), and include Yellowtail Ascites Virus (YTAV), first isolated from yellowtail in Japan (Sorimachi and Hara 1985) and Tellina virus (TV) (Underwood et al. 1977), initially isolated from mollusks.

14.5.3 Replication of IPNV

IPNV replicates in a variety of cell lines from teleost fishes at temperatures below 24 °C (Dobos 1995), an intrinsic property of the virus (Dobos 1995). One replication cycle in RTG-2 cells is completed within 16 h at 24 °C and by 24 h at 15 °C when cells are infected with 6.7 and 8.5 MOI, respectively (Malsberger and Cerini 1965). Replication rate is dependent on temperature as mentioned but also MOI (MacDonald and Kennedy 1979). The RdRp is virion-associated and proteolytic pretreatment is not needed for the RpRp to be active (Cohen 1975). Uncoating is not necessary for initiation of transcription and, following virus entry, the initial transcription occurs inside the core-like particle (Shatkin and LaFiandra 1972) with the negative strand as a template for mRNA synthesis. This renders the viral genome unexposed to cytosolic, innate sensors. These initial steps are crucial for keeping the dsRNA unexposed to the innate immune sensors and will allow time for initial steps of the virus replication cycle.

14.5.4 Innate Immune Responses to IPNV Infection

Many aspects of innate immune responses to IPNV infection are not known in detail. Interferon responses and apoptosis are probably the best two studied innate responses to
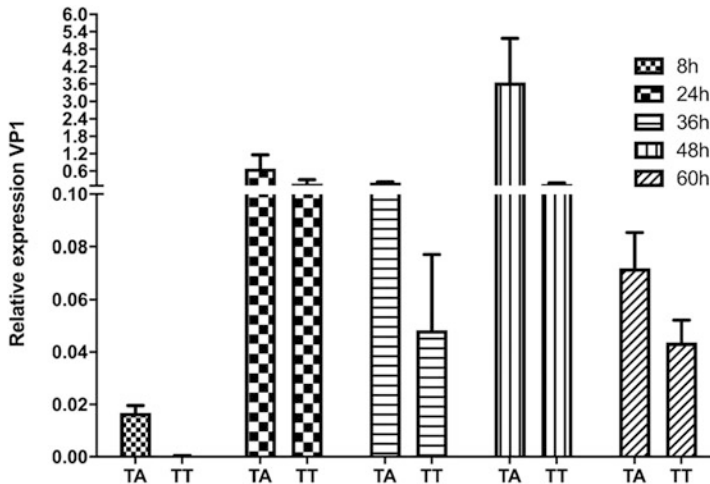
Fig. 14.8 Recombinant IFN-α1 effect on viral replication of TA and TT-infected TO cells. Viral replication levels (VP1 expression) at different time post infection (hours post infection; hpi) of TO cells, pretreated (24 h) with recombinant IFN-α1. The TA strain shows earlier increase of replication levels (24 hpi) and peak at 48 hpi at 23-fold higher replication levels than the TT-strain at this time point. $N = 3$–4 replicates per time point. From Gadan et al. (2013)

IPNV. Pretreatment with IFN-α (Robertsen et al. 2003; Skjesol et al. 2009) and overexpression of Mx protein (Larsen et al. 2004; Lester et al. 2012) result in inhibition of virus replication, suggesting that type I IFN responses are important in controlling IPNV replication. However, some virus strains are able to replicate under high levels of interferon and Mx gene expression both in vivo and in vitro (Gadan et al. 2013; Munang'andu et al. 2013; Saint-Jean and Perez-Prieto 2006), and strain virulence for Sp serotypes of IPNV seems to impact on or reflect the virus sensitivity to IFN-α. We have found that virulent strains of IPNV, recognized by two amino acid residues ($T_{217}A_{221}$) in the VP2 protein, are less sensitive to IFN-α than low virulent ($T_{217}T_{221}$) strains (Fig. 14.8). These findings indicate that some IPNV strains are able to counteract or a refractory to type I IFN responses and are supported by reports showing that concentrations as high as 200 U/ml of type I IFNs do not block but only able to reduce virus replication and prevent CPE (Svingerud et al. 2012).

### 14.5.5 The Interplay Between IPNV Infection and IFN Response

Different in vitro and in vivo studies have reported the suppression type I IFN signaling and VP4 and VP5 proteins (Collet et al. 2007; Skjesol et al. 2009) as well as all structural proteins (Lauksund et al. 2015) have been implicated in these responses, but the details are unknown. The mechanisms involved seem to be complex as the suppressive effect may

differ between in vivo and in vitro systems. One clear example is the induction of the IFN and Mx expression in the ovary during IPNV infection, which are suppressed in vivo and stimulated in the in vitro ovary cultures (Chaves-Pozo et al. 2010). Differences have also been observed in the ability of susceptible and resistant strains of Atlantic to mount interferon responses following IPNV infection, with higher responses observed in the susceptible population (Robledo et al. 2016). Further, the interplay between IPNV and the type I IFN differs between cell lines obtained from different species (Collet et al. 2007; Saint-Jean and Perez-Prieto 2006). In addition, IPNV isolates of different serotypes like the E1S isolate (Ab serotype) and the North American VR299 strain induce higher gene expression levels of type I IFN than European Sp isolates, while the latter result in higher TNFα mRNA expression (Gamil et al. 2015). Both cytokines are potent inducers of inflammation. While inflammatory responses usually are considered to be of benefit to the host, cytokine storms can promote pathology and be detrimental to the host (Vogel et al. 2014). The implication of these two cytokines, and others, in inducing or protecting the infected host from pathology or infection during IPN disease is not known and should be subject to more detailed studies.

14.5.6 Protein Shutdown

IPNV-infected cells respond with a global inhibition of protein synthesis. The response is not cell type or MOI-specific and has been observed in AGK, RTG-2 (Chen et al. 2014), and TO cells (Fig. 14.9). The response involves eIF2α phosphorylation induced through PKR activation. An interesting finding has been that while there is global shutdown of the cellular protein synthesis, virus replication and virus protein synthesis are not affected

Fig. 14.9 Global protein synthesis in TO cells following IPNV infection. Cells were infected with 1 MOI and labeled with S35 30 min prior to sampling. Numbers represent time in hours post infection. Mock = uninfected cells (own studies)
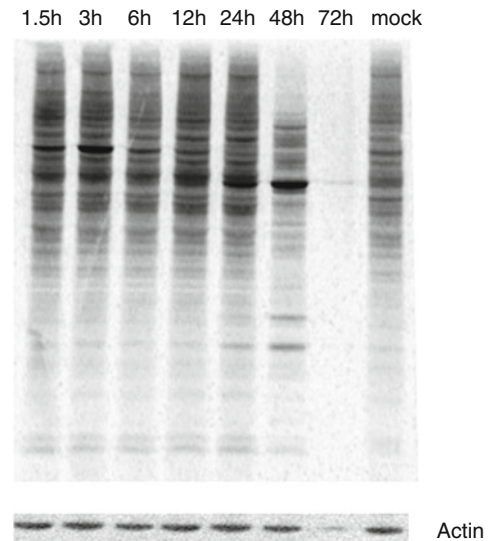
(Gamil et al. 2016) and the indications have been that IPNV benefits from eIF2α phosphorylation rather than being negatively impacted, probably by inhibiting the production of antiviral effectors including IFN. These findings would raise a question if IPNV is resistant to eIF2α phosphorylation. We still do not know the underlying mechanisms employed by the virus to translate its protein when the translation machinery in the cell is shutdown. In a recent study, however, an IRES was identified in the 5′-UTR and was shown to function in a temperature-dependent manner (Rivas-Aravena et al. 2017). This finding suggests that IPNV uses a cap-independent strategy for protein translation which explains the resistance of IPNV protein synthesis to the translational shutdown.

The involvement of PKR in the observed cellular responses during IPNV infection is also interesting. PKR is constitutively expressed in cells and upregulated by increased type I IFN levels. Studying PKR activation is difficult in fish/salmon since there are few antibodies available. We have found that inhibition of PKR has negative impact on virus replication observed as reduced virus yields. This effect is concomitant with decreased eIF2α phosphorylation (Gamil et al. 2016). We have suggested that the translation of virus proteins may be controlled by uORF encoding VP5, enhanced when eIF2α is phosphorylated. This is still a hypothetical approach and requires further investigation.

14.5.7 Apoptosis Responses

The apoptosis response in the IPNV-infected cell is still poorly understood. Some studies reported atypical apoptosis preceding the necrotic changes (Hong et al. 1998) while others found that infected cells are mostly necrotic (Espinoza et al. 2005). These seemingly conflicting findings were obtained using different IPNV isolates, and different assessment methods and direct comparison are therefore difficult. We have observed differences in the ability of different IPNV isolated to induce apoptosis with the marine isolates inducing higher apoptosis. This may point toward intrinsic properties that allow the marine isolate to induce more rapid and pronounced apoptosis (Gamil et al. 2015). However, these isolates are adapted to growth at a relatively higher temperature which may have contributed to the differences observed. From our studies, we found that necrosis precedes apoptosis in infected cells in vitro and appears to be the main mode of cell death induced following IPNV infection. We have suggested that the timing of occurrence of the two cell death pathways may reflect a fight for survival between the virus and the host cell with the necrotic changes induced by the virus for release while apoptosis is induced by the cells to limit virus spread. Nevertheless, the role of apoptosis as a protective mechanism is still not well understood, and it is not clear whether the virus plays an active role in delaying apoptosis or not. Moreover, the signaling mechanisms involved are still not understood although involvement of TNF alpha and bad/bid-mediated apoptotic pathway was suggested (Wang et al. 2011). Further, the interferon-regulatory factor-1 was found to be involved in the induction of phosphatidylserine receptor in response to IPNV infection indicating its possible participation in inducing both IFN and apoptotic pathways (Kung et al. 2014).
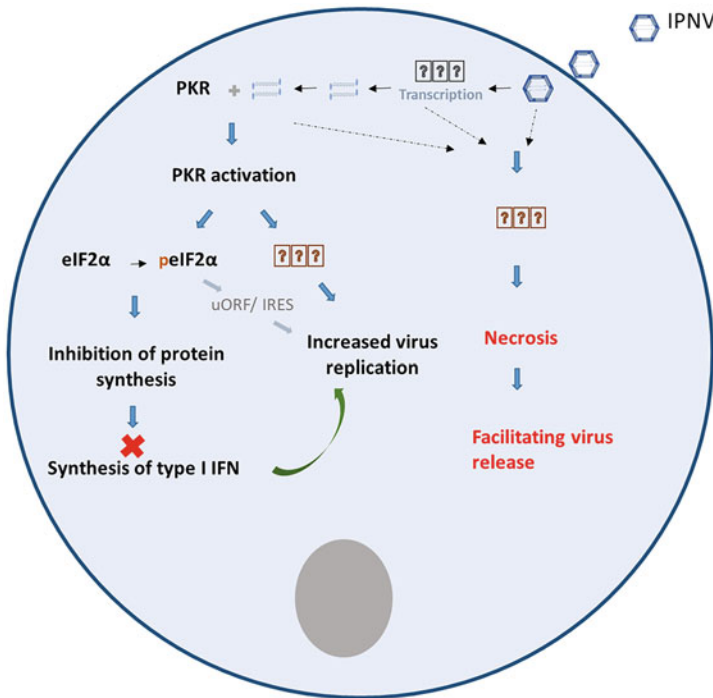
Fig. 14.10 Suggested model for IPNV interaction with host cell protein. After attachment and entry of the virus into the cell, the virus nucleic acid is transcribed and dsRNA is produced. The produced dsRNA is then recognized by PKR in the cytoplasm of infected cells. The interaction between the virus nucleic acids and PKR leads to PKR activation which benefits virus replication in two ways: (1) Phosphorylation of eIF2α and subsequent inhibition of protein synthesis that blocks the production of type I IFN and its effector proteins. There is also a possibility that the translation of virus proteins is enhanced under eIF2α phosphorylation due to the presence of uORF and/or IRES. (2) Unknown downstream signaling that facilitates virus replication. IPNV infection also results in necrosis that helps virus spread but neither the initiation nor the execution mechanism is known. Own drawing (Amr Gamil)

Overall, many aspects of the interplay between IPNV and the host cell responses are not clearly understood. An illustration of the sequence of events, known and unknown, has been depicted in Fig. 14.10 as a summary of the different stages of an IPNV infection.

14.6 Conclusions

The interplay between both SAV and IPNV infection and the host/host cell is complex and both viruses employ (some degree) of inhibition of protein synthesis, as one strategy to counteract the antiviral responses and limit the effect of the interferon and other antiviral effectors on the production of progeny. A better understanding of virus-host crosstalk could

open avenues for better infection control. It potentially plays a role in designing vaccine formulations that modulate and direct adaptive responses to the pathogen. Another point might be to explore antiviral interventions based on elicitation of innate responses that limits virus replication and gives the host an upper hand when it comes to controlling the infection and development of disease.

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The Ontogeny of the Fish Immune System

15

Kurt Buchmann

Abstract

The early development of the fish immune system and its associated organs, tissues, cells and molecules can be recognized shortly after fertilization of the egg. Even though maternal effector molecules (e.g. Ig and complement) and mRNA of maternal origin encoding protective factors (complement factors, AMPs) are found in the egg stage, the developing larva has its own capacity to express immune genes shortly after fertilization. The first stem cells appear in haematopoietic intra-embryonic cell masses located in association with the developing somites and the yolk sac, and the development of thymus and kidney is noted even before the egg hatches. The development is dependent on the species and environmental parameters as differences are found between the different teleost groups due to their evolutionary background and adaptation to very different ecosystems. Various primitive myeloid and lymphoid cell types appear in the embryonic cell masses, and putative T cells colonize the thymus and putative B cells, the Bursa equivalent which is the kidney in many species. The kidney is considered the main B-cell producing organ and contains haematopoietic tissue and lymphocytes. A series of genes encoding innate and adaptive immune factors are expressed even before hatching with an accelerated development post-hatch. During the yolksac stage, the fish may prevent pathogen evasion by covering its surface with an armament of innate factors and PAMP stimulation elevates their expression. Genes encoding inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α, iNOS, SAA, cathelicidins, and hepcidin are expressed in the yolksac larva a few hours after being exposed to pathogens. T cells and

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MHCII expression are localized in the thymus and IgT in the gill epithelia. The transition from a primarily innate response pattern in yolksac larvae to increased involvement of adaptive immune genes in older fry is critical as it may initially be associated with higher susceptibility to pathogen invasion. Thus, it is more difficult to infect yolksac larvae due to the strong innate and mechanical protective shield (Castro et al. Sci Rep 2015;5:15458). The development of immunocompetence, and the ability to raise a protective immune response, depends on the fish species, age, size and environmental factors, but even fry can raise a protective response following vaccination.

Keywords

Ontogeny · Thymus · Head kidney · Innate immunity · Adaptive immunity

Abbreviations

AMP	Antimicrobial peptide
C	Complement
CRP	C-reactive protein
Dpf	Days post-fertilization
Dph	Days post-hatching
ELISA	Enzyme-linked immunoassay
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MBL	Mannose-binding lectin
MHC	Major histocompatibility complex
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptors
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
RAG	Recombinase-activating gene
SAA	Serum amyloid protein A
TCR	T-cell receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor

15.1   Introduction

All organisms are continuously being exposed to potential pathogens during their entire life and need to establish various types of armament for protection. This applies to fish as well in which mechanical, chemical and cellular tools are being applied by the individual. The tools applied differ between the different developmental stages (egg, yolksac larva, fry, fingerling, adult), but they all contribute to the survival of the species. Immunological research has mainly focused on the protective elements produced by the developed fish in which both innate (Bayne and Gerwick 2001) and adaptive factors have been widely described. However, the immunological techniques developed in this process have guided our understanding of the first appearance of immune factors (organs, cells, molecules) in the early life cycle stages. These factors play a decisive role for when the fish may establish an adaptive response and thereby obtain lasting immunity involving immunological memory and responsiveness to vaccination. Classical histological techniques (Botham and Manning 1981; Chilmonczyk 1983) supplemented with ultrastructural techniques (Castillo et al. 1991, 1998) were originally applied to detect primordial immune organs in fish embryos, and basic vaccination and challenge methods were used to evaluate the appearance of protection (Johnson et al. 1982a; Mulero et al. 2008). During the latest decades, the development of gene expression techniques, supplemented by immunohistochemistry (IHC), has made it possible to record the immune genes activated at the different developmental stages and precisely describe the involvement of innate and adaptive factors. The egg is partly protected by molecules of maternal origin (Zhang et al. 2013), and also, the earliest yolksac larval stage contains innate and adaptive factors of maternal origin (Løvoll et al. 2006; Swain and Nayak 2009; Seppola et al. 2009) although the larva quickly initiates its own production of immune molecules a few days after fertilization. Our knowledge on these parameters is based mainly on experimental studies using commercial fish species, but they clearly show that the evolution and ecology of a certain fish species determine the details of the immunological armament and the timeline of the ontogeny. The studies conducted to elucidate the ontogenetic development of immune factors in fish larvae have focused both on well-characterized innate immune molecules such as pattern recognition receptors (PRRs) (Hansen et al. 2011) including Toll-like receptors (TLRs) (Rebl et al. 2010; Palti 2011), pathogen-binding molecules such as complement factors, acute-phase reactants (e.g. SAA, CRP, iNOS) (Villarroel et al. 2008) and adaptive elements (RAG, B cells, T cells, Igs) (Hansen and Kaattari 1995, 1996). Light and electron microscopical techniques have provided a firm and solid basis for the anatomy of the early development of immune organs (Grace and Manning 1980) and gene expression methodology supplemented by detection of effector molecules by immunohistochemistry, and ELISA has played a major role when dissecting the molecular basis for immunity in larval and young fish.

15.2 Development of Immune Organs

The central immune organs in fish are thymus and kidney, whereas the spleen is a secondary immune organ (Zapata et al. 2006). Aggregations of lymphoid cells appear in various compartments of the fish host and play a role in host defence throughout the entire life of the fish, but the ontogenetic development varies between fish hosts (Bertrand and Traver 2009; Bertrand et al. 2010). Although some general principles for development in the early life stages are evident, the evolution and the ecology of the individual fish species influence the timing. Below, the process is outlined for a few selected fish species with differing ecology for illustration of the main principles and deviations in the protective mechanisms applied by the early developmental stages of fish.

15.2.1 Rainbow Trout (*Oncorhynchus mykiss*) (Family Salmonidae)

The development of immune organs in the rainbow trout embryo is—as in all ectothermic animals—dependent on temperature. The unfertilized egg (Fig. 15.1a) and the fertilized egg (Fig. 15.1b) containing the developing larva via the hatched yolksac larva (Fig. 15.1c) running through metamorphosis to the fully developed fry stage (Fig. 15.1d) illustrate how the most vulnerable stages of the fish display entrance points for pathogens in the environment (Tatner and Manning 1983, 1985). The early developmental stage may be at least partly protected by maternal transfer of several complement factors transferred from the female spawner to the egg (Løvoll et al. 2006). This applies for other protective molecules as well—including lysozyme (Grinde et al. 1988)—which is noted also in other salmonids (Yousif et al. (1991). After fertilization, the embryo displays a very early immune system. The thymus rudiment appears five days before hatching as an outgrowth from the dorso-anterior pharynx epithelium (Grace and Manning 1980), and a few days after hatch, the thymus in trout is a clearly well-defined organ with active lymphopoiesis (Fig. 15.2). At this time point, the kidney is populated by IgM-positive lymphocytes (Razquin et al. 1990), but even earlier time points (14 days after fertilization) can display B lymphocytes in the organ (Castillo et al. 1993). A structural analogue of the bursa of Fabricius was recently discovered in Atlantic salmon along the posterior part of the intestine (Løken et al. 2020), but the extent of B-cell maturation in that organ in rainbow trout awaits further description. CD8+ T cells (Fig. 15.2) appear in the thymus of rainbow trout yolksac larvae 17 days after hatching (Chettri et al. 2012), and genes associated with T cells (encoding TcR, CD4 and CD8) have been found expressed in the egg and the early post-hatch period (Heinecke et al. 2014). Already 7 dpf Fischer et al. (2005) recorded expression of the CD8 gene even before appearance of the thymus, suggesting that gene activity precedes organ development. The first expression of immunoglobulins can be detected very early in the trout egg. The IgM gene was expressed 4 days after fertilization, but a marked increase was recorded following hatching at 60dpf. The first transcripts of IgT were noted on 26–36 dpf, but a higher and more stable expression of the IgT gene (Hansen

et al. 2005; Zhang et al. 2010) was seen just after hatching at 46 dpf (Heinecke et al. 2014). The newly hatched larva has a dense surperficial layer of IgT+ cells in the gill epithelia (Fig. 15.3), which suggest a central role of IgT in the early immunity of rainbow trout larva. The MHCII-encoding gene became significantly upregulated after hatching, as was found for genes encoding CD4, CD8 and TCR. Despite a low pre-hatch expression of these genes associated with adaptive responses, the study suggested that the initial preparation for establishment of adaptive responses really starts after hatch, which was further supported by IHC analyses showing CD8- and MHCII-positive cells in the thymus (Heinecke and Buchmann 2013; Heinecke et al. 2014). However, a series of complement factors and acute-phase reactants (such as SAA) were seen both pre- and post-hatch indicating that the yolksac larva is highly dependent on innate effector molecules. Evidence for antigen-presenting cells (Langerhans cells) in the early rainbow trout embryo was shown by Lovy et al. (2011). Langerhans-like cells were demonstrated by electron microscopy in kidney and thymus at 2 weeks post-hatching prior to the development of the spleen. This fits the timing of B and T lymphocyte appearance in the lymphoid tissues.

## 15.2.2 Seabass *Dicentrarchus labrax* (Family Moronidae)

One of the main aquacultured perciform fishes is the European sea bass *D. labrax*, possessing a well-characterized T-cell receptor (Partula et al. 1995; Picchietti et al. 2008; Miccoli et al. 2021). IHC studies demonstrated the ontogenetic appearance of T cells on day 25 post-hatching (Scapigliati et al. 1995) with indications of cell specialization from the T-cell distribution that revealed compartmentalization of the early thymus (Picchietti et al. 2008). A decreasing amount of IgM of maternal origin was detected by ELISA in sea bass eggs from day 0 to day 5, but from 15 days post-fertilization (dpf) the larva was able to produce antibodies in increasing amounts leading to significant levels in 50-day-old larvae with fully developed lymphoid organs (Breuil et al. 1997). Gene expression studies combined with IHC (Miccoli et al. 2021) showed a differential occurrence of different TCRs. The gamma chain of the TCR was undetected from hatching to 73 days post-hatching (dph) but appeared in thymus and intestine at 74 to 88 dph. Expression of the beta chain was detected at 21 to 24 dph and increased during subsequent development.

15.2.3 Gilthead Sea Bream *Sparus auratus* (Family Sparidae)

Another Mediterranean aquaculture species, sea bream *S. auratus,* shows lymphocytes in the thymus 47 dph, in the head kidney 54 dph and in spleen after 77 dph (Josefsson and Tatner 1993). Genes encoding RAG are detectable 21–27 dph, and almost simultaneously, the TCR and IgM genes are measurable at 27–48 dph. A series of innate immune genes were detectable and expressed during the hatching process accounting for production of TLR, inflammatory cytokines, antiviral factors and antimicrobial peptides (AMPs) (Mulero
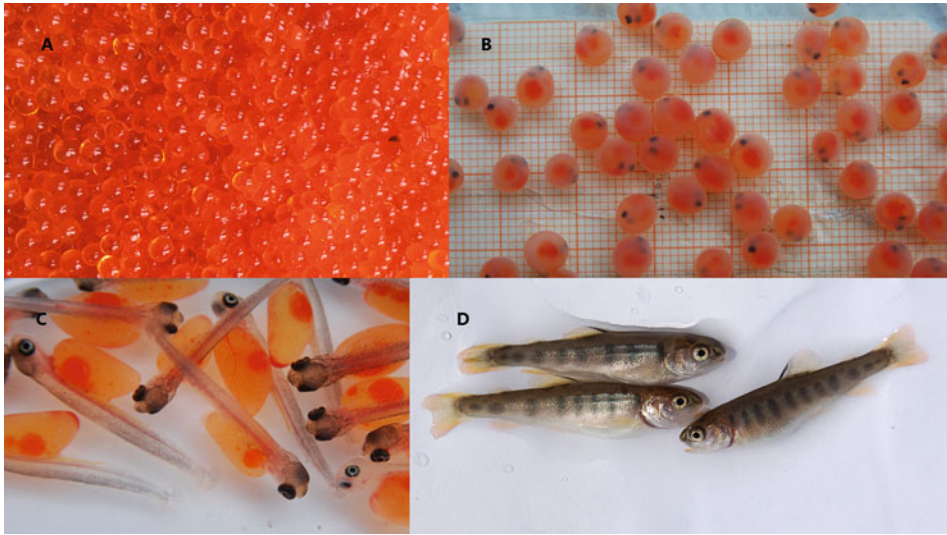
Fig. 15.1 (**a**) Unfertilized rainbow trout eggs. (**b**) Fertilized, eyed rainbow trout eggs containing embryos. (**c**) Rainbow trout yolksac larvae following hatching. (**d**) Rainbow trout fry after metamorphosis. Photos by Kurt Buchmann
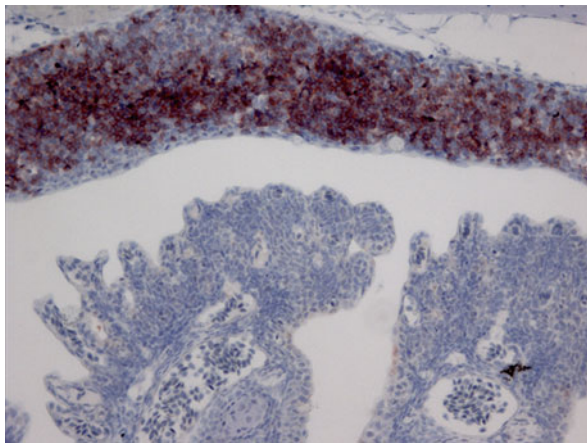
Fig. 15.2 Light microscopy of a thymus in rainbow trout yolksac larva with prominent presence of CD8+ T cells (reddish-brown stain). Based on a section of a disease-free rainbow trout larva fixed in neutral formalin, paraffin-embedded, immunostained using a monoclonal antibody reacting with rainbow trout CD8. The section was haematoxylin-stained before embedding in the aquaphilic Aquatex mounting medium. Photo by Kurt Buchmann and Jiwan K. Chettri

et al. 2008). The innate repertoire was further elucidated by Li et al. (2021), demonstrating that eggs and larvae of sea bream exhibited a significant production of lysozyme. Protection afforded by vaccination (reflecting immunocompetence of the larva) against

Fig. 15.3 Light microscopy of a thymus in rainbow trout yolksac larva with prominent presence of IgT+ cells (reddish-brown stain). Based on a section of a disease-free rainbow trout larva fixed in neutral formalin, paraffin-embedded, immunostained using a monoclonal antibody reacting with rainbow trout IgT. The section was finally haematoxylin-stained before embedding in the aquaphilic Aquatex mounting medium. Photo by Kurt Buchmann and Jiwan K. Chettri
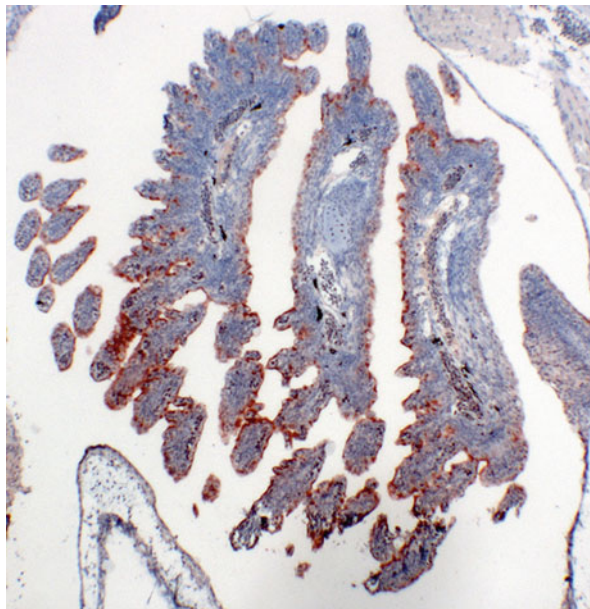
Photobacterium damselae was investigated, but surprisingly vaccination of young larvae by immersion or oral routes increased susceptibility, as judged from subsequent immersion challenge in live bacteria (Mulero et al. 2008). This may suggest that the immune capacity of the early-stage larvae may be exhausted upon excessive stimulation.

15.2.4 Common Carp *Cyprinus carpio* (Family Cyprinidae)

Common carp *C. carpio* have lymphocytes in the thymus by 3 dph, and B lymphocytes appear in the head kidney at 6 dph (Botham and Manning 1981). Gene expression studies by Huttenhuis et al. (2006a) provided a finer resolution of the development. Although complement and immunoglobulin of maternal origin were found in the unfertilized egg, the developing fertilized carp egg had significant expression of innate immune factors, including complement factor genes, during the early development of the fish larva. SAA, alpha-2-macroglobulin, C1, C3 and MBL (mannose-binding lectin)-associated serine protease (MASP) were noted 12 h post-fertilization and before hatching. When the egg reached the hatching stage, it was possible to localize myeloid cells associated with the aorta, vena cardinalis and kidney (Huttenhuis et al. 2006b). The early larva expressed IL-1β, inducible nitric oxide synthase (iNOS) and SAA when exposed to lipopolysaccharide (LPS), and regulation of complement factor C3 and alpha2-macroglobulin, demonstrating the ability to raise an innate defence reaction just after hatching. The appearance of intraepithelial lymphocytes and myeloid cells (monocytes and macrophages) showed that lymphocytes (putative T cells) colonized the intestine 3 dpf and myeloid cells 7 dpf (Huttenhuis et al.

2006c). Ig-positive cells appeared later, several weeks after fertilization and hatch. B and T cells (lymphopoietic cells) were looked for in early developmental carp stages by studying the expression of the RAG gene. It was first seen in carp thymus 4 dpf and from 6dpf in kidney clearly indicating an early initiation of adaptive processes (Huttenhuis et al. 2005). These events were confirmed by developmental studies on a related cyprinid species, the zebrafish *Danio rerio* (Willett et al. 1999; Lam et al. 2004; Wang and Han 2013). The latter authors stimulated larvae with LPS and followed expression of complement factors C1r/s, C3, C4, C6, B and MBL. The production increased until 21 dpf for most of the genes, but the alternative pathway was shown to dominate during the process where the adaptive response genes were less expressed. A series of other innate PRRs, including phosvitin, can protect the embryo and may have bactericidal properties (Wang et al. 2011). Common carp larvae also exhibit a clear ability to express central immune receptors. TLRs recognize PAMPS, and carp larvae express TLR21 at early developmental stages 1 and 10 dpf (Li et al. 2018b). This TLR is believed to bind viral molecules. Other receptors are cytoplasmic pattern recognition receptors (PRRs), such as nucleotide-binding oligomeriza-tion domain (NOD)-like receptors (NLRs). A novel NLRC gene (CcNLRC) in common carp was described by Li et al. (2018a). It was constitutively expressed in healthy tissues but showed two expression peaks at 1 dpf and 24 dpf. Stimulation with pathogen-associated antigens induced a significant up-regulation of the carp NLR expression in liver, spleen, foregut, hindgut and skin, suggesting that these receptors are involved with innate immune defence in carp larvae. This appearance of immune receptors coincides with the production of perforins by immune cells in carp larvae. Perforins are pore-forming glycoproteins with the ability to penetrate target cell membranes and are generally pro-duced by cytotoxic cells such as T cells. Li et al. (2018c) showed that carp larvae 22 dph expressed three forms of these molecules. Expression was stimulated by PAMPs such as poly I:C and an *Aeromonas hydrophila* bacterin, supporting the notion that 22 dph carp larvae respond actively to infection.

15.2.5 Atlantic Cod *Gadus morhua* (Family Gadidae)

Lymphoid organs appear in the cod egg shortly after fertilization (Schrøder et al. 1998), and the embryonic cells in the cod egg initiate their own expression of immune genes following fertilization. However, unfertilized eggs contain transcripts encoding lysozyme and the AMP cathelicidin. This indicates maternal transfer of antibacterial transcripts, which are likely to provide an early protection of the embryo (Seppola et al. 2009). Also, IL-8-encoding transcripts were found in the egg, whereas IL-6 expression increased later at the time of hatching (Lanes et al. 2012). Further, lysozyme was detected in the unfertilized egg supporting the findings that the female spawner transfers antibacterial material to the egg for early protection (Seppola et al. 2009; Lanes et al. 2012). When the egg was fertilized and started to divide, the first expression in the embryo of additional genes for antiviral proteins and the acute-phase reactants pentraxin and hepcidin was found. Active expression

of IgD and IgM was seen post-fertilization (during segmentation) by the embryonic cells, which suggests the onset of B-cell development and immunoglobulin involvement in early protection. Innate immune genes increased their transcription when hatching indicating a programmed establishment of protection which pathogen exposure elevates. When the yolksac larva metamorphosed, transcript levels were elevated showing maturation of the immune system. The role of pentraxins, such as C-reactive protein CRPI and CRPII, was further demonstrated by Magnadóttir et al. (2018) studying the transcription and modification of these pentraxins during the early ontogeny phases of the cod larva. Central organs (liver, kidney, nervous system) in the cod larvae expressed various forms of the pentraxins as early as 3 dph.

15.2.6 *Hippoglossus hippoglossus* (Atlantic Halibut) (Family Pleuronectidae)

The Atlantic halibut (*Hippoglossus hippoglossus*) is a coldwater species with an extended embryonic development and a prolonged yolksac larval stage. Therefore, a different developmental timeline is expected when comparing to other fish species occupying other habitats, such as salmonids and cyprinids. Extensive analyses by Patel et al. (2009) showed that the kidney was present at hatching, whereas the thymus could be localized at 33 dph. The spleen appeared later at 49 dph. Expression of immune genes was measured at 66 dph when IgM+ cells could also be detected in the anterior kidney and spleen from this time. The developed halibut is able to activate a series of central adaptive immune genes (including T-cell markers) (Øvergård et al. 2013), but as in other fish species the early stage of the halibut (the yolksac larva) is equipped with a protective shield of molecules. One of the strategies is to cover the surface exposed to environmental threats with lectins. The halibut larva develops very slowly, and Magnadóttir et al. (2019a) described the presence of a ladder-like lectin present on the halibut larvae at 119 to 1050 dph. This time period still represents the early stages of the fish. Skin, gills and gut surfaces were positive for the lectin suggesting a major role in protection of mucosal surfaces, but if the lectin plays a role in complement activation (Magnadóttir et al. 2019b) needs further confirmation.

15.3   Maturation of the Adaptive Immune Response

The protective immune response is operating already in the unfertilized fish egg as a series of transcripts, and effector molecules of maternal origin are present in the egg delivered by the female spawner. In addition, the eggshell, represented by the chorion and membranes, provides a mechanical protection against environmental factors including potential pathogen colonization at the very earliest stage. Following fertilization, transcription of the innate immune genes, and to some extent genes associated with the adaptive response, can be readily measured in the developing embryo, and after hatching, the yolksac larva

accelerates production of protective molecules. The reactions are, at the early developmental stage, dominated by the innate immune molecules, but it is central to pinpoint the time points at which the adaptive immunity takes a larger part in the overall protection. The immune factors involved in this process are the T and B cells with the ability to diversify their antigen receptors (T-cell receptor and antibodies, respectively), expand their repertoire and bind to a variety of antigens resulting in clonal expansion of lymphocytes (Castro et al. 2017). The timeline for these processes is likely to vary considerably dependent on the ecology of the fish species. To date, more than 30,000 fish species have been described and their appearance during evolution left them in a diversified range of ecological niches. The adaptation to these differing environmental conditions explains the wide variation in timing of immune development. Their tolerance to environmental factors, especially temperature, can be viewed in connection with the embryonic development. The cyprinid zebrafish is a well-studied representative of warm water fish and rainbow trout, a valuable model for coldwater fish. Cells move from the zebrafish thymus to peripheral organs such as the kidney already 146 h after fertilization (3 days after hatching), but an active adaptive immune system may first operate effectively several weeks later. Central questions concern the number and diversity of antigens such a young individual is able to raise a response against. In this context, focus should be placed on the VDJ combinations as these create the basis for the antibody repertoire. It is noteworthy that this is very stereotyped at the earliest appearance in zebrafish (Jiang et al. 2011) where different individual fish larvae share the same idiotypes. By using high-throughput sequencing for characterization of the antibody repertoire at five different developmental stages, the authors characterized the diversifying antibody repertoire. A small number of V (variable) D (diverse) J (joining) gene segment combinations were found in use and dominating at the earliest time points. Later, the stereotypy decreased as the diversity increased markedly. However, even older and adult fish share VDJ combinations, but the diversification of the repertoire in these stages probably involves elevated occurrences of nucleotide deletions and insertions (Jiang et al. 2011).

One of the main constraints is that the very young fish contain a very limited number of lymphocytes, which in itself will set a roof of possible clonal expansions of T- and B-cell clones. It can then be speculated if antibodies with relatively low specificity, the so-called natural antibodies, are part of an early antibody strategy. Certain lymphocyte populations, B1 lymphocytes, may be responsible for this production.

The rainbow trout ontogenetic immune development can be elegantly studied in unfertilized (Fig. 15.1a), eyed egg containing a developing embryo (Fig. 15.1b), the yolksac larva (Fig. 15.1c) and later stages after first feeding (Fig. 15.1d). This approach showed that the reactions to different pathogen types, such as Gram-negative bacteria versus rhabdovirus, differ markedly (Castro et al. 2015). The antiviral response towards VHSV developed gradually, whereas fry established a marked response towards the bacterium *Aeromonas salmonicida* shortly after exposure.

However, two sets of immune-related genes playing a role in both bacterial and viral infections could be differentiated. More than 40 genes responded at three developmental

stages to both viral and bacterial infections. These encoded among others AMPs, APP, complement factors, lectins, MASP, chemokines and proteases. A larger fraction (247) of the activated gene pool responded to both pathogen types only at later larval stages.

15.4   Protective Responses and Immunological Memory in Fish Larvae and Fry

The basic development of immune organs and appearance of different lymphoid and myeloid cell types in fish are clearly a prerequisite for establishing a response to invading pathogens or raising a protective response to a vaccine. However, the time when a fish is able to obtain protection following vaccination may not follow the appearance of the different cell types, as an effective protective response is based on an intricate network of humoral and cellular processes organized and regulated by the cytokine network (Mulero et al. 2008). In order to establish the developmental stage when the young fish are immunocompetent and respond to vaccination by raising protection, it is necessary to conduct in vivo and practical immunization and challenge studies. Early studies on different salmonid species were performed with immersion of fish into *Yersinia ruckeri* and *Vibrio anguillarum* bacterins, and the impact of host species, host age, host size and temperature was clearly established (Johnson et al. 1982a, b). Protection could be induced by vaccination. Rainbow trout fry at 0.5 g attained some protection by immersion vaccination in a *Vibrio anguillarum* bacterin, but the efficacy was clearly increased in larger fish. Rainbow trout immersion vaccinated with a *Yersinia ruckeri* bacterin responded significantly when they reached a size of 2.5 g (Johnson et al. 1982a). Later studies showed that even fry at 750 mg can raise a response providing protection against *Y. ruckeri* up to 6 months (Buchmann et al. 2003), but generally larger fish fry sustain protection longer compared to vaccinated smaller fish (Johnson et al. 1982b). Although adaptive immune factors become activated at this early stage, it is not clear to what extent innate and adaptive responses contribute to protection lasting a few months. Primary immersion vaccination has a limited duration, and in order to reach prolonged protection, booster vaccination is needed. Thus, the protection seen in the smallest fish may, to a large extent, depend on innate immune factors, whereas in larger fry more sophisticated adaptive mechanisms may be involved (Chettri et al. 2012). In all cases, the immunization dosage and temperature can influence the studies and the subsequent application of vaccination procedures at the farm level. It is also noteworthy that it is the fish size rather than age that determines the immunological capacity at early developmental stages. This may be explained by the total pool of immune cells (lymphocytes and myeloid cells), which is correlated with fish size (Jaafar et al. 2016).

15.5 Conclusion and Future Outlook

The work conducted up until now has shown that unfertilized eggs, fertilized eggs and the yolksac larvae hatching from the eggs of teleosts contain active immune effector molecules such as complement factors, cathelicidins and lysozyme, securing protection of the earliest developmental stages against a range of pathogens present in the aquatic environment challenging the fish. Maternal transfer of effector molecules and immune gene transcripts secures a basic protection independent of the embryo's own activity. However, transcription of innate immune genes encoding lectins, complement factors, AMPs and acute-phase reactants such SAA is initiated quite early after fertilization. The constitutive production of the immunoglobulins IgT and IgD, considered to be adaptive immune molecules, may serve a similar purpose and provide a protective layer of pathogen-binding molecules in the external surface zone. The adaptive responses, involving IgM, MHC, TCR, CD8, etc., are also established quite early in development and accelerate when the yolksac larva metamorphoses into the later fry stage. The heavy innate armament seems to be highly protective during the first period after hatching. However, the constitutive and rather broadly acting innate defences may be energetically expensive and perhaps not sufficiently effective when the older fish meets a wide plethora of biotopes equipped with a high number of pathogens. Here, the adaptive immune capabilities will show superior, more flexible responses and reach a wider range of target epitopes. However, the abilities of various pathogens to circumvent or evade adaptive responses may make the young fry more vulnerable during the transition phase from a predominantly innate period to the mainly adaptive stage. Thus, pathogens may bind to surface molecules of immune cells and enter the host (e.g. by being engulfed by phagocytes) but still survive this process. The most early developmental stages respond the least to immunization and vaccination and may have a very limited immunological memory. However, keeping in mind that the innate response mechanisms are the prevailing weapon of the egg, yolksac larva and earliest fry, it may be worthwhile to focus on these factors and ways to stimulate them in future research into development of prophylactic measures for securing the health of fish fry.

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Antibacterial Immune Responses

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Abstract

Teleost immune responses towards bacterial infections follow initially a general pattern of reactions, including pathogen recognition, signal transduction, initiation of inflammatory reactions, production and release of innate effector molecules and subsequent establishment of adaptive responses with the involvement of T and B cells. Detailed descriptions of these factors and their regulation, biological function and genetic background are treated in various chapters of this book. However, a wide range of variations exist over this theme. The highly varying surface structure of the different bacterial types, encountered by fish, affects the outcome of the reactions—at both the early and late phases of infection. The fish host surface plays a role for pathogen attraction, adhesion and possible invasion of the host. Gram-negative fish bacteria such as *Vibrio anguillarum*, *Aeromonas salmonicida*, *Yersinia ruckeri*, *Edwardsiella ictaluri*, *E. tarda*, *Flavobacterium psychrophilum*, *F. columnaris* and *F. branchiophilum* elicit a different response compared to Gram-positive bacteria such as *Renibacterium salmoninarum*, *Lactococcus garvieae*, *Streptococcus agalactiae*, *S. iniae*, *Mycobacterium marinum* and *M. fortuitum*. The surface structures account for many of the pathogen-associated molecular patterns (PAMPs) interacting with the pattern recognition receptors (PRRs) in the host. In addition, the immune evasion strategies applied by the major bacterial groups explain the subsequent differences in host reaction patterns. The first infection phase involves PRRs such as TLRs and a fast expression of genes encoding AMPs together with SAA and other acute-phase reactants (precerebellin, hepcidin, lysozyme). The high upregulation of inflammatory cytokine

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genes (e.g. IL-1β, IL-8, TNF-α) is associated with a fast recruitment of leucocytes to the focus of infection. Different types of granulocytes, including neutrophils, possessing well-documented killing capabilities, are believed to explain a part of the initial elimination of pathogens. An array of acute-phase reactants (hepcidin, precerebellin, complement factors, CRP, MBL) represent some of the innate effector molecules. Production of antimicrobial peptides and reactive O and N species by leucocytes present in the affected tissue play a major role in the immediate killing of the invaders. Antigen-presenting cells (resident or systemic) at peripheral or central immune locations with aggregations of lymphocyte centres engulf and degrade the bacterial pathogen and present antigens with their MHC molecules to T cells carrying TCR whereafter B cells are stimulated to produce specific antibodies. Important factors influencing the response are the ontogenetic changes seen during the process from hatched yolk-sac larva via fry to the fingerling stage where the adaptive elements become increasingly dominating. Granuloma formation occurs when bacterial killing mechanisms are insufficient and sequestration of the pathogens may limit spread of the bacteria, which emphasizes the limitations of the piscine immune system. It has the capacity to recognize pathogens, activate both innate and adaptive components and eventually eliminate the invader. This is the basis for the successful application of a range of antibacterial vaccines in fish farming. Thus, the adaptive immune response of fish involves immunological memory securing reaction for an extended period, but the immune evasion strategies of the different bacterial types determine the type of reaction, the fate of the bacteria and the extent of protection.

Abbreviations

AMP	Antimicrobial peptides
C	Complement
CRP	C-reactive protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
MBL	Mannose-binding lectin
MHC	Major histocompatibility complex
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
SAA	Serum amyloid protein A

TCR   T-cell receptor
TLR   Toll-like receptor
TNF   Tumour necrosis factor

16.1 Introduction

Bacteria are among the first organisms to colonize surfaces in the environment, and fish skin, fins and gills are excellent substrates for these microorganisms. The extensive studies on the microbiota associated with fish surfaces clearly show that a high number of taxa are attracted to and able to attach to the fish. In this context, it is surprising to recognize the limited number of bacterial species being capable of invading the healthy fish host under normal circumstances. Nevertheless, selected bacteria can elicit disease in fish following penetration of the external barrier of the fish. However, the genetic composition of the fish determines if the naïve fish is susceptible or naturally resistant to infection (Fraslin et al. 2020). They possess mechanisms to resist and even circumvent the mechanical, physical and chemical barriers associated with the fish host epithelia composed of compact layers of epithelial cells equipped with densely packed mucous cells continuously releasing mucus containing a range of antibacterial compounds. PAMPs of the pathogens interact with PRR on the fish surface and when entering the host organism additional immune reactions are activated (Chettri et al. 2011). These are described in detail in various chapters of this book elaborating on the host–pathogen interaction involving humoral and cellular factors of the innate and adaptive immune systems. TLR, precerebellin, hepcidin, complement factors, CRP, MBL, SAA, AMPs, MHC and B and T cells are among the decisive elements determining the course of infection and fate of the pathogen. The direct pathogen exposure of the external fish surfaces initiates a series of innate responses of both cellular nature and humoral nature. Cells produce a range of effector molecules including AMPs such as cathelicidins (Zanetti 2004) and reactive O and N species. Subsequently, the adaptive responses accelerate. Antigen-presenting cells and lymphoid cells are present (Scapigliati 2013), and immunoglobulins, such as IgT, may be produced at these sites (Xu et al. 2013; Zhang et al. 2017; Yu et al. 2018). The intricate interactions at all levels are made possible through an efficient signalling network of cytokines binding to specific receptors in relevant physiological compartments (Zou and Secombes 2016). Important factors influencing the response are the ontogenetic changes seen during the process from hatched yolk-sac larva via fry to the fingerling stage. Innate effector molecules such as complement factors (Løvoll et al. 2006) may be maternally transferred via the egg. This stage and the yolk-sac larva show an intrinsic expression of innate immune genes (Heinecke et al. 2014), but during the ontogenetic development the adaptive elements become increasingly dominating (Chettri et al. 2012). In addition, environmental factors such as temperature

(Raida and Buchmann 2007; 2008a, b) and salinity (Chettri et al. 2015a, b) may significantly affect the host responses together with stressors associated with inferior physico-chemical parameters or biological factors such as crowding (Tort 2011). The very different surfaces of the different bacterial types invading the fish determine the outcome of the reactions— at both the early and late phases of infection, and the classical Gram staining representing a coarse measure of the bacterial surface composition is thereby a relevant way of addressing the question. The different bacterial pathogens may also target different exposed surfaces (skin, fins, gills, buccal cavity, gastrointestinal tract, olfactory sac, sideline system), and these special microhabitats of the fish host surface influence the subsequent reactions. They play a role for pathogen attraction, adhesion and invasion of the host and Gram-negative fish bacteria, such as species within the genera *Vibrio*, *Aeromonas*, *Yersinia, Edwardsiella* and *Flavobacterium,* elicit a different response compared to Gram-positive bacteria, such as species within the genera *Renibacterium, Lactococcus, Streptococcus* and *Mycobacterium*. In addition, various elegant immune evasion strategies, applied by these major bacterial groups, explain the subsequent differences in host reaction patterns (Grayfer et al. 2014). The list of host species subjected to immunological investigations is rapidly expanding, and the number of pathogens recognized is increasing even more. The ability of teleosts to raise a protective immunological response towards bacterial pathogens has been used by the pharmaceutical industry to present a range of vaccines for fish farming enterprises (Plant and LaPatra 2011; Brudeseth et al. 2013). Different vaccine delivery methods are in use, ranging from injection via immersion to oral administration of antigens. Evidence for antigen-sampling and antigen-presenting cells in fish mucosal surfaces (Kato et al. 2018), the presence of lymphoid tissue in gills (Koppang et al. 2010) and a significant production of immunoglobulins (Xu et al. 2013) may explain the efficacy of immersion vaccines although injection is absolutely the most effective for induction of protective immunity (Chettri et al. 2013). However, it raises possibilities for extensive development of vaccines also for smaller fish unsuitable for injection vaccination. This chapter addresses a few selected bacterial pathogens and the main immune reactions they raise in fish. This will leave room for further studies on the plethora of immune reactions and modulation mechanisms found in these pathogen–host systems.

16.2 Immune Responses Against Gram-Negative Bacteria

16.2.1 *Vibrio anguillarum*

Bacteria in the family Vibrionaceae are Gram-negative straight or curved motile rods moving by use of mainly polar flagella and occur in the aquatic environment where fish serve as hosts (Fig. 16.1). An important genus is *Vibrio* comprising a wide range of bacterial species identifiable by their biochemical characters (Actis et al. 1999). *V. anguillarum* causes classical vibriosis, one of the first bacterial fish diseases to be described (Bergman 1909), associated with acute haemorrhagic septicaemia, anorexia,

fin, skin and inner organ haemorrhages, ulcers and abscesses, which originally necessitated the use of antibiotics in order to control the infection in aquaculture (Aoki et al. 1984). The surface structures of the bacterium, attached to the outer membrane, allows differentiation based on serotype systems recognizing the O antigens (polysaccharides in the lipopolysaccharide layer LPS), K antigens of the capsule polysaccharides, H antigens of the flagellar proteins and F antigens of fimbria proteins. The surface antigens are the basis for establishing a serotype system. This has revealed that different serotypes of the bacterium are highly selective in their choice of host fish species (Larsen et al. 1988, 1994). Also, fish recognize the different surface antigens and immunization of fish with *V. anguillarum* antigens induces production of humoral protective factors transferable by adoptive transfer (Viele et al. 1980). Both the adaptive and innate immune systems of the fish respond to these bacterial antigens, which secure some protection of the host, because the surface structures are widely used by the bacterium during the invasion of the naïve fish host (Khansari et al. 2019). Thus, *V. anguillarum* applies its motility and chemotactic abilities to locate and colonize the host whereafter bacterial proteases and haemolysins are used for penetration, invasion and establishment. Subsequently, the bacterium is able to produce iron-binding proteins, termed siderophores, allowing the pathogen to bind the necessary iron for propagation. Gene expression studies of juvenile rainbow trout exposed to *V. anguillarum* serotype O1 reveal a clear picture of reactions raised by the fish host (Karami et al. 2020). The bacterium penetrates the fish surface and reaches the inner organs and in the fish a higher number of bacterial transcripts are noted in the spleen, compared to liver. Fish with clinical signs show a higher transcript level compared to fish without disease signs, emphasizing that different susceptibility to infection occurs among individuals in the fish population. When analysing reactions in gills, the first genes to be upregulated are interleukin (IL)-1β, IL-6, IL-8, IL-22, cathelicidin (Cath)1, Cath2 and SAA. The early responses involving AMPs such as cathelicidins represent an important barrier against bacterial pathogens including *V. anguillarum* in a range of fish species (Meloni et al. 2015, Furlan et al. 2018). Later stages in the course of infection show involvement of genes encoding membrane-bound IgD (IgDm), secreted IgD (IgDs) and TCR together with genes encoding cytokines (IL-2, IL-4/13A, IL-6, IL-8, IL-10, IL-12, IL-17C2, IL-22, TNF) and effector molecules IgM, Cath1 and Cath2. Upregulation of cytokine genes for IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17C1, IL-22, TNF, interferon (IFN)γ and effector molecules C3, Cath1, Cath2 and SAA was observed in spleen samples with the highest increases for SAA, Cath2 and IL-6. At the early stage, genes encoding IgDm, IgDs, IgM, IgT, TCR and TGF-β were downregulated in spleen. Also, in liver upregulation of genes encoding Cath2 and SAA was found. Cytokine genes encoding IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17C1, IL-17C2, IL-22 and TNF-α and the gene encoding IgM were also significantly upregulated in infected fish livers, whereas IgDm, IgDs and IgT genes were downregulated in this organ. Lysozyme and SAA genes remained upregulated at later stages of the infection course. Fish reactions comprise both innate and adaptive elements, but the specificity and immunological memory activated by *V. anguillarum* antigens are demonstrated by the well-documented antibody titre increase after infection or vaccination

(Shao et al. 1988; Holten-Andersen et al. 2012b). A practical result is the successful application of vaccines for vibriosis control in fish farming (Håstein et al. 2005, Caipang et al. 2008, Holten-Andersen et al. 2012b). However, although the adaptive response becomes activated by the infection it is clear that the innate reactions (e.g. AMP, SAA, complement) play a major role both at early and late stages of the infection course. There is a high degree of genetic disposition to *V. anguillarum* infection in various host species. This indicates that it is possible to establish breeding programmes for genetically improved fish populations. Quantitative trait loci (QTL) identify fish with increased resistance towards *V. anguillarum* infection in olive flounder (Wang et al. 2014; Sakamoto and Chen 2015), in tongue sole (Tang et al. 2016), in turbot (Zhang et al. 2019) and in rainbow trout (Karami et al. 2020). The genes responsible for the natural resistance in the fish are not identified, but evidence points to involvement of at least MHC and immunoglobulin genes in natural resistance in the studied fish.

16.2.2 *V. salmonicida*

Within the family Vibrionaceae, the bacterium *V. salmonicida* causes haemorrhagic septicaemia in salmon and infected fish appear lethargic, dark, move abnormally and are anorectic. The infection causes cold-water vibriosis (Hitra disease) capable of eliciting high mortality in salmon farms. The infected fish show haemorrhages in the skin, at fin bases, and inner organs and the heart and muscles are affected. The bacterium causes a marked inflammation, ascites and oedema often seen as raised scales. The enlarged spleen reflects that a strong immune reaction is activated by the infection. Indeed, the extensive use of effective vaccines against the disease since the late 1980s signifies that protection is conferred following immunization with a significant production of specific antibodies as one of the decisive immune mechanisms (Steine et al. 2001).

16.2.3 *Aeromonas salmonicida*

This Gram-negative non-motile rod (Fig. 16.2) occurs in freshwater, brackish water and saltwater (Europe and North America) where it causes furunculosis (Fig. 16.3), preferentially in salmonids, although other fish species may be susceptible. The subspecies *A. salmonicida* subsp. *salmonicida* causes typical furunculosis in salmonids associated with haemorrhagic septicaemia and high mortality. The enlarged spleen and kidney in infected fish indicate that the fish react strongly to the infection. Virulent *A. salmonicida* strains possess an outer A layer, which protects the pathogen against various immune factors, and the bacterium spreads to the musculature causing necrosis, haemorrhages and abscesses. Experimental infection studies indicate that entrance of the bacterium occurs through the fish surfaces (Adams et al. 1987) although minute injuries of the skin may accelerate the process (Marana et al. 2016). Also, external physico-chemical factors such as

Fig. 16.1 *Vibrio anguillarum*. TEM, comma-shaped cells with polar flagella shown. Courtesy of Dr. J. L. Larsen

salinity may affect entrance of the pathogen into the fish (Chettri et al. 2015b). Immunological studies on juvenile rainbow trout exposed to the bacterium show that a series of innate and adaptive immune factors are activated shortly after infection. The cytokine genes activated (in the fish surface and the inner organs such as spleen and liver) shortly after infection of rainbow trout include a large number of pro-inflammatory cytokine genes encoding molecules such as IL-1β (and the regulatory counterpart IL-10), TNF-α, IFN-γ, IL-6 and IL-8 (Marana et al. 2021). Genes encoding innate antibacterial effector molecules comprising cathelicidins 1 and 2, lysozyme, SAA and complement factor C3 become highly upregulated in gills, spleen and liver shortly after infection. These innate responses involving cytokines and chemokines may have a double function as judged from the observation that chemokines, such as CK11, have a strong antimicrobial effect (Munoz-Atienza et al. 2019). There seems to exist a high contribution of innate and non-specific responses in the vaccine-induced protection (Olivier et al. 1985). This mixed response was also indicated by Kumari et al. (2015) showing expression in *A. salmonicida*-exposed Atlantic salmon mucosal tissue of the type 1 response genes encoding T-bet, IFN-γ, IL-22 and natural killer enhancement factor. The disease has been successfully controlled in salmon farming since the early 1990s when oil-based vaccines became available (Lillehaug et al. 1992; Midtlyng et al. 1996a, b). The protection induced is highly correlated to the antigen dosage (Marana et al. 2017a). The adaptive responses induced by vaccination are reflected by upregulation in immune organs of primarily the IgM gene, which may explain the relatively high protective effect of vaccination accompanied by a rise in the antibody titre (Adams et al. 1988; Bricknell et al. 1999; Romstad et al. 2012, 2013, 2014; Marana et al. 2019). It should be noted that the oil-adjuvanted *A. salmonicida* vaccines induce a

series of additional responses and side effects associated with inflammation and granulo-
matous lesions (Midtlyng et al. 1996b; Mutoloki et al. 2004, 2006a, b). These processes
include upregulation of genes encoding complement factors C1q and C6, mannose-binding
protein, lysozyme C, C-type lectin receptor, CD209, cathepsin D, CD63, LECT-2, CC
chemokine and metallothionein. Growth performance of salmon is also affected by the
reactions induced by oil-adjuvanted vaccines (Melingen and Wergeland 2002). The
vaccines are based on formalin killed bacteria (bacterins) and refinement of the production
process and application of subunit vaccines may be a path towards vaccines with lower
degrees of side-effects (Marana et al. 2017b). The involvement of a Th17 response was
indicated by regulation of TGF-β, IL-17A and its receptor (Mutoloki et al. 2010), but a
corresponding type of reaction was also seen in rainbow trout exposed by bath to a pure
suspension of *A. salmonicida* without any adjuvant included. The juvenile trout showed
elevated expression of IL-17A, IL-17C and TGF-β mainly in liver (Marana et al. 2021).
This emphasizes the broad immune reaction raised by this pathogen. However, the natural
susceptibility or resistance is heritable. Various strains or species show highly variable
susceptibility to *A. salmonicida* (Holten-Andersen et al. 2012a), which prepares a solid
basis for genetic breeding approaches with focus on innate and adaptive responses (Marana
et al. 2021).

16.2.4 *Yersinia ruckeri*

The causative pathogen of enteric redmouth disease (ERM) is the rod-shaped Gram-
negative enterobacterium *Yersinia ruckeri*. Several serotypes of the pathogen have been
described, and even within one serotype, several biotypes may be defined (Tobback et al.
2007). *Y. ruckeri* serotype 1 comprises two biotypes (Wheeler et al. 2009), one carrying
flagella (Fig. 16.4) and one without (Fig. 16.5). The pathogen may enter through several
portals in the fish surface but reaches the inner organs within minutes (Khimmakthong et al.
2013). The acute disease is a septicaemia associated with high mortality, and fish become
lethargic and anorectic. The inner organs (spleen, liver, intestine, adipose tissue, muscula-
ture) become affected by haemorrhages. Bleeding also occurs in and around the mouth and
in skin, fins and fin bases, and exposure to *Y. ruckeri* antigens clearly activate central
immune organs of the host (Kumar et al. 2018) with a marked inclusion of both innate
(Raida and Buchmann 2009) and adaptive (Raida and Buchmann 2008a, b) immune
mechanisms. The susceptible fish respond with a strong induction of inflammatory
cytokines (Raida et al. 2011a). Correspondingly, experimental infection of naïve rainbow
trout with live *Y. ruckeri* bacteria elicited in spleen and head kidney, a few days post-
infection, a marked upregulation of genes encoding the cytokines IL-1β, TNF-α, IFN-γ and
IL-10 (Desmukh et al. 2013). These were associated with changes in the immune cell
density in these organs where melano-macrophages and CD8+ cells and IgM+ cells are
located together. Lymphocytes showed an initial decrease in density a few days post-
infection but increased subsequently after one week (Desmukh et al. 2013). Subsequent
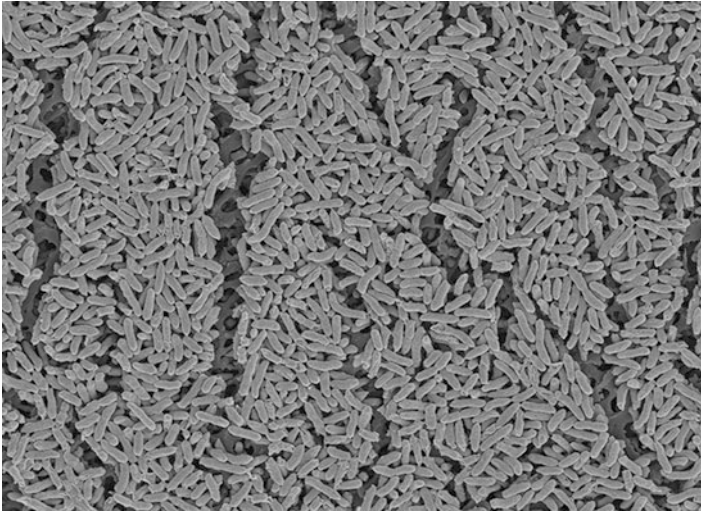
Fig. 16.2 *Aeromonas salmonicida*. SEM. Courtesy of Dr. O. S. Møller

Fig. 16.3 Rainbow trout exhibiting skin ulcer due to experimental infection with *Aeromonas salmonicida*

and expanded studies (Zuo et al. 2020) showed upregulation of cytokine genes encoding IL-1β, IL-6, IL-8, IL-10 and IFN-γ and effector molecule genes encoding SAA, Cath1 and Cath2 in spleen, liver and gill. Genes encoding IL-17C2, TNF-α and C3 were upregulated in spleen and liver, and IL-22 in spleen. The effector molecules IgM and lysozyme were produced in gills and liver. The liver carried a lower bacterial burden compared to spleen but in highly susceptible fish, showing clinical signs at early time points after bacterial exposure, the liver genes encoding IL-1β, IL-4/13A, IL-8, IL-17C1, IL-17C2, IFN-γ, TGF-β, TNF-α, Cath2, lysozyme and SAA were upregulated to a higher extent than in spleen. The involvement of immunoglobulin genes in the reaction agrees with the fact that

rainbow trout raise a strong IgM response measurable by ELISA 3–4 weeks post-immunization (Raida et al. 2011b). The strong host response is the basis for an extensive use of vaccines against ERM. The prototype bacterins were developed and tested half a century ago (Anderson and Nelson 1974; Johnson et al. 1982; Newman and Majnarich 1982), and immersion, bath, injection and oral administration methods have subsequently been documented in depth (Cossarini-Dunier 1986; Desmukh et al. 2011; Chettri et al. 2013). Immersion was the first method to be used and has been validated by a wide range of subsequent studies. ERM in fry and fingerlings in trout farms can be controlled success-fully by this immunoprophylactic management method based on a 30-sec immersion in a suspension containing 5×10^8 killed *Y. ruckeri* bacteria per ml (Desmukh et al. 2013; Chettri et al. 2013). The immunization method confers protection for several months, but a booster immersion is needed for sustained protection (Chettri et al. 2015a; Schmidt et al. 2016). However, the secondary response due to repeated immersion vaccination is rather additive than multiplicative (Jaafar et al. 2018). Larger fish raise a lasting protection if injection vaccinated intraperitoneally with 0.1 ml vaccine containing 5×10^9 formalin-killed bacteria per ml, and adjuvants may increase the efficacy significantly (Jaafar et al. 2015). The different biotypes differ in their antigenic profile, and although some cross-protection occurs between biotypes, the vaccination result may be optimized by selection of strains and types relevant for vaccine production (Desmukh et al. 2013). The protection is clearly dependent on adaptive immune mechanisms in the fish host, but the application of immune-stimulating adjuvants elevates the protection for both immersion and injection vaccines (Jaafar et al. 2015; Skov et al. 2018). Oral administration of *Y. ruckeri* bacterin would be a convenient method, but recent studies have indicated that the recommended administration of low antigen dosages over 2×5 days may induce some immunological tolerance, which may leave the treated fish more susceptible to infection (Jaafar et al. 2018; Marana et al. 2020).

16.2.5 *Edwardsiella*

These bacteria are Gram-negative, motile rods and belong to the family enterobacteriaceae as *Yersinia*. Two species within the genus *Edwardsiella* cause problems in fish. *E. ictaluri* causes enteric septicaemia in catfish kept at higher temperatures. The infected fish become lethargic and show abnormal swimming movements. The acute form of the disease elicited by bacterial invasion through the intestine is associated with intestinal bleeding and ascites, whereas the chronic form is caused by the pathogen entering through the olfactory organ, spreading to the brain and eventually to other parts of the head before producing openings in the head. *E. tarda* has a wider host span and occurs in catfish, eels and tilapia. Initial clinical signs are red foci in the skin connected to underlying abscesses in the muscle tissue filled with bacteria. Internal organs including kidney and liver showing microabscesses may be affected as well. The Sutchi catfish *Pangasianodon hypophthalmus,* commonly cultured in Asia, has been exposed to an experimental *E. tarda* infection (intramuscular
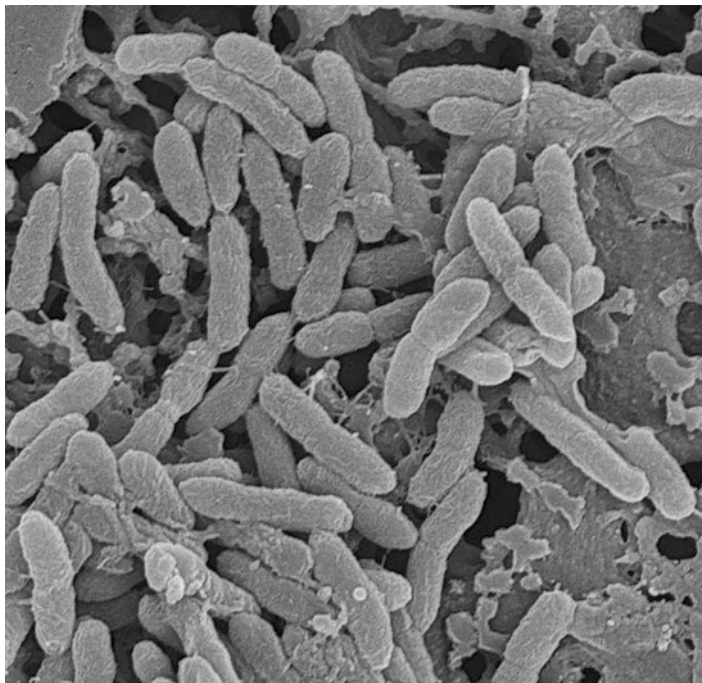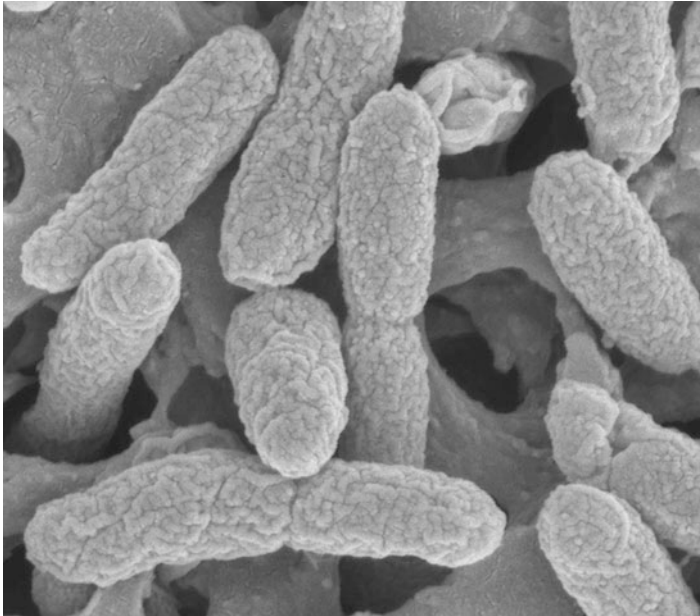
Fig. 16.4 *Yersinia ruckeri* serotype O1, biotype 1. Flagella present. SEM. Courtesy of Dr. O. S. Møller

injection) whereafter the fish raised a host response involving expression of genes encoding the pro-inflammatory cytokine interleukin-1β (IL-1β), the acute-phase protein transferrin and complement factor C3 in liver, kidney, spleen and peripheral blood cells (Hoquea et al. 2020). Thus, the highly invasive nature of the two bacterial species has the potential to elicit a strong immune reaction (both innate and adaptive responses are involved), but the pathogen may survive direct exposure to host serum. Evidence has been presented showing that a surface factor of the bacterium interferes with complement activity in flounder serum (Li et al. 2015). However, despite this immune-modulating mechanism fish may raise a protective response and a number of studies have shown that catfish fry and fingerlings can be protected by live-attenuated *E. ictaluri* vaccines (LAVs) (Shoemaker et al. 1999).

16.2.6 *Flavobacterium psychrophilum*

Experimental infections of rainbow trout have indicated that the pathogen possesses immune-regulating abilities (Henriksen et al. 2015). In the early phase of infection, an inflammatory reaction was indicated by the upregulation of the gene encoding IL-1β, whereas the IgT gene was downregulated. A positive correlation was found between the

Fig. 16.5 *Yersinia ruckeri* serotype O1, biotype 2. Flagella absent. SEM. Courtesy of Dr. O. S. Møller

F. psychrophilum load and the FoxP3a gene expression, suggesting a downregulating mechanism in the bacterium as this molecule is a marker for regulatory T cells. In addition, after 48 h, a ten-fold downregulation of IL-17C1 was found. At an even later infection stage, the IgT gene and the MHC II gene were found downregulated although a positive correlation was found between bacterial load and genes encoding IL-17C1, SAA, CD4 and MHC I. The T-cell receptor gene and the IL-4/13 cytokine gene were downregulated in the infected group. However, although the presence of *F. psychrophilum* seems to depress the genes involved in an effective adaptive response it is possible to detect specific antibodies in trout several days after infection or immunization (Högfors et al. 2008; Henriksen et al. 2015). Consequently, several studies have explored the possibility to produce vaccines and a commercially available vaccine has been tested with success on rainbow trout fingerlings (Hoare et al. 2018). One major challenge is to induce immunological protection in fish fry in which the immune system is inadequately developed. Some innate immune genes are expressed even in the fish embryo in the egg and yolk stage of the fish, but the efficiency and ability to include adaptive elements in the protection occur at a later fry stage (Heinecke et al. 2014). In addition, the total number of leucocytes including lymphocytes is low in fry restricting the potential number of antigen-specific reactivities (Jaafar et al. 2016). This poses a challenge for the development of vaccines for fry in general, and alternative approaches may assist optimization of fish health in these cases. Genetic

breeding can be an option. The genetic background of the fish plays a major role, and genome-wide association studies may pave the way towards less susceptible fish (Marancik et al. 2015). This has wide prospectives for practical breeding purposes as demonstrated by the detection of a QTL for *F. psychrophilum* resistance in rainbow trout described by Wiens et al. (2013) and Vallejo et al. (2014).

16.2.7 *Flavobacterium columnare*

This Gram-negative slender rod-shaped bacterium (with a length up to 10 μm) occurs mostly in warm freshwater where it primarily colonizes the surface and elicits columnaris disease in several species of freshwater fishes. Ulcerations of the dorsal part of the fish are associated with inflammatory reactions indicating upregulation of immune processes, but they are not sufficiently effective as the infection may spread to inner organs. When rainbow trout is exposed to infection by immersion into a solution of live *F. columnare* bacteria, the epithelia are stimulated immediately and display hyperplastic changes within hours. Innate responses reflected by expression of the iNOS, and cathelicidin genes occur at the same early time points. Immunoglobulin gene expression (IgT, IgD and IgM) is increased during the course of infection, suggesting that B cells play a significant role in protection (Tongsri et al. 2020). This suggests that an adaptive response is raised in the fish by the infection leaving the possibility of producing a protective vaccine. Vaccines have been tested, and a recombinant vaccine was shown to confer significant protection in immersion vaccinated channel catfish (Lange et al. 2019). These fish also showed an increased skin antibody production following immunization.

16.2.8 *Flavobacterium branchiophilum*

This is a related species, which infects primarily gills of salmonids eliciting bacterial gill disease associated with severe hyperplastic changes in gill structure. No vaccines are available against this infection, but a genetic disposition factor for infection has been indicated at the farm level (Bulfon et al. 2020). A comparative study showed that susceptible fish—in contrast to resistant fish—exhibited a lower lysozyme activity in mucus and reacted with a stronger inflammatory response, when exposed to *Lactococcus garvieae*, as judged by the expression of the genes encoding the pro-inflammatory cytokine IL-1β and its corresponding regulator cytokine IL-10. In contrast, innately resistant fish showed high respiratory burst activity in head kidney cells and higher lysozyme activity in mucus, suggesting that these factors are involved in vulnerability to infection (Bulfon et al. 2020).

16.2.9 *Rickettsia*

These Gram-negative organisms are obligate intracellular organisms, and *Piscirickettsia salmonis* is one of the best studied pathogens in this group. The bacterium spreads among fish in marine farms, causes septicaemia in salmonids and may have devastating effects on mariculture production. Although all organs are affected, it is especially the targeting of the haematopoietic tissue associated with inflammation, oedema, necrosis and a granulomatous response, which is problematic. The bacterium survives phagocytosis by macrophages as it persists in cytoplasmic vacuoles resisting the action of lysosomes. This may be achieved by a pathogen-induced modulation of lysosome density and function (Perez-Stuardo et al. 2019). As in many other pathogen–host systems, there is a potential for genetic selection of rainbow trout with a higher natural resistance to *R. salmonis* infection (Yoshida et al. 2018). Since the year 2000, another rickettsial disease, termed red mark syndrome (RMS), has spread among rainbow trout farms. The causative agent is considered a Rickettsia-like organism termed *Midichloria,* which has been shown to spread among cohabitated fish (Jørgensen et al. 2019). Discolouration of the fish appears 2–3 months post-exposure, as more or less confluent red skin spots, leading to downgrading of the product. Fish farmers claim that fully recovered fish become immune and controlled experimental studies have demonstrated a marked immune reaction in the affected part of the fish skin. Affected fish show a significant upregulation of inflammatory cytokine genes encoding IL-1β, IL-8, IFN-γ and the regulatory cytokine IL-10. Upregulation of effector molecule genes encoding SAA, IgT, IgD and IgM demonstrates the establishment of an initial innate response, which is then followed by an adaptive response involving several antibody classes. Correspondingly, immunohistochemical analyses show a clear infiltration of the skin with T-like lymphocytes (CD8+) and B lymphocytes (IgD+, IgM+) (Jørgensen et al. 2019).

16.3   Immune Responses Against Gram-Positive Bacteria

16.3.1 *Renibacterium salmoninarum*

This Gram-positive short non-motile rod-shaped bacterium elicits bacterial kidney disease (renibacteriosis) in salmonids. Exposure of a fish host to the bacterium clearly stimulates the immune system, but the immune reactions established are not able to eliminate the bacterium efficiently. It is able to resist ingestion by macrophages due to a highly immunomodulating and protective surface molecule termed p57 (Grayson et al. 2002). The bacterium proliferates in internal organs and kidney causing extensive necrosis. Despite the development of an inflammatory response involving macrophages, with expression of genes encoding IL-1β, MHC II, inducible cyclo-oxygenase (Cox-2) and inducible nitric oxide synthase (iNOS), the pathogen was able to survive (Grayson et al. 2002).

16.3.2 Gram-positive Cocci

16.3.2.1 *Lactococcus garvieae, S. iniae* and *S. agalactiae*

Several species of Gram-positive cocci elicit infections in fish. It is relevant to distinguish between coldwater streptococcosis and warm water streptococcosis. The latter group has a prominent species *Lactococcus (Streptococcus) garvieae* infecting a range of different fish species occurring in Europe, Asia, Africa and Australia. Other species such as *S. iniae* and *S. agalactiae* may infect tilapia and rainbow trout. These Gram-positive cocci are serious pathogens and elicit hemorrhagic septicaemia and high mortality, and the pathological reaction associated with the disease indicates a marked inflammation and involvement of most immune factors. Vaccination may confer some protection to the fish, but due to a high degree of strain differences and antigenic variation very specific vaccines should be used in order to achieve protection. Immunization of tilapia with various recombinant *S. agalactiae* proteins confers protection against challenge with the live bacterium. Resistance is based on specific antibody production following upregulation in the spleen and head kidney of genes encoding cytokines TNF-α, IL-1β, IFN-γ, chemokines and immune cell-related molecules such as CD8α and MHC factors (Ma et al. 2020).

16.3.3 Mycobacteria

16.3.3.1 *Mycobacterium marinum* and *M. fortuitum*

These bacteria are Gram-positive, non-motile and acid-fast rods. They elicit a slowly progressing granulomatous disease in a wide range of fish species. Infections of salmonids, tilapia, turbot, halibut, cod and sea bass have been described in depth, but a series of other species are susceptible as well. The bacteria possess immune-modulating mechanisms. Despite the activation of humoral and cellular responses, the infection persists and leads to severe pathological reactions reflecting the overt immune reactions. These include exophthalmia, depigmentation and skin abnormalities. Due to the inferior effect of immune reactions on the bacterium, the host eventually develops granulomas around the sequestrated bacterial foci. By the use of a zebrafish model, Harjulaa et al. (2020) performed a transcriptomic analysis and highlighted several immune pathways activated by injection of *M. marinum*. The inflammation induced by the infection involved a range of chemokines and was associated with an acute-phase response that included neutrophil degranulation and mast cell activity. Innate responses are clearly involved in the response. This was shown by upregulation of genes encoding factors such as SAA, hepcidin, lysozyme, AMPs and complement factors, whereas regulation of genes associated with immunoglobulin production represented the adaptive response.

16.4   Conclusion

The bacterial pathogens, which successfully circumvent the antibacterial substances in mucus and cross the external barrier of the fish surface, and thereby gain entrance into the fish organism, represent a diverse spectrum of different categories of bacteria. However, compared to the number of bacterial taxa present in the environment of the fish and in the surface microbiota of the fish, the number of pathogens is relatively low. This indicates that fish surfaces possess a number of protective mechanisms, which are able to clean away bacteria before they reach the interior. In addition, the specificity of the host–pathogen relationships shows that species/serotypes/biotypes have a selective pathogenicity. Thus, one pathogen strain may be extremely pathogenic or lethal to a certain host species or host strain, whereas other fish types perform well, even in an environment with a high infection pressure. This makes research across the fields of fish pathology, immunology and genetics highly relevant. Older classical breeding studies, together with recent studies based on improved DNA technology, have shown that genetic breeding of pathogen-resistant fish is one of the most promising ways to elevate health status of farmed fish. Together with developments within fish vaccine technology, this approach will pave the way to more sustainable fish farming, securing improved health and welfare among fishes. The outer protective shield of the fish is the epithelium with its production of mucus and antibacterial compounds. Already at this point, the successful pathogen must possess mechanisms to circumvent these basic protective pathways and genes encoding these factors are likely to be involved in natural resistance. Following entry into the host and subsequent dissemination to various target organs, further host immune mechanisms are deployed. Cellular and humoral immunological mechanisms are in play from the early encounter between bacteria and host tissue, where they exploit their potential to inactivate and eliminate the invading bacteria. The extensive network of chemokines and cytokines acts as an efficient communication between these protective elements, but as described in the cytokine chapter and the chapter on MHC new discoveries in cytokine research and innate and adaptive immune mechanisms are still adding new knowledge and understanding to the field. The innate immune responses, activated by a series of receptors described in the innate response chapter (Chap. 2), may silence the pathogens and thereby render the fish free of infection. However, various pathogens possess mechanisms to resist attack from defences of a cellular and humoral nature whereby the bacteria remain in the host. Prolonged infections require T- and B-cell responses (Chap. 5), dependent on the specific pathogen, for their final elimination. Highly resistant bacterial types, such as mycobacteria, are only partly controlled and sequestrated in granulomas. Also, in these cases a combination of research into genetic breeding and vaccinology will be a solution to the problem. These efforts rely on a thorough understanding of all elements in fish immunology. In particular, identification of the bacterial mechanisms and tools applied for survival in the host will be of value for selection of future vaccine targets. The present treatise on the principles within the field provides a valuable summary of recent research results. However, the fish immune system

is at present merely partly described and the future research efforts foreseen during the next years are likely to expand our knowledge significantly. This will inevitably improve the health of farmed fish.

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Antiparasitic Immune Responses

17

Kurt Buchmann

Abstract

Antiparasitic immune responses employ a common set of host immune factors but display a large variation over the theme. The number of fish species counts far more than 33,000, and due to the high host specificity of parasites, the number of fish parasites is probably even higher. In addition, the parasite group comprises protozoans (amoebae, flagellates, ciliates) and metazoans (myxozoans, monogeneans, cestodes, trematodes, nematodes, acanthocephalans, crustaceans) representing a diversity of life forms. These parasite types apply a broad field of mechanisms to modulate the host response optimizing survival of the parasite species in question. Despite these differences among parasite groups, a common set of immune reactions are activated upon infection although variations occur due to the highly varying surface molecules and excretory/secretory compounds released during the infection process. These are recognition of PAMPS by PRR, subsequent signal transduction and initiation of inflammatory responses, the expression of innate protective effector molecules, antigen presentation and establishing adaptive T- and B-cell responses. The host immune reactions can control the infection, but immune-modulating mechanisms have evolved to secure an optimal survival of the parasite type securing survival and spread of the species. The local release of immune-modulating substances in the microhabitat may explain why established parasites survive, while younger invading parasite stages fail to reach their destination. This opens the perspectives for the production of antiparasitic vaccines in the future.

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Abbreviations

PAMP   Pathogen-associated molecular pattern
PRR   Pattern recognition receptor
TLR   Toll-like receptor
AMP   Antimicrobial peptide
APP   Acute-phase protein
Ig   Immunoglobulin
C3   Complement factor C3
SAA   Serum amyloid protein A
ILC   Innate lymphoid cell

17.1   Introduction

The term "Parasite" is derived from the Greek words "para" (next to) and "sitos" (grain, food) reflecting that the parasitic organism is associated with the host and is absorbing nutrients from the host. During this process, various forms of tissue injury may occur, whereby the host elicits an immune reaction although the extent may vary considerably between parasite types and species. In this context, it should be kept in mind that these pathogens comprise a highly heterogeneous group, ranging from minute protozoans to large helminths, interacting with the host in different ways. The life cycle strategies of some parasite species operate by affecting the host minimally but manipulating the immune response maximally. Other parasites will in their larval stage benefit from a decrease in the physiological performance of the host, whereby they optimize predation, which ensures that the larva reaches the final host for maturation. Some highly pathogenic parasites have a high reproductive potential and will survive as they swiftly spread to other populations or subpopulations of hosts. In all cases, parasites may modulate the host response, whereby the expulsion/rejection of the established parasite is circumvented and survivability of the parasite is optimized. However, due to the well-described host response—although reduced towards the established parasites—new infections may be prevented, a condition termed "concomitant immunity". Due to the extended exposure of the host to parasite immunogens, it is well established that the fish is able to distinguish self from non-self. The fish reacts specifically towards antigens and brings immunological memory into play. Both innate and adaptive elements play a central role in all the reactions starting with recognition of the pathogen by host receptors, signal transduction leading to the production

of chemokines and cytokines, and initiation of inflammatory reactions with the recruitment of immune cells (granulocytes and lymphocytes) to the infection focus. The subsequent involvement of specific T cells and B cells and eventual sequestration, encapsulation or elimination of the parasite has been described in many parasite–host systems.

17.2 Parasite Types

Although the fish host deploys most of its defence weapons when being exposed to foreign antigens, it is worthwhile to remember that fish parasites comprise a highly heterogeneous group ranging from protozoans (amoebae, flagellates, coccidians, ciliates) to metazoans (myxozoans, monogeneans, cestodes, trematodes, nematodes, acanthocephalans, crustaceans). They may target all host organs, and thereby, it is evident that a full description of immunity in fish against parasitic infections is difficult, if not impossible, to reach in a single book chapter. The topic is extremely broad and displays an immense number of variations over innate and adaptive immunity mechanisms. In addition, as the number of described fish host species exceeds 33,000, and as many of the parasite species are relatively host-specific, one can infer that the total number of parasite species, and thereby host–parasite relations, must be very high. The different parasitic pathogens target different host organs. First of all, the external surfaces, which are directly exposed to pathogens in the environment, such as the skin, fins, buccal cavity and gills, are the preferred sites of many pathogens. Other species target the internal surfaces such as the oesophagus, stomach and intestine. Internal organs comprising the vascular system, heart, brain, eyes, liver, spleen, kidney, swim bladder, urinary bladder, ureters, stomach and intestine provide space for various parasites invading tissues and lumen. The fish hosts investigated so far appear to apply the general set of immune factors described in this book in their response, but the subsequent extent of activation and downstream reactions relies on the nature of the pathogen and the injury induced. Generally, the immediate response is dependent on the extent of host injury. Both cellular and humoral elements are in play when a fish become infected with a parasite, but the extent of activation is dependent on the level of invasion in tissues and any immune-evading mechanisms exhibited by the parasite.

17.3 Model Systems to Elucidate Immune Reactions in Fish Against Parasites

Important information can be obtained from studies on wild or aquacultured fish naturally infected with one or more parasites. However, the history of infection, including dosage and time post-exposure, is in these cases unknown and can limit interpretation of data because acute and chronic reactions may differ towards a particular parasite species. The optimal systems should be based on controlled experimental infection of naïve and disease-free fish. Acute and chronic reactions should be addressed, and reinfection studies should

follow. The infection dosage and the physico-chemical conditions during exposure and post-challenge will then be known, and recorded changes in immune reactions can be ascribed to the particular pathogen and age of infection.

Relatively, few parasite–host models fulfil these criteria, but in the following section the main immunological reactions post-infection are described for selected species.

First of all, the parasite has to locate and then successfully colonize the target host and the organ. The mechanical barriers comprising the mucus layer, produced by a dense layer of mucous cells (goblet cells) in epithelia of gills, skin, fins or the gastrointestinal tract, may limit further colonization. The specificity of the parasite may also determine if the parasite will establish an infection. Various parasite species show a highly expressed preference for certain host species, whereas they are repelled by others. Chemical clues of the host (mucus, carbohydrates, serum proteins, lectins) may determine if the parasite stays within the host or leaves. Several innate receptors, termed pattern recognition receptors (PRRs), may interact with molecules on the surfaces of the pathogens, pathogen-associated molecular patterns (PAMPs), whereby they play a prominent role in this early encounter between parasite and host. If the parasite does not become expelled or deterred by the external barrier, the infection process may proceed. The host may be colonized or penetrated whereafter the underlying tissue reacts with an expression of one or more of the pro-inflammatory cytokines such as IL-1β, TNF-α, IFN-γ, IL-6, IL-8 and associated and regulatory cytokines such as IL-10 (see Chap. 11). At the same time, the expression will be initiated for genes encoding a range of innate factors including acute-phase reactants (such as CRP, SAA, hepcidin, precerebellin and complement factor C3). Fast-reacting innate cell types (macrophages, granulocytes including neutrophils) and innate lymphoid cells (ILCs) are generally attracted to the site of infection mainly guided by inflammatory mediators including complement factor fragments and by IL-8 and other chemokines. The cells adhere to and penetrate the endothelial cell linings of capillaries and move into tissues to interact with the pathogen in a process termed extravasation. Following an initial production of fast-reacting innate factors, capable of reducing a part of the parasite population attached to the host, the adaptive responses are activated. This process is of longer duration, and weeks to months are needed before a protective stage is reached. Specificity and immunological memory are the hallmarks of this response, and although it takes time to develop the reactivity, it may be sustained for years. Both immunoglobulin-producing B cells, helper T cells and directly reactive T cells are involved in the process guided by a series of cytokines such as IL-4/13. The different life cycle strategies of the parasite types determine to a wide extent the outcome of the infection.

17.4 Protozoans

Host reactions may be severe but dependent on the depth of parasite invasion. A light attachment of a few single-celled parasites on the gill or skin surface of a fish has a low impact on the fish. If the parasite penetrates the external barrier and injures the epidermis

and underlying tissues the reaction will inevitably be far more pronounced. However, the high reproductive rate of some non-invasive protozoans allows a massive increase in the parasite population, which will impact the fish.

### 17.4.1 Amoebae

17.4.1.1 *Neoparamoeba perurans*

A range of studies have elucidated the responses of Atlantic salmon gills towards gill amoebae (Sokolowska and Nowak 2020). The basic observation is epithelial hyperplasia, at least partly associated with the expression of the pro-inflammatory cytokine IL-1β, where epithelial cells proliferate extensively resulting in gill clubbing (Adams and Nowak 2003). Some early studies indicated that a previous infection may confer some protection against reinfection (Findlay et al. 1995). These promising observations urged immunological investigations. Consequently, a range of experimental vaccines have been produced and tested, but none of them have as yet conferred successful protection of vaccinates (Sokolowska and Nowak 2020). Innate mechanisms may play a central role in the pathological reaction observed microscopically, but the lack of an effective antiparasitic response may be associated with downregulation of immune genes associated with antigen processing (Young et al. 2008).

17.4.2 Flagellates

17.4.2.1 *Ichthyobodo necator*

The ectoparasitic flagellate *Ichthyobodo necator* (Fig. 17.1) attaches to the fish epithelium and insert its posterior part into the epithelium. This injury elicits a marked skin reaction, which initially is dominated by inflammatory reactions but switches into a more benign reaction, resembling a Th2 response. The injuries are generally less extensive because the parasite attack is restricted to the upper cell layers. This ectoparasitic flagellate targets skin, fins and gills of freshwater fish and elicits a clear immune response. However, it is highly dynamic and may change from the onset of infection to later stages in the infection course. Experimental rainbow trout infections (Chettri et al. 2014) followed by the use of IHC and qPCR of affected skin showed a significant increase in skin with regard to the density of IgM-positive cells concomitant with a marked increase in the IgM expression. IgT- and CD8-positive cells declined in number together with a reduced expression of genes encoding IgT and CD8 T-cell marker. Innate immune genes encoding lysozyme, SAA and cathelicidin 2 were upregulated. The involvement of cytokines (IL-1β, IL-4/13A, IL-6, IL-8, IL-10), the cell marker CD4 and the transcription factor GATA3 showed a significant increase after infection and suggested a partial switch towards a Th2 response.

Fig. 17.1 *Ichthyobodo necator*, an ectoparasitic flagellate on skin, fins and gills of trout. The basal part of the protozoan is inserted in between the epithelial cells. Courtesy of Dr. Ole S. Møller

0.01 mm
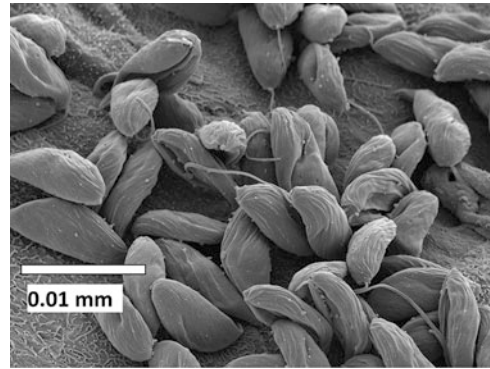
17.4.2.2 *Trypanoplasma borreli* and *T. danilewski*

These kinetoplastid flagellates target cyprinids in both farms and natural waters. They are transmitted by a leech vector. In the aquatic environment, these blood-feeding helminths secure an effective transfer of the delicate blood parasites from one host to another. In the leech, the parasite exists in the trypomastigote stage before it is transferred to the teleost host following incision of the host skin and opening of underlying vessels. When present in the vascular system of the fish, the parasites are exposed to the entire armament of the carp immune system, which necessitates the establishment of an effective immune-modulating barrier in the parasite. The mechanisms comprise first of all skewing of the immune response towards a Th2 reaction and macrophage polarization (Joerink et al. 2004), but a series of intricate systems are in play. Peripheral blood leucocytes in carp are the first host cells to interact with *T. borreli,* and evidence for a Tlr20 recognition of the parasite has been presented (Pietretti et al. 2014). In addition, a soluble immune type receptor (SITR) produced by macrophages seems to play a role in the nitric oxide-mediated response towards it (Ribeiro et al. 2011). Among other factors, thrombocytes in carp are important immune molecule-producing cells and are thereby an important element in the response of carp. It is therefore noteworthy that *T. borreli* induces a marked depletion of thrombocytes from the fish circulation. The parasite may eliminate these host cells via a nitric oxide-induced apoptosis (Fink et al. 2015).

17.4.2.3 *Cryptobia salmositica*

This haemoflagellate, infecting salmonid fish, may apply a vector for transmission. The freshwater leech vector *Piscicola salmositica* delivers the pathogen during its feeding on the host blood, but controlled experiments have confirmed direct transmission from fish to fish (Woo and Wehnert 1983). One of the main virulence factors of this flagellate is the 200 kDa metalloprotease degrading host fish collagen (Zuo and Woo 1997), and both innate and adaptive host responses involve this factor. The immune reactions in salmonids against the haemoflagellate *Cryptobia salmositica* are very host-specific. Even related salmonid hosts show different innate responses towards the parasite, and the alternative complement cascade is responsible for protection in resistant fish (Forward and Woo

1996). Even diluted plasma from the naturally resistant fish host lyses the parasites within one hour. In addition, a certain plasma level of α_2 macroglobulin, a naturally occurring antiprotease, allows the host to tolerate a rather high parasitaemia. The metalloprotease is neutralized by the protein emphasizing that the proteases play a key role as virulence factor. Various types of granulocytes become prominent in the blood of parasitized fish within a few weeks after infection indicating a strong innate cellular response. Susceptible but surviving fish are capable of mounting an adaptive immune reaction, which is reflected by a rise in parasite-specific antibody titre within a few weeks post-infection or immunization with an attenuated strain of the parasite (Sitja-Bobbadilla and Woo 1994; Chin and Woo 2005). Involvement of cellular immune responses is shown by the elevated occurrence of lymphocytes and monocytes in the blood of vaccinated fish 6 weeks post-immunization. In these fish, complement-fixing antibodies play a role in protection probably by application of the classical complement cascade (Li and Woo 1995).

17.4.3 Ciliates

17.4.3.1 *Philasterides dicentrarchi*

Various species of scuticociliates cause severe problems in aquacultured fish populations, but *P. dicentrarchi* is particularly pathogenic for farmed turbot. Infection elicits an immune response, which in certain cases may be protective (Piazzon et al. 2013). This has inspired development of various vaccine types, with and without adjuvants. Turbot peritoneal cells responding to the vaccines induced expression of genes associated with cell adherence and cell migration, and an M2 macrophage response was indicated (Fontenla et al. 2016). Repeated exposure to live ciliates may reflect the natural immunity induced when compared to intraperitoneal (i.p.) injection of parasite antigens. The encounter between host surfaces and the infective parasite initiates an inflammation in turbot, and gene expression analyses of internal organs in repeatedly exposed fish indicate the involvement of adaptive immune responses. Protection may be associated with a significantly increased IgT level in the host mucus measured 40 days following the last exposure to the parasite (Valle et al. 2019).

17.4.3.2 *Ichthyophthirius multifiliis*

Some highly reproductive parasites, such as the parasitic ciliate *Ichthyophthirius multifiliis* (Fig. 17.2), may elicit protective immunity in the fish host unless the infection is lethal (Hines and Spira 1974; Matthews 2005; Dickerson and Findly 2017). In highly exposed environments, the parasite can easily kill the fish host, but before reaching this stage it may have propagated many folds, whereby the species may spread to new naïve hosts (Hines and Spira 1973; Jaafar et al. 2020). The infective stage, the theront, is quite small, measuring 30–40 μm (Li and Buchmann 2001), and reactions towards the infection bears a series of similarities to antibacterial responses. The establishment of a protective immune response against the infection has been known for more than a century (Buschkiel 1910),

Fig. 17.2 Infective stage, the theront, of the parasitic ciliate *Ichthyophthirius multifiliis.* Courtesy of Dr. Ole S. Møller
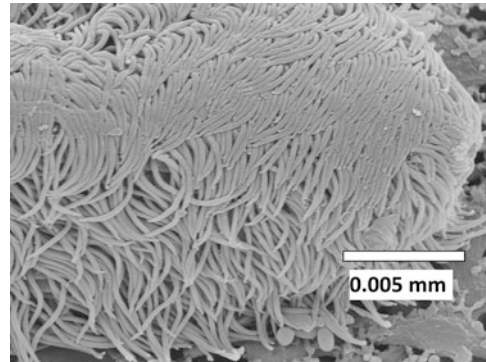
and the infection of fish and the subsequent development of immunity towards this parasitic ciliate has been studied extensively for decades (Bauer 1953; Hines and Spira 1973; Matthews 2005). Infected fish may succumb, but if they survive the infection immunity towards reinfection may develop (Sigh and Buchmann 2001). The microhabitat (epidermis) of the immunized fish presents a hostile micro-environment, which forces the invading theronts to escape the skin within a few hours (Dickerson and Clark 1998; Jørgensen et al. 2018). These observations urged research groups to develop and investigate experimental vaccines based on live, killed and/or formalin-inactivated parasitic stages (Goven et al. 1980; Burkart et al. 1990; Ling et al. 1993; Alishahi and Buchmann 2006; Xu et al. 2008; Moreira et al. 2016), recombinant protein antigens (He et al. 1997; Jørgensen et al. 2018) and DNA vaccines (Jørgensen et al. 2017; Xu et al. 2019). Despite some protection conferred by these experimental vaccines, there are still no available commercial vaccines on the market. However, the experiments have allowed investigations into the nature of the protective immune mechanisms deployed in the host reaction. Classical approaches to investigation of immune reactions in the fish comprised histopathological techniques showing extensive cellular responses comprising epithelial hyperplasia, mucous cell hyperplasia, attraction of various granulocytes including neutrophils, basophils, eosinophils and eosinophilic granular cells (Hines and Spira 1973; Ventura and Paperna 1985; Cross and Matthews 1993; Jørgensen et al. 2018). Lymphocytes are also attracted to the site of infection, at least partly by inflammatory cytokines (Sigh et al. 2004a). Some may be non-specific in nature (Graves et al. 1985), whereas others are CD8-positive T cells and immunoglobulin-producing B cells (Olsen et al. 2011). These cellular accumulations suggest that both innate and adaptive responses protect the fish against infections. The partial protection towards *I. multifiliis* infection, which was documented in rainbow trout recovering from a *Gyrodactylus derjavinoides* infection (Buchmann et al. 1999), indicates that non-specific responses provide some protection. Both non-specific cytotoxic cells (Graves et al. 1985) and neutrophils (Jørgensen et al. 2018) may be among the reactive effector cells involved. Cells at the infection focus produce inducible nitrogen oxide synthase responsible for the release of reactive N-species. In addition, complement factors (Sigh et al. 2004b; Gonzalez et al. 2007a) and plasma proteins such as SAA (Gonzalez et al.

2007b) are important innate protective elements involved in disease resistance. Among the adaptive host factors, immunoglobulins are well-known elements in protection against this ciliate (Clark et al. 1988; Lin and Dickerson 1992; Xu et al. 2013; Wang et al. 2019). The adaptive response was indicated by the expression of MHCII and IgM genes in skin and head kidney indicating a systemic response (Sigh et al. 2004b). Establishment of the systemic response may be initiated in internal immune organs (head kidney, spleen), but it is noteworthy that antigen-presenting cells occur in the mucosal surfaces of rainbow trout (Kato et al. 2018) where T-cell assemblies also occur (Koppang et al. 2010; Olsen et al. 2011). The external mucosal surface of the fish covers skin, fins, buccal cavity and gills, and it is likely that local responses are of particular importance as even the discrete olfactory organ is protected (Yu et al. 2018). This suggests that the lasting reaction is established, at least partly, at an early time point during infection. Host antibodies react specifically with surface glycoproteins in the surface of the pathogen, and cross-binding of the so-called i-antigen immobilizes the parasites in vitro (Wang et al. 2002). This suggests this mechanism plays a role behind the protective response, as cross-binding of ciliate surface antigens by host antibodies may trigger the escape reaction from immunized fish skin (Dickerson and Clark 1998). Both IgM and IgT bind to the parasite surface (Jørgensen et al. 2011; Xu et al. 2013), but the latter Ig class is associated with the protection of mucosal surfaces in fish and a likely candidate to be the first antiparasitic adaptive molecule in immune fish (Buchmann 2019). When applying zebrafish as a model to study reactivity in teleosts towards this ciliate, it was found that genes associated with adaptive immunity but skewed towards a Th2 type response were activated. However, a clear involvement of neutrophilic granulocytes, also at later stages of the infection, was documented (Jørgensen et al. 2018). The introduction of transcriptomic analyses including RNA sequencing and quantitative real-time PCR of fish infected with *I. multifiliis* has enlightened this field. The reactions established at the first encounter between the host and the parasite have been elucidated by Syahputra et al. (2019). The authors showed that rainbow trout genes encoding Toll-like receptor signalling pathways, especially TLR8, and the chemokines CCL4, CCL19, CCL28, CXCL8, CXCL11, CXCL13 and CXCL14 become significantly upregulated at this early time point. Subsequently, the initiation of the inflammatory response can be measured, facilitated by activation of mediator genes encoding IRF7, p38, JNK and MAPKs. Rainbow trout pro-inflammatory cytokine genes are then highly upregulated followed by a series of innate effector molecule genes. The expression levels of genes encoding cytokines (IL-1β, IL-4/13A, IL-12, IL-22, IL-2, IL-6, IL-17A/F2, IFN-γ, IL-8, IL-17C1, TGF-β, IL-10, IL-17C2 and TNF-α) in gills, spleen and liver of the trout have been studied (Jaafar et al. 2020). The gills and spleen showed higher expression of cytokine genes, whereas all cytokine genes, except those encoding IL-8, TGF-β, IL-17C2 and TNF-α, were significantly downregulated in liver of all challenged fish. The expression of genes encoding effector molecules including secreted IgD (IgDs), membrane-bound IgD (IgDm), IgM, IgT, cathelicidin-1, cathelicidin-2, TCR-β, lysozyme, C3 and SAA was differentially expressed. The IgT gene was slightly upregulated in the gills of all fish groups, whereas it was downregulated in spleen and liver. The gene expression of IgM was

elevated in the gills of all fish and in the internal organs of surviving fish. The expression of cathelidin-1 and SAA genes was significantly upregulated in the gills, spleen and liver of all the challenged groups, whereas cathelicidin 2 gene expression was upregulated in gills and liver. The gene-encoding lysozyme was significantly upregulated in spleen and liver—but not in gills. The C3 gene was particularly upregulated in spleen and liver in surviving fish. The potential of even signalling molecules such as the chemokine CK11 to exert antimicrobial effects (also towards *I. multifiliis* theronts) adds to the notion that innate responses serve as the underlying protection towards the parasite (Munoz-Atienza et al. 2019). Despite the strong involvement of innate immune genes in the reaction of fish towards *I. multifiliis,* it is clear the adaptive elements that allow the establishment of specific recognition and immunological memory—and thereby lasting protecting—are in play. Immune pathway genes active in B-cell and T-cell responses are significantly upregulated in the gills of all infected fish (Jørgensen et al. 2018; Saleh et al. 2019; Syahputra et al. 2019; Jaafar et al. 2020), which support the notion that vaccination against ichthyophthiriosis is feasible. Future approaches to allow the protection of aquacultured fish against this disease may also be found in the interface between immunity and genetics. A genetic component in host immunity was suggested by Gleeson et al. (2000), and controlled infection studies applying microarray technology has been helpful for elucidation of this issue. By using this technique, quantitative trait loci (QTL) were described through location of 57,501 single nucleotide polymorphisms (SNP) in the rainbow trout genome. Fish-resisting infection showed resistance genes located at chromosomes 16 and 17 (omy 16 and omy 17), which calls for further analyses of genes on these genomic hot spots (Jaafar et al. 2020).

17.5   Metazoans

17.5.1 Myxozoans

These cnidarian parasites related to hydrozoa comprise a wide range of species, which target various host organs. The early stages in the host occur as amoeboid plasmodia spreading between internal organs before reaching the target for development into the sporogonic stage. Investigations on host immune reactions towards myxozoan parasites have mainly focused on economically important species, but clear indications of induction of protective immunity have been presented. This applies among others for *Tetracapsuloides bryosalmonae* (eliciting proliferative kidney disease PKD in salmonids), *Myxobolus cerebralis* (causing whirling disease in salmonids), *Sphaerospora molnari* in common carp, *Myxobolus honghuensis* in gibel carp *Carassius auratus gibelio*, *Myxidium giardi* in eels and *Enteromyxum leei* in sea bream. The PKD-eliciting parasite causes a state of chronic pathology and transcriptional changes (Abos et al. 2018). Both B-cell responses and T-helper cells are altered, but at least three types of immunoglobulins (IgM, IgD and IgT) are produced in response to the infection. Lymphocytes dominate the response, but the

Fig. 17.3 Sporogenic stage of the myxozoan *Myxidium giardi* sequestrated on eel gills with a minimum of tissue destruction
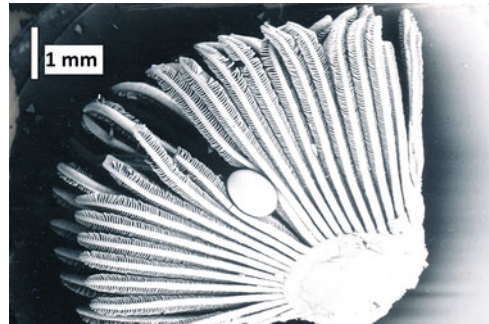
phagocytes are depressed and may account for increased sensitivity to other infections (Bailey et al. 2020). Although the extrasporogenic stages in internal tissues of, e.g. *M. giardi,* may elicit pathological reactions, and affect physiology and structure of the host, some sporogenic stages are sequestrated at external surfaces, such as gills, with a minimal effect on the host (Fig. 17.3). Correspondingly, *Myxidium* sp. encapsulated in eel skin elicit an epidermal dysplastic reaction, while a thin fibroblast and collagen layer encapsulates the sporogenic stage of the parasite (Manera et al. 2016).

17.5.1.1 *Tetracapsuloides bryosalmonae*

This myxozoan-eliciting PKD has a severe impact on fish health. Early histopathological investigations on diseased rainbow trout showed that the parasite colonizes the kidney interstitial tissue and develops from the extrasporogenic stage to the sporogenic stage. Its presence elicits a severe hyperplastic response with destruction of renal tissue including renal tubules and its vascular system. A fibrotic reaction and regeneration of tissue may follow in later stages (Bailey et al. 2020). Gene expression studies on the immune response of rainbow trout towards this parasite have shown that TLRs are actively involved in the innate response. As seen in many other infections, genes encoding antimicrobial peptides such as cathelicidins 1 and 2 were upregulated. The genes encoding inflammatory cytokines were downregulated, whereas immunosuppressive cytokines such as IL-10 are upregulated. Although myeloid and lymphoid cells take part in the response to some extent, it is noteworthy that their activity is suppressed and various mechanisms are found associated with immunosuppression. Regulatory T cells are actively involved as judged from the upregulation of their marker genes *foxp3a* and *foxp3b* in infected fish. Immunoglobulins are actively taking part in the response, and one symptom may be hyperimmunoglobulinaemia. The three Ig classes IgM, IgT and IgD are all involved in the humoral response, but especially IgT seems to coat the extrasporogonic stages indicating a role of these classes despite the modulation of the Ig production (Abos et al. 2018). The immune reaction is dynamic and may be skewed during the course of infection. Th1 type cytokines dominate at some infection stages, whereas a Th2 profile takes over in advanced clinical cases.

17.5.1.2 *Sphaerospora molnari*

This myxozoan has a severe impact on gill and skin health of common carp, but it has a presporogenic proliferating stage in the vascular system of the host. Despite exposure to the humoral and cellular defence systems in the carp blood, the parasite survives for several weeks. This clearly suggests that a series of immune-modulating mechanisms are applied by the parasite. Despite the presence of proliferating B cells and the production of specific IgM, the parasites isolated from carp blood were not coated by antibodies. The high upregulation of the gene encoding the immunoregulating cytokine IL-10 may partly explain the evasion (Korytar et al. 2019). In addition, the massive armament of protease genes in the parasite should be regarded as major virulence factors. Their presence may explain the efficient removal of the antibody coat readily produced when the parasite moves in the blood of the host (Hartigan et al. 2020).

17.5.1.3 *Enteromyxum leei*

This myxozoan parasite targeting the intestinal epithelia of gilthead sea bream *Sparus aurata* represents a major problem for Mediterranean mariculture. Protective immunity may develop in the host following recovery of a primary infection (Picard-Sanchez et al. 2019), but at present no effective vaccines are available. Evidence points to a systemic IgM response as an important factor in acquired immunity. IgT is present in the host but seems to play a minor role. Other immune factors with potential to induce protection are $\alpha2m$, a protease inhibitor, counteracting proteinase activity. Likewise, LCP1 (protease inhibitor of the cystatin superfamily of cysteine proteinase inhibitors) was found upregulated in the responding fish host (Picard-Sanchez et al. 2019).

17.5.1.4 *Myxobolus honghuensis*

A transcriptomic analysis has been performed on the effect of this myxozoan infecting the oesophagus of the gibel carp (*Carassius auratus gibelio*) and revealed that a series of innate and adaptive immune factors were highly regulated in infected fish. Genes encoding mucins, TLRs, lectins, SAA, cathepsins and complement factors were activated due to infection. Cytokines and chemokines were clearly involved in the regulation suggesting massive involvement of leucocytes including T and B lymphocytes and production of IgM and IgT (Zhao et al. 2019).

17.5.2 Monogeneans

Monogeneans are generally divided into the monopisthocotyleans (surface browsers) and polyopisthocotyleans (blood feeders). The different feeding strategies expose these types to very different immune responses. Monopisthocotyleans attach by sclerotized anchors of different shapes (Fig. 17.4) to the fish, and the mere mechanical penetration will elicit an inflammatory reaction in skin and gills of the host. Rainbow trout mounts a protective response against infections by *Gyrodactylus derjavinoides* reflected by a significant

Fig. 17.4 Two specimens of the monopisthocotylean monogenean, *Gyrodactylus derjavinoides*, attached (by their opisthaptors carrying anchors and hooklets) to the fin of a rainbow trout *Oncorhynchus mykiss*

0.1 mm
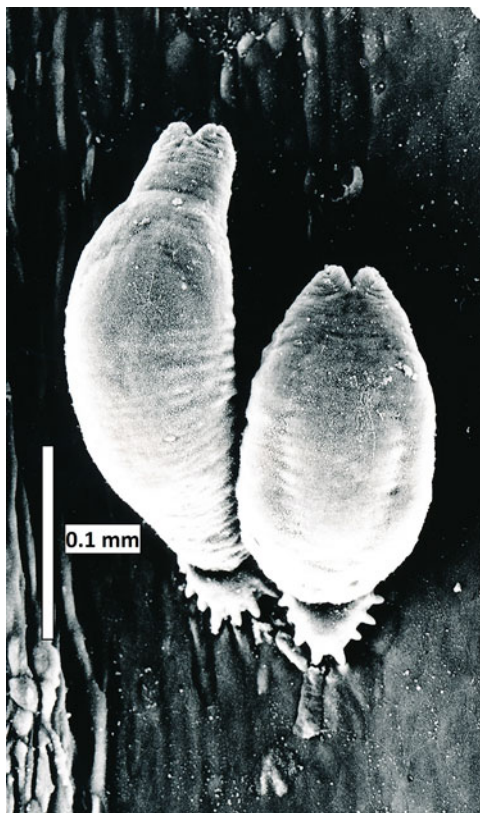
elimination of the parasite population on skin and fins (Lindenstrøm and Buchmann 2000). It may last for at least 70 days, and although adaptive elements may be involved, it is clear that innate immunity and non-specific immunity play a role (Buchmann et al. 1999). Gene expression studies showed that factors related to inflammation and innate responses (IL-1β, TNF-α, IL-8, iNOS and cyclooxygenase) were upregulated but also adaptive elements such as MHCII and TCRβ (Lindenstrøm et al. 2004). Similar studies on Baltic salmon infected by *Gyrodactylus salaris* on skin and fins showed a response in the fins reflected by increased the expression of genes encoding interferon, Mx, immunoglobulin and MHC genes (Kania et al. 2010). When the local reaction, comprising cellular and humoral responses, becomes too aggressive, the parasite *G. derjavinoides* may move to non-reactive sites (e.g. the corneal surface). When *G. derjavinoides* is exposed directly to fish serum (from both naïve and previously exposed fish), complement factor C3 is deposited on the monogenean tegument and the complement cascade is activated possibly through the alternative or lectin-binding pathway (see Chap. 20) leading to fast killing of the parasite (Buchmann and Bresciani 1999; Harris et al. 1998). Similarly, in vitro exposure of *G. derjavinoides* to host macrophages leads to fast colonization and killing of the parasite (Buchmann and Bresciani 1999). Adaptive responses in the mucosal surfaces

Fig. 17.5 Polyopisthocotylean monogenean, *Axine belones*, showing attachment clamps with a minimal mechanical impact on gill lamella of the garfish *Belone belone*. Courtesy of Eric Buchmann
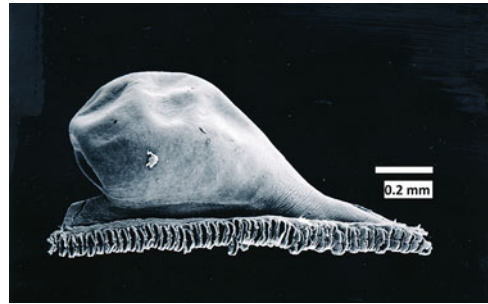
towards monopisthocotyleans exist, and heavy loads of the eel parasite *Pseudodactylogyrus* on host gills lead to a detectable host antibody production (Buchmann 1993).

Polyopisthocotyleans may attach by delicate clamps reducing the mechanical impact on the gill surface. Despite the large size of the monogenean, such as *Axine belones*, the attachment by the use of more than fifty clamps minimizes the pull in the gill lamellae (Fig. 17.5). Their direct feeding on full blood occurs through a disruption of the gill epithelia and capillaries. This will elicit some inflammation, but the epithelial host response is limited when compared to the host epidermis of fish infected by the broadly browsing and injuring monopisthocotyleans. Despite the limited contact between the host and the polyopisthocotylean parasite, a measurable antibody response (IgM) has been documented in trout infected with *Discocotyle sagittata* (Rubio-Godoy et al. 2003). The content of the blood meal contains, besides numerous red blood cells, a vast battery of reactive molecules and immune cells. This demands the establishment of a series of immune evasion strategies to counteract the action of immunoglobulins, complement factors, antimicrobial peptides (AMPs), proteases and acute-phase reactants. The parasite *Eudiplozoon nipponicum* produces proteases, such as cathepsin L, having an ability to cleave immunoglobulins at least in vitro (Jedličková et al. 2018). The production of various peptidase inhibitors in *E. nipponicum,* parasitizing the gills of common carp, has been well characterized (Ilgova et al. 2020). The inhibitors may serve several purposes, but a recombinant cysteine peptidase inhibitor termed Stefin of *E. nipponicum* induces a general immunosuppression by downregulating TNF-α and IL-10 when investigated in a mammalian in vitro model (Ilgova et al. 2020) and the secreted Kunitz domain protein (EnKT1) interferes with complement activation (Jedličková et al. 2019).

17.5.3 Cestodes

Cestodes (tapeworms) in fish invade various organs and tissues. Adult forms occur in the lumen of the pyloric caeca and the intestine *(Eubothrium* and *Bothriocephalus*). Other forms occur as larval stages (plerocercoids) in the body cavity or the musculature

Fig. 17.6 *Triaenophorus nodulosus*, plerocercoid stage of the pike tapeworm recovered from liver of the intermediate host, the perch

(*Triaenophorus*) (Fig. 17.6) or other organs such as *Gilquinia squali* in the immune-privileged corpus vitreum of the eye of the gadid whiting. Metacestodes including stages of *Dibothriocephalus* (syn. *Diphyllobothrium*), *Ligula intestinalis* and *Schistocephalus solidus* may occupy the peritoneal cavity and/or internal organs. The plerocercoid stage of *D. dendriticum* attracts host leucocytes but resists the host attack when a fibrotic capsule appears around the parasite. These cestodes may apply a series of immunomodulators in order to pacify the reactive immune factors released from the host (Hoole 1994). Likewise, stickleback infected by *S. solidus* react by involving TLR and macrophages in the initial phase of the invasion, whereas modulation of the reaction occurs in later stages (Haase et al. 2016). When opening the encapsulation during dissection, fully viable cestodes can be recovered reflecting that the immune modulators of the cestode effectively protect the worm. Adult forms of *Bothriocephalus acheilognathi,* parasitizing the carp intestine, elicit a marked inflammatory reaction in the intestinal mucosa where the bothria attach and compress the tissue eliciting an inflammatory reaction (Scholz et al. 2012). However, the parasite produces substances believed to modulate immune reactions in the host intestine optimizing survivability of the parasite (Hoole and Nisan 1994). The pyloric caeca of brown or rainbow trout are often parasitized by the cestode *Cyathocephalus truncatus*, carrying an apical sucker-like bothrium attaching firmly to the mucosa. This induces attraction of host leucocytes, but the parasite may even circumvent the extensive cellular reaction accompanied by fibrosis. The reaction produced locally in the tip of the pyloric caecum secures the attachment of the parasite, which may be difficult to remove during dissection (Hermanns and Körting 1987). Despite this marked cellular reaction, the scolex remains intact when dissected free from the encapsulation, reflecting that the cestode has evolved mechanisms to resist the attack.

17.5.4 Trematodes

The digenean trematodes (digenetic flukes) represented by eye flukes within the genera *Diplostomum* and *Tylodelphys* penetrate (in the cercarial stage) the surface of the fish host and migrate, following shedding of the tail, relatively fast (minutes to hours) as with the so-called diplostomule stage to the eye of the fish. Extensive inflammatory reactions in the target organ (the eye) are prevented as they would be devastating for the host and destroy the vision of the host. The entrance of innate or adaptive immune cells into the lens is limited, at least under normal circumstances. Immunoglobulins or other humoral factors do not get access to the interior of the lens and thereby the parasite remains in a non-hostile host site, an immunological refuge. The *Tylodelphys clavata* metacercaria occupies the vitreous humour (*corpus vitreum*) with a corresponding minimal inclusion of immune cells (Fig. 17.7). The lens is considered the preferred microhabitat of several *Diplostomum* species (such as *D. pseudospathaceum* and *D. spathaceum*). The metacercaria itself is highly susceptible to colonization and killing by rainbow trout leucocytes (Whyte et al. 1990), but due to the lack of immunological responses in the eye the metacercaria does not produce a protective cyst wall. Thereby, the metacercarial stage in the lens or the vitreous humour can remain alive and relatively active in a non-encysted stage for years. The diplostomule stage (the invading and migrating parasite without a tail) must be exposed, at least for a few hours, in the vascular system of the host before reaching the eye microhabitat, but a series of innate effector molecules may exert their effect on the parasite. Repeated infections of stickleback show that innate responses (TLR, macrophages, complement) are activated at the early phase of the infection, but the establishment of immunological memory is indicated by the expression of MHCII genes and the cytokine IL-4 (Haase et al. 2016). This corresponds to the observation that immunization reduces the number of diplostomules reaching their target in rainbow trout (Höglund and Thuvander 1990; Whyte et al. 1990). However, although antibody titres increase, experimental studies suggest that macrophage and their production of reactive oxygen radicals together with the level of complement factors, lysozyme and other acute-phase reactants can explain the partial elimination of diplostomules before they reach the eye.

Other trematode cercariae such as *Cryptocotyle lingua* penetrate the skin of the host (e.g. cod, plaice) but need to protect themselves (by forming a sclerotized proteinaceous cyst wall) against the massive cellular and humoral response in the host skin. This mechanical barrier of the parasite resists the host attack, and the metacercaria remain alive for extended periods awaiting the ingestion by a predator (seagull, dog) whereafter it excysts and infects the final host. The penetrating *C. lingua* cercariae elicit a humoral host response including measurable immunoglobulin production (Wood and Matthews 1987), which may, together with complement fixation, stimulate the parasite to secrete the protective proteinaceous cyst wall. The hardness of this cyst wall is reflected by its resistance to pepsin digestion at low pH in the stomach of the avian final host. A number of other trematode species, often in their adult stage, inhabits the stomach or intestinal lumen of the fish host but with a minimal contact. The two parasite suckers (Fig. 17.7) are

Fig. 17.7 Metacercaria of the digenean trematode *Tylodelphys clavata* isolated from the *corpus vitreum*—an immunological refuge—of rainbow trout
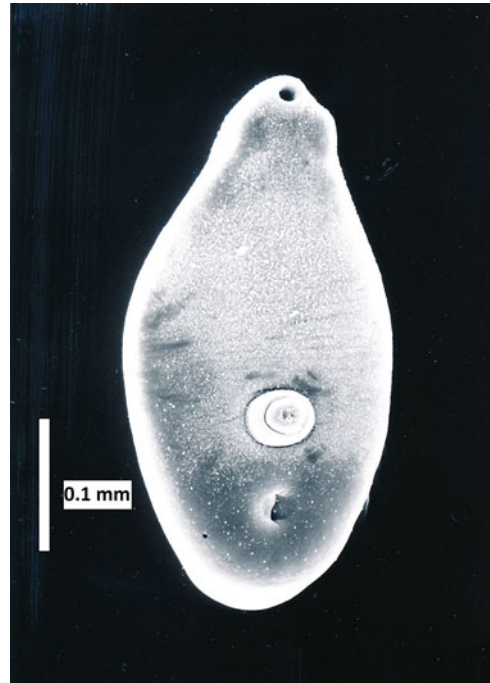
the focal contact points between parasite and host, whereby the need for immune-evading strategies probably is limited to these attachment organs.

Blood flukes within the family sanguinocolidae, located in the blood stream, are, in contrast to the eye flukes, highly exposed to immune attack by the host. Reactions target the penetrating cercariae, the adult parasites, the eggs produced and to some extent the emerging miracidia (larvae hatching from eggs deposited in gill tissue before release to the environment). Eggs deposited in gills and visceral sites (liver, spleen, pancreas) attract various leucocytes (eosinophils, neutrophils, macrophages) resulting in formation of granulomas, with subsequent infiltration with fibroblasts and deposition of collagen (Richards et al. 1994). Immunogens from adult blood flukes and cercariae elicit lymphocyte proliferation (Richards et al. 1996a, b), and common carp raises an antibody response when directly injected (i.p.) with live cercariae. In contrast, naturally infected carp remain unresponsive, which indicates that the blood flukes apply immune-evading mechanisms to stay alive. To unravel the survival strategies of these parasites, it will be beneficial to refer to the well-studied blood flukes parasitizing mammals, such as the genus *Schistosoma* comprising *S. mansoni* and *S. haematobium* of humans. The mechanisms applied to resist host attack (antibodies, complement) include disguising their surface with a series of host molecules (e.g. Schroeder et al. 2009). The complement factors of fish also seem to play a central role when fish hosts are responding to invading cercariae (Roberts et al. 2005) and

despite immunomodulation exerted by the parasite some partial protection is induced through immune priming by previous infections.

17.5.5 Nematodes

Nematodes occur mainly as adult forms in the lumen of the gastrointestinal tract although adult forms of some species inhabit various organs and tissues. This applies for the species *Cystidicola farionis* occupying the swim bladder wall of salmonids, including brown trout and rainbow trout. In addition, species within the genus *Philometra* occupy the vascular system. Larval stages of anisakis nematodes are often found in the body cavity or fish organs such as musculature, liver and gonads (Fig. 17.8). The different microenvironments encountered ranging from the gastrointestinal lumen, via the gut wall, the peritoneal cavity, internal organs and musculature present highly different concentrations of immunoreactive substances and cells. This imposes quite different pressures on the worms and their physiology. The anisakis larval stages are protected by a larval sheath and may thereby resist massive exposure to reactive immune molecules and cells. Bahlool et al. (2012) demonstrated the accumulation of CD8-positive T cells around the *A. simplex* larvae invading liver and pyloric caeca of experimentally infected Baltic salmon. Innate and adaptive host responses are involved during the larval migration in the host organs, but release of excretory–secretory compounds modulates the effects on the parasite (Bahlool et al. 2013). Rainbow trout experimentally infected by *A. simplex* regulate several immune genes in both liver and spleen. Genes encoding innate effector molecules such as complement factor C3, serum amyloid protein A, SAA and precerebellin were significantly upregulated together with the adaptive marker for CD4 positive T cells. In contrast, IgM and co-receptor CD8 genes were downregulated (Haarder et al. 2013). The Baltic cod, a subpopulation of the Atlantic cod, *Gadus morhua*, may be infected, by third-stage larvae of the anisakis nematode *Contracaecum osculatum* targeting the host liver. The parasites become encapsulated and protected in a fibrotic cell layer (Buchmann 2012) and immunological analyses indicate that the parasite suppresses reactions locally in the liver, when compared to the expression level in spleen in the same host (Marnis et al. 2020). It was noted that genes encoding effector molecules CRP, IFN-γ, lysozyme G1, hepcidin and C3 were downregulated in the liver reflecting a survival strategy of the worm. Other nematodes, e.g. the dracunculoides, apply other evasion strategies. The swim bladder nematode *Anguillicoloides crassus* resides as adult in the lumen of the swim bladder of eels where macrophages and granulocytes accumulate in the affected part of the swim bladder wall (Molnar et al. 1993). It feeds directly on eel blood taken from the capillaries and must neutralize a vast amount of innate and reactive molecules and cells ingested with the blood meal. The close contact between the host immune system and the worm leads to a strong antibody reaction (Buchmann et al. 1991). The antibodies seem to have little effect on the worm, whereas reactions target the invading larvae more seriously. Its larval stage migrates during the infection process from the intestine (released from the ingested

Fig. 17.8 Larva of the anisakis nematode *Contracaecum osculatum* leaving the fibrotic capsule in cod liver
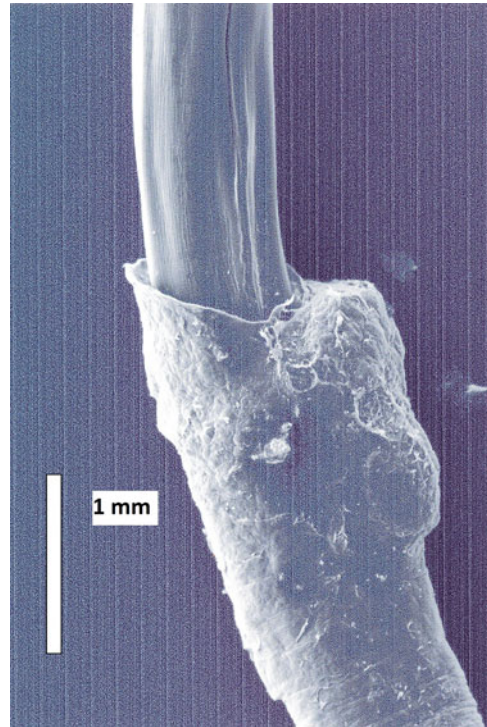

intermediate host, a cyclopoid copepod), through the body cavity and penetrates the swim bladder wall to reach the lumen. Immunized eel reactions can arrest and kill the migrating larvae during their way through the wall—although the adult worms remain viable in the swim bladder lumen. This so-called concomitant immunity is typical in parasitology, reflecting that the host is reacting, but the established parasite has established one or more immune-evading processes.

17.5.6 Acanthocephalans

These thorny headed worms colonize primarily the gut of the host and insert their proboscis into the intestinal wall. The helminths do not ingest host tissue but absorb nutrients from the intestinal lumen via their tegument. Some species such as *Echinorhynchus gadi* (Fig. 17.9), localized in the intestine of Atlantic cod, attach rather superficially to the mucosa and elicit minimal reaction and pathology. Deeper penetration of the proboscis into the intestinal mucosa, e.g. *Acanthocephalus lucii* into the pike intestine, elicits a stronger cellular reaction (Dezfuli et al. 2018). The penetration may in certain species such as *Pomphorhynchus laevis* be very deep and elicit a strong cellular reaction, eventually establishing a fibrotic capsule, which may be used by the parasite to attach firmly within

Fig. 17.9 Proboscis, armed
with hooks, of the
acanthocephalan
Echinorhynchus gadi
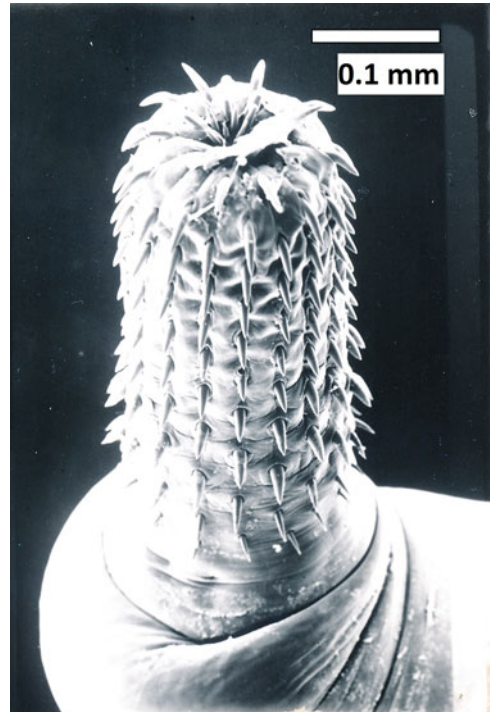
the gut wall (Taraschewski 2000). The front part of the parasite remains intact and unaffected by the massive cellular infiltration, with fibrotic layers making dissection of the worm difficult. Studies on the immune reactions raised against the infections are few, but histopathological investigations show infiltration with macrophages and mast cells (Bullock 1963; Dezfuli et al. 2018; Chine et al. 2020).

17.5.7 Crustaceans

The crustacean parasites are like monogeneans in settling and attaching to the host surface (gills, skin fins) and apply various forms of mechanical and/or chemical attachment. The copepod parasites *Lernaea cyprinacea* in carp and *Sphyrion lumpii* on redfish attach by inserting the cephalothorax deeply into the host tissue (e.g. the musculature). The cellular reaction involving granulomatous tissue, accumulation of monocytes, granulocytes and fibrosis around the attachment structures secures the attachment even further, and the reactive cells attracted to the site of infection have little effect on the parasite covered by a chitinous exoskeleton (Erlingsdottir et al. 2020). The more superficially located salmon louse (*Lepeophtheirus salmonis*) has several stages attaching to the skin of various salmonid species including Atlantic salmon, rainbow trout and sea trout. The parasite goes through an ontogenetic process comprising eight instar stages. After going through

Fig. 17.10 Adult stage of *Lepeophtheirus salmonis*, the salmon louse

1 mm
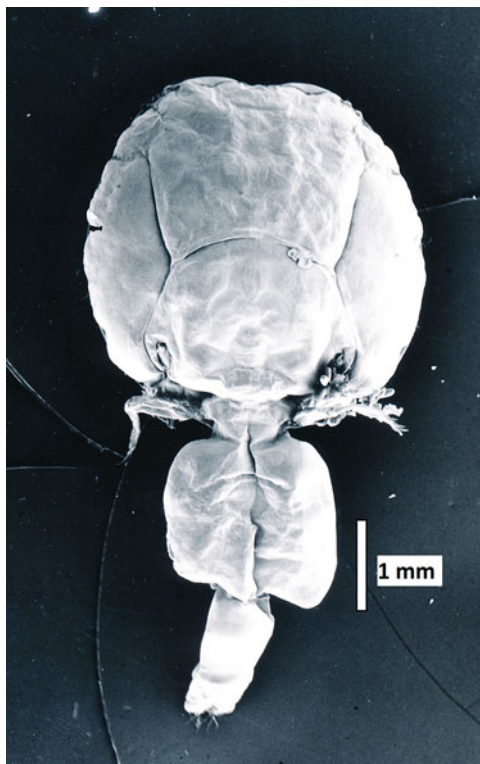
two planktonic naupliar stages before reaching the infective copepodid stage, the parasite settles on host skin. Then, it passes through additional moults to reach the adult stage (Fig. 17.10). Feeding occurs by scraping the epidermis and underlying tissue through the mouth tube, whereby reactions are, inevitably, mechanically provoked. However, the host reactions differ between the stages. First of all, the infective copepodid stage attaches and faces the antiparasitic substances in host mucus. Following a series of moults the chalimus, the preadult and the adult stages are reached. A controlled experimental infection of rainbow trout (Dalvin et al. 2020) demonstrated a partial but significant rejection of the parasite during the developmental process. During the process, the parasite burden was reduced from 80 copepodids (infection dosage) to 32 preadults and subsequently to 11 adults per fish. This reduction could at least partly be attributed to the host immune response. However, the surviving adults seem to apply a number of escape mechanisms, among others production of salivary secretions, to reduce the antiparasitic immune response. Previous studies have pointed to a parasite-induced skewing of the response in salmon towards a Th2-like reaction (Skugor et al. 2008; Braden et al. 2015). A corresponding modulation of responses was also noted during the late infection stage of rainbow trout. The presence of copepodid, chalimus and preadult stages activated a series of innate immune genes concomitant with progression of epidermal hyperplasia.

Granulocytes, macrophages and lymphocytes were attracted to dermal layers, especially locally under the attached parasite. During the progress of infection, a decreased expression of both innate and adaptive immune genes was observed (Dalvin et al. 2020). Immunohistochemistry demonstrated SAA-producing cells in dermis and epidermis reflecting that acute-phase protein-secreting cells were recruited to the site of infection. This presence of innate effector molecules was associated with the expression of genes encoding inflammatory cytokines (TNF-α, IL-1 β, IL-6, IL-8) and antimicrobial peptides (cathelicidin 2). The Th2 skewing was indicated by the expression of genes encoding the cytokine IL-4/13 and immunoglobulins IgD and IgM. Activation of the TGF-β gene indicated that regeneration processes were involved in reestablishing the integrity of the host epidermis following injury induced by the feeding parasite (Dalvin et al. 2020). The reduced reaction at the late stage of infection was interpreted as a parasite-induced immune depression possibly affected by parasite secretions.

17.6   Conclusions

The fish host immune reactions towards parasitic pathogens are extremely diverse. This is because the immune system of the more than 33,000 fish species differs to some extent and the number of parasite species that infect fish is even higher. However, one of the main reasons is that parasitic organisms (even when excluding bacteria, fungi and viruses) display an extreme variation with regard to chemical composition, anatomy, physiology and life history. Evolutionary processes have secured the existence of a wide variety of parasite life cycle strategies and the associated deployment of various immune modulation mechanisms leading to survival of the parasite species. Despite this challenge, when teleost hosts elicit responses towards parasites, it is also evident that a number of common reactions take place in all systems. The immunological fields described in the present treatise on fish immunology are all engaged in these host–parasite interactions. The first encounter between the pathogen and host initiates the interactions between PAMPs and PRRs, leads to the expression of cytokines involving both innate and adaptive pathways. The cellular involvement (macrophages, granulocytes, lymphocytes) is dependent on MHC, immunoglobulin and complement production. Due to the unsuccessful elimination of some parasites, a full range of different immunopathological reactions in host fishes may result from the infections. Further, the close relation between the fish host and the aquatic environment leaves the fish vulnerable to environmental changes. These can act as stressors with a potential to moderate the host immune responses towards the parasites. The existence of concomitant immunity in fish against young infective stages of a certain parasite, despite the presence in the same host of a developed parasite of the same species, shows that it is possible to establish protective immunity in fish against parasites. This infers that the production of antiparasitic vaccines is a possible path forward—although it may be a challenging task due to the existence of widespread immune modulation mechanisms. These should be explored and targeted in future vaccines.

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Immunopathology

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Abstract

Immunopathology is broadly defined as the study of immune responses associated with disease, including the study of the pathology of an organism, organ system or disease with respect to the immune system. As other chapters in this book discuss topics such as the immune responses of fish to infectious agents and the effects of stressors on the immune response, this chapter addresses a subcategory of immunopathology. Immunopathology is defined here as the study of pathological changes in organs of the immune system resulting from a variety of aetiologies, including infectious agents (viruses, bacteria, fungi and parasites) and non-infectious conditions (nutrition-related, toxicant-related and neoplasia). Whereas numerous studies on fish have focused on the host response to various stressors, relatively few have investigated immunopathology. This review utilises examples of infectious and non-infectious diseases in teleost fishes to summarise our knowledge of teleost immunopathology. For some diseases, the

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presence of an infectious agent or toxin may directly or indirectly cause destruction of immune organs. In other diseases, an intense inflammatory response, or conversely, suppression of the immune response, may contribute to disease progression in these organs. Other factors such as the virulence of a pathogen biotype, degree of toxicity or carcinogenicity of an exogenous element or compound, the species and age of exposed fish and environmental conditions such as water temperature and water quality can also contribute to determining the outcome of a disease. Additional research is needed to investigate the mechanisms responsible for immunopathology observed in many diseases.

Keywords

Fish immunopathology · Pathology · Immune organs · Melanomacrophage centres · Fish disease · Immunotoxicity · Neoplasm

Abbreviations

CCVD	Channel catfish virus disease
CyHV-2	Cyprinid herpesvirus 2
DBTC	Di-n-butyltindichloride
DNA	Deoxyribonucleic acid
HE	Haematoxylin–eosin (histological stain)
HIRRV	Hirame rhabdovirus
HVHN	Herpesvirus haematopoietic necrosis
IcHV-1	Ictalurid herpesvirus 1
IHNV	Infectious haematopoietic necrosis virus
ILT	Interbranchial lymphoid tissue
ISAV	Infectious salmon anaemia virus
ISKNV	Infectious spleen and kidney necrosis virus
MALT	Mucosa-associated lymphoid tissues
MMC	Melanomacrophage centre
OMVD	*Oncorhynchus masou* virus disease
PAHs	Polyaromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PHC	Petroleum hydrocarbon
qPCR	Quantitative polymerase chain reaction
RAG	Recombination-activating gene
RNA	Ribonucleic acid
SalHV-2	Salmonid herpesvirus 2
SVCV	Spring viraemia of carp virus
T3SS	Type III secretion systems

TBTO	Bis(tri-n-butyltin) oxide
USA	United States
VHSV	Viral haemorrhagic septicaemia virus
WHO	World Health Organization

18.1 Introduction

Immunopathology is a discipline at the interface between immunology and pathology. Here, we will focus on the pathology of various organs belonging to the immune system. While there is a lot of research done on fish immune response to different stressors, relatively little is known about fish immunopathology. Furthermore, studies on the immune response of teleosts using histology have been complicated due to lack of standard interpretation; for example, there is still no acceptance of a standard classification of fish granulocytes, particularly in fish histology using haematoxylin–eosin staining (Koppang et al. 2007; Adams et al. 2017).

This chapter summarises our knowledge on fish immune organ pathology based on aetiology of the conditions, including infectious—viral, bacterial, fungal, parasitic and non-infectious—nutritional, toxicant-related and neoplasms. Anatomy of immune organs is covered in another chapter (see Chap. 1) as are immune responses to difference pathogens (see Chaps. 13, 14, 16 and 17).

18.2 Viral Infections

Both RNA and DNA viruses commonly affect teleost fish. Major fish RNA viruses belong to the families *Reoviridae*, *Birnaviridae*, *Picornaviridae*, *Nodaviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Rhabdoviridae*, *Retroviridae* and *Togaviridae*. The major DNA viruses include members of the families *Iridoviridae* and *Adenoviridae* and the former family *Herpesviridae* (now *Alloherpesviridae*; Davison 2010). In general, viruses either cause hypertrophy of cells (e.g. lymphocystis virus or megalocytivirus) or cause multifocal areas of necrosis up to complete destruction of haematopoetic tissues. The affected fish can either develop clinical disease or may remain asymptomatic carriers. Affected fish may also become debilitated and suffer secondary bacterial infections. The amount of cell damage caused by the virus depends on the virus strain or genotype and the susceptibility of the host. Within the *Iridoviridae*, the megalocytiviruses are characterised by unique inclusion bodies within hypertrophic cells, the presence of which is pathognomonic for diseases caused by these viruses on histological examination. For example, the megalocytivirus infectious spleen and kidney necrosis virus (ISKNV) causes cell hypertrophy and necrosis in spleen and renal interstitial tissue of infected zebrafish (*Danio rerio*) (Xu et al. 2008). Infection with the same virus in Nile tilapia (*Oreochromis niloticus*) farmed in Ghana,

Africa, resulted in mild necrosis in the spleen and the renal haematopoietic tissue (Ramírez-Paredes et al. 2021). The ISKNV infections in tilapia were associated with haematopoietic cell nuclear and cytoplasmic pleomorphism with marginalisation of chromatin and fine granulation as well as vacuolation and increase in the number of melanomacrophage centres (MMCs) (Ramírez-Paredes et al. 2021). MMCs are located in a number of teleost immune organs, including the spleen, kidney and sometimes the intestinal submucosa and the thymus, with tissue distribution and size depending on factors such as fish species, age and health status (Agius and Roberts 2003; Steinel and Bolnick 2017). Because these melanomacrophages can contain different types of pigments, e.g. melanin, lipofuscin, ceroid, hemosiderin and others, re-naming these structures as "pigmented macrophage aggregates" has been suggested.

18.2.1 Rhabdovirus

Several members of the family *Rhabdoviridae* produce lesions in fish haematopoietic tissues. Prominent in this group are infectious haematopoietic necrosis virus (IHNV), which infects salmonids; viral haemorrhagic septicaemia virus (VHSV), which infects diverse freshwater and marine species; spring viraemia of carp virus (SVCV), which principally infects cyprinid fishes although it has been reported from non-cyprinid hosts; and hirame rhabdovirus (HIRRV), which infects several marine and freshwater species (Borzym et al. 2014; Woo and Cipriano 2017). IHNV infections are typically most severe in young fish and can cause acute and extensive necrosis of haematopoietic tissues of the kidney and spleen (Fig. 18.1, Bootland and Leong 2011). Initial changes in the anterior kidney may consist of small, lightly stained foci of apparent macrophages and degenerating lymphoid cells, and may progress to necrosis so severe that kidney tissue may consist primarily of necrotic debris (Bootland and Leong 2011; Leong and Kurath 2017). In the kidney, marked necrosis of vascular endothelium of the renal portal tissue and (in later stages) renal tubules can also occur (Yasutake 1970); other tissues commonly infected

Fig. 18.1 Kidney, interstitial necrosis (asterisk) due to infection with infectious haematopoietic necrosis virus (IHNV) in a rainbow trout (*Oncorhynchus mykiss*), HE stain, bar = 25 μm
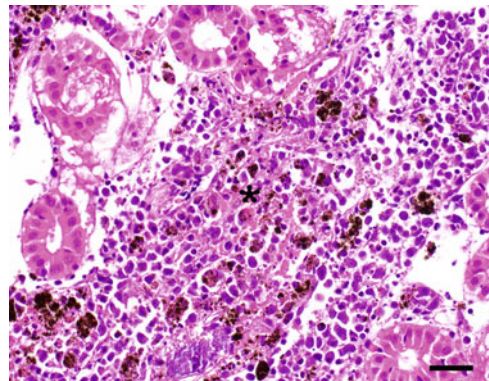
include liver, pancreas and digestive tract (Leong and Kurath 2017). Tissue lesions caused by VHSV are often systemic and are primarily necrotising, but patterns of the lesions can depend on the virus genotype and fish species (reviewed in Lumsden 2017). For example, VHSV genotype Ia in rainbow trout (*Oncorhynchus mykiss*) may produce necrotic lesions in kidney and liver, but other organs such as spleen, heart and brain can also be affected. In contrast, turbot (*Scophthalmus maximus*) infected with VHSV genotype Ib and several Great Lakes fish species infected with genotype IVb showed the most severe lesions in the heart, although liver and haematopoietic tissues were also affected. Severe vasculitis was also observed in many infected tissues of the Great Lakes fish (Al-Hussinee et al. 2011). Common histopathological changes observed in SVCV infections include oedema, inflammation, haemorrhage and necrosis in various tissues including spleen, kidney, heart, muscle and air bladder (Dixon and Stone 2017). Lesions frequently described in haematopoietic tissues of the kidney and spleen of several species of experimentally infected fish include haemorrhage, kidney interstitial necrosis with mononuclear cell infiltration, necrosis of splenic ellipsoids, depletion of splenic white pulp, and proliferation of the reticuloendothelium and enlargement of MMC in the spleen (Gaafar et al. 2011; Sano et al. 2011; Misk et al. 2016). Infections with HIRRV also cause necrosis of kidney haematopoietic tissue as well as necrosis of splenic ellipsoids and haematopoietic tissue (Oseko et al. 1988, 1992; Zhang et al. 2017).

18.2.2 Herpesvirus

Several herpesvirus diseases affect haematopoietic tissues of fishes; these diseases often have a relatively narrow host range. Examples include channel catfish virus disease (CCVD) caused by Ictalurid herpesvirus 1 (IcHV-1), herpesvirus haematopoietic necrosis (HVHN) caused by cyprinid herpesvirus 2 (CyHV-2) and *O. masou* virus disease (OMVD) caused by salmonid herpesvirus 2 (SalHV-2). Channel catfish virus disease produces infections in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*) and hybrids of the two species; infections are most severe and acute in young fish (0–4 months old) (Hanson and Khoo 2017). Pathological changes in naturally infected fish include oedema in the kidney and spleen and necrosis of haematopoietic tissues of these organs, an increase in lymphoid cells in the kidney, splenic congestion and the presence of macrophages laden with degenerated erythrocytes in the spleen (Major et al. 1975; Hanson and Khoo 2017). Infrequently, multinucleate syncytia may be present in necrotic foci in the kidney interstitium, and eosinophilic intranuclear inclusions may be observed in presumptive lymphocytes (cells with enlarged nuclei and a very thin rim of basophilic cytoplasm) within blood vessels of the posterior kidney (Hanson and Khoo 2017). Herpesvirus haematopoietic necrosis virus disease is highly pathogenic to goldfish (*Carassius auratus*), crucian carp (*C. carassius*) and gibel carp (*C. auratus gibelio*) and may also infect certain other cyprinid fishes (Thangaraj et al. 2020). Lesions typically observed in goldfish naturally infected with CyHV-2 include severe necrosis of haematopoietic tissues of the

kidney and spleen, with some degenerating cells adjacent to areas of necrosis showing enlarged nuclei with emarginated chromatin and amphophilic or smudged inclusions (Goodwin et al. 2006; Thangaraj et al. 2020). Goldfish with subclinical CyHV-2 infections may show only single-cell necrosis in haematopoietic tissue of the pronephros, with the presence of CyHV-2 demonstrated in areas of single-cell necrosis by in situ hybridisation, suggesting that clinically normal fish can serve as carriers of the virus (Giovannini et al. 2016). *O. masou* virus disease is known to affect several salmonid species of the genus *Oncorhynchus* in Japan (Yoshimizu et al. 2017). Initial infection with SalHV-2 may be systemic and frequently lethal, with virus multiplication in endothelial cells of blood capillaries, haematopoietic tissues and hepatocytes (Yoshimizu 2012). In 1- to 3-month-old masu salmon (*O. masou*), coho salmon (*O. kisutch*) and chum salmon (*O. keta*), the kidney may be the principal early target organ for the virus, resulting in haematopoietic tissue necrosis, with later necrotic lesions observed in the liver, spleen and pancreas (Yoshimizu et al. 2017). In naturally or experimentally infected rainbow trout, the most severe and consistent lesions occurred in the spleen and consisted of necrosis and haemorrhage of ellipsoidal cells and splenocytes, but necrotic lesions were also observed in kidney haematopoietic tissue, intestine, liver and heart (Furihata et al. 2005). About 4 months after the initial appearance of OMVD clinical signs, varying proportions of surviving fish (up to 100% in some cases) develop epitheliomas around the mouth or on the body surface, and occasionally in the kidney (Yoshimizu 2012; Yoshimizu et al. 2017). Tumours may persist up to 1 year post-infection, and SalHV-2 has been isolated from the tumours (Yoshimizu 2012; Yoshimizu et al. 2017).

18.2.3 Orthomyxovirus

Infectious salmon anaemia virus (ISAV) is a member of the family *Orthomyxoviridae* that causes serious disease in farmed Atlantic salmon (*Salmo salar*); the disease is characterised by anaemia, haemorrhage and circulatory disturbances, including kidney interstitial haemorrhages, renal tubular necrosis and oedema (Reimschuessel and Ferguson 2006; Aamelfot et al. 2014; Falk and Aamelfot 2017). Major target cells for ISAV are the endothelial cells lining blood vessels of all organs, but the presence of the virus does not induce inflammation or cause cytopathic effects in infected cells (Aamelfot et al. 2012; Falk and Aamelfot 2017). Virus particles also bind to (but apparently do not replicate within) circulating erythrocytes, which may then attach to endothelial cells (Aamelfot et al. 2012). Severe anaemia and haemorrhagic necrosis observed in the liver, kidney or gut may not be directly caused by ISAV infection, but may be indirect or secondary results of haemophagocytosis of infected erythrocytes, impaired blood circulation and hypoxia in tissues of severely anaemic fish (Aamelfot et al. 2012, 2014). In Atlantic salmon experimentally infected with ISAV, the size of interbranchial lymphoid tissue (ILT) was reduced and a high degree of diversity of T cells was observed with no expression of RAG-1 and RAG-2 in Atlantic salmon (Aas et al. 2014).

18.3   Bacterial Infections

Many systemic bacterial infections of fishes affect tissues and organs of the immune system, including the kidney, spleen, thymus, gill and mucosa-associated lymphoid tissue (reviewed in Inglis et al. 1993; Ferguson 2006; Woo et al. 2011; Austin and Austin 2012; Bruno et al. 2013; Woo and Cipriano 2017; Smith 2019). Fish pathogenic bacteria utilise many virulence mechanisms that enable invasion of host tissues, production of pathological effects and evasion of host defences. A detailed discussion of virulence-associated factors of bacterial fish pathogens is beyond the scope of this chapter, but some important virulence mechanisms for bacterial adherence and colonisation include adhesins, bacterial motility and factors facilitating intracellular survival. Some essential mechanisms for bacterial multiplication and invasion include iron uptake systems; extracellular products such as proteases, haemolysins and phospholipases; endotoxins; and protein secretion systems such as type III secretion systems (T3SS), complex bacterial structures that enable gram-negative pathogens to inject bacterial effector proteins directly into the host cell cytoplasm and thereby bypass the external milieu. Virulence factors of bacterial pathogens have been reviewed by several authors (e.g. Sudheesh et al. 2012; Méndez et al. 2012; Defoirdt 2014; Frey and Origgi 2016; Ben Hamed et al. 2018; Wrobel et al. 2019; Lemos and Balado 2020; Sarkar et al. 2021).

Bacterial pathogens frequently localise in the haematopoietic (interstitial) tissue of the kidney in part because of its extensive blood supply and trapping abilities; bacteria are often observed in association with the portal vasculature (Ferguson 1984; Reimschuessel and Ferguson 2006). Rapid haematogenous spread of bacteria such as *Aeromonas salmonicida* subsp. *salmonicida* and *Aliivibrio* (formerly *Vibrio*) *salmonicida* into the kidney interstitium and other tissues can result in the presence of large numbers of bacteria with little tissue reaction during the early stages of infection (Bruno et al. 2013). Acute bacteraemias associated with organisms such as *Aeromonas* spp., *Pseudomonas* spp., *Vibrio* spp., *Photobacterium damselae* ssp. *piscicida*, *Edwardsiella ictaluri* and *Yersinia ruckeri* can cause significant necrosis of the kidney interstitium. Tissue necrosis may be associated with the release of a variety of bacterial exotoxins, which can be divided into three classes based on their modes of action: (1) action on the surface of host cells, (2) formation of pores in cell membranes and (3) injection of toxins with an enzymatic activity targeting a particular cytosolic protein or cell organelle (Sarkar et al. 2021). Haemorrhages, often induced by bacterial haemolysins, as well as oedema and infiltration of inflammatory cells, can also occur. In acute inflammation, neutrophils may predominate, whereas infiltration of macrophages and lymphocytes is more common in chronic infections (Reimschuessel and Ferguson 2006). A robust inflammatory response is not always present, however. Evidence suggests that T3SS effectors of *Aeromonas salmonicida* subsp. *salmonicida* can induce temporary immunosuppression via depression of B- and T-cell lymphoproliferation in the anterior kidney early in infection (Dautrempuits et al. 2006), along with reduction in antigen-presenting cell activation and leucocyte migration to sites of infection (Burr et al. 2005; Vanden Bergh and Frey 2014).

Additionally, injection of T3SS effectors of *Aeromonas salmonicida* subsp. *salmonicida* into immune cells can alter cell signalling and block phagocytosis (Burr et al. 2005; Vanden Bergh and Frey 2014).

In subacute and chronic bacterial diseases affecting the kidney haematopoietic tissue, inflammation often becomes a prominent feature. For example, granulomatous inflammation, characterised by the presence of macrophages of various types (McClean and Tobin 2016; Shah et al. 2017), is frequently observed in the kidney interstitium of fish chronically infected with bacteria such as *Piscirickettsia salmonis* (Rozas and Enríquez 2014), *Francisella noatunensis* (Soto and Hawke 2017), *Edwardsiella piscicida* (Fogelson et al. 2016), *Renibacterium salmoninarum* (Elliott 2017), *Mycobacterium* spp. and *Nocardia* spp. (Lewis and Chinabut 2011). Phagocytised bacteria are often present within the macrophage cytoplasm; some species can survive and perhaps multiply within these cells (e.g. Gutenberger et al. 1997; Ellis 1999; Booth et al. 2006; Soto et al. 2010; Bakkemo et al. 2016; Pérez-Stuardo et al. 2019). Research is beginning to identify mechanisms enabling fish pathogenic bacteria to survive within phagocytes; examples include inhibition of lysosomal fusion by *Mycobacterium marinum* (El-Etr et al. 2001) and *Photobacterium damselae* subsp. *piscicida* (Elkamel et al. 2003), and avoidance or interference with TNF-α-dependent killing pathways by *Renibacterium salmoninarum* (Grayson et al. 2002). Genes in the *Francisella* pathogenicity islands (FPI) are important for intra-macrophage survival of *Francisella noatunensis* subsp. *orientalis* (reviewed by Soto and Hawke 2017), inhibition of the respiratory burst in Atlantic cod (*Gadus morhua*) leucocytes is key to survival of *F. noatunensis* subsp. *noatunensis* within phagocytes (Vestvik et al. 2013), T3SS effector proteins are required for multiplication of *Edwardsiella ictaluri* within macrophages (Thune et al. 2007), and both T3SS and type VI secretion system (T6SS) effectors have important roles in survival and replication of *E. piscicida* within macrophages (Hu et al. 2019; Qin et al. 2020). The term "granulomatous inflammation" encompasses a spectrum of lesion types (Shah et al. 2017), depending on the pathogen, the host fish species, and the infection stage and duration. Granulomatous inflammation may range from a loose aggregation of macrophages admixed with other inflammatory cells such as lymphocytes (diffuse granulomatous inflammation), to focal organised granulomas with central necrosis (and sometimes calcification) surrounded by macrophages and other inflammatory cells and encapsulated by fibrous connective tissue. Activated macrophages in sites of granulomatous inflammation are often epithelioid with round to ovoid nuclei, abundant granular eosinophilic cytoplasm and indistinct cell boundaries (Cronan et al. 2016; Shah et al. 2017). Epithelioid macrophages in mycobacterial granulomas in zebrafish and mammals express E-cadherin and other proteins characteristic of epithelial cells and form tight junctions with each other (Cronan et al. 2016); these macrophage adherens junctions may reduce host immune cell access to the granuloma and help to protect the pathogen (Cronan et al. 2016; Nathan 2016). Conversely, confinement of a pathogen in the interior of a granuloma can serve to effectively "exclude" it from the host via enclosure in a pseudoepithelium formed by macrophages with tight junctions, thereby protecting host tissues from pathogen spread (Nathan 2016).

Granulomas associated with *Pasteurella skyensis* in Atlantic salmon (*S. salar*) usually include multinucleate giant cells (fused macrophages) surrounding a central area of caseous necrosis (Jones and Cox 1999; Bruno et al. 2013). Giant cells may also be observed in mycobacterial granulomas in fish, but evidence from experimental infections in plaice (*Pleuronectes platessa*) suggests that they may be found primarily during early granuloma formation (Timur et al. 1977) and their presence may be less consistent than in mammalian mycobacteriosis (Lewis and Chinabut 2011; Gauthier and Rhodes 2017).

Many of the pathological effects of systemic bacterial infections observed in the spleen are similar to those described in the interstitium of the kidney and include oedema, necrosis, haemorrhage and acute and chronic inflammatory processes, often resulting in splenomegaly (reviewed in Noga 2006). Bacterial pathogens trapped at other sites such as the macrophage network in the renal portal system may localise in the spleen, likely transported there within macrophages. Release of toxins by pathogens such as *Aeromonas* spp. that are trapped by splenic ellipsoids during haematogenous spread may destroy ellipsoid structures with resultant loss of vascular integrity and presence of large numbers of plasma cells in chronic cases. Chronic disease can result in an increase in splenic white pulp, particularly around the ellipsoids and MMCs. In certain bacterial infections, such as rainbow trout fry syndrome caused by *Flavobacterium psychrophilum*, the spleen is particularly affected (Fig. 18.2). Distinctive features of the disease in the spleen include hypertrophy of the organ, intercellular oedema, haemorrhage, necrosis, fibrinous inflammation, loss of definition of the splenic border (with replacement by a loose eosinophilic layer) and the presence of numerous filamentous bacteria within the cytoplasm of phagocytes as well as free in spleen tissue (Rangdale et al. 1999; Decostere et al. 2001; Bruno et al. 2013); however, those may be hard to see in histological sections without
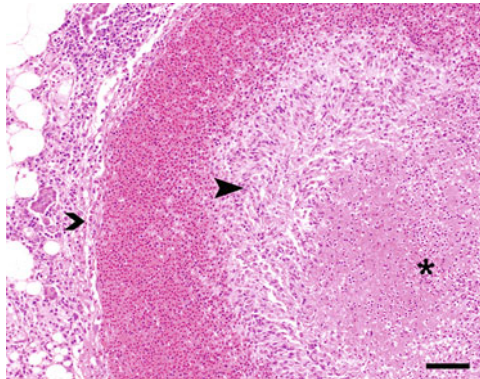
Fig. 18.2 Spleen, necrotising and granulomatous splenitis in a rainbow trout (*Oncorhynchus mykiss*) due to infection with *Flavobacterium psychrophilum*, central necrosis (asterisk), surrounded by macrophages and epithelioid macrophages (closed arrowhead); the serosa is thickened due to macrophages and lymphocytes and fibrin deposition (open arrowhead), inflammatory cell infiltrate in surrounding fat and pancreas tissue, HE stain, bar = 50 μm

special stains. Evidence from in vitro research suggests that *F. psychrophilum* may decrease survival and inhibit phagocytic ability of macrophages (Semple et al. 2020). In addition, pathogenesis of *F. psychrophilum* may depend on a functioning type IX secretion system (T9SS), as a mutant with a core T9SS gene deletion was deficient in adhesion, gliding motility and extracellular proteolytic and haemolytic activities, and exhibited reduced virulence in rainbow trout (Barbier et al. 2020).

Bacterial diseases affecting the thymus have not been widely investigated. The superficial location of the thymus may increase its vulnerability to infection, and the presence of pores on the surface of the teleost thymus suggests further ease of exposure to antigens (Noga 2006). Necrosis of the epithelium overlying the thymus is common in young fish with severe bacterial infections of the branchial cavity and is frequently followed by invasion and destruction of the thymic parenchyma (Noga 2006).

With the exception of organised areas of gill ILT and the newly described salmonid bursa in the anal region (see Chap. 2), the mucosa-associated lymphoid tissues (MALTs) of teleosts consist of diffuse populations of B and T cells that respond to mucosal infections (see Chap. 12). Although immune responses to bacterial infections in fish have been described (see Chap. 16), pathological changes specific to bacterial infections of organised MALT have received little attention. However, localised and systemic bacterial infections can affect the mucosal and submucosal tissues of the gastrointestinal tract, gills and skin, causing pathological changes in areas of diffuse MALT.

The MMC may play a significant role in the pathogenesis of certain bacterial diseases. Macrophages in the kidney transport intracellular bacteria such as mycobacteria, *Nocardia* or *Renibacterium salmoninarum* to MMCs in the kidney or spleen, creating foci of infection in these organs (Agius and Roberts 2003). Disruption and lysis of melanomacrophages and subsequent dispersal of pigment in tissues are a histopathological characteristic of bacterial kidney disease caused by *R. salmoninarum* (Elliott 2017). The MMCs also play an important role in chronic infections with *Vibrio anguillarum* and *Yersinia ruckeri*, where serious haemolytic anaemia occurs, and large amounts of hemosiderin from lysed erythrocytes are deposited in MMCs in the spleen (Agius and Roberts 2003; Bruno et al. 2013).

18.4 Fungal Pathology

Studies on the pathology induced by fungi and fungi-like organisms have historically been confounded due to problems with identification and a propensity to label all external fungi as "*Saprolegnia*" (Wolke 1975). Further, there was a widespread assumption that all fungi affecting fish were secondary opportunistic invaders, which delayed the recognition of *Aphanomyces invadans* as a primary pathogen (Baldock et al. 2005). Initially, *Ichthyophonus* spp. and *Dermocystidium* spp. were also considered to be fungi and both were reported from multiple organs including the kidney; both provoke a granulomatous
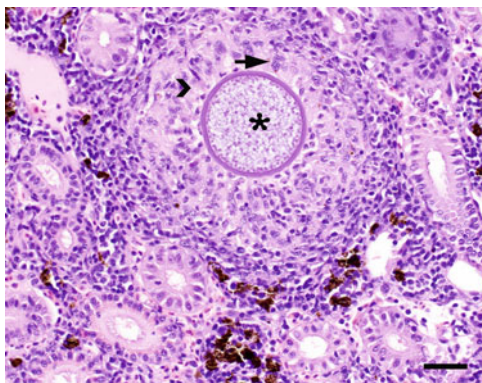
Fig. 18.3 *Ichthyophonus hoferi* infection in a grayling (*Thymallus thymallus*) causing granulomatous interstitial nephritis, multinucleated spores (asterisk) with a thick eosinophilic wall, surrounded by epithelioid macrophages (open arrowhead) and multinucleated giant cells (arrow), HE stain, bar = 25 μm

inflammatory response in all affected organs. In the kidney, the large granulomas displace or destroy both renal and haemopoietic tissues (Fig. 18.3, Agius and Roberts 2003).

Neither *Ichthyophonus* spp. nor *Dermocystidium* spp. are now considered to be fungi, but a separate clade of eucaryotic protists, the Mesomycetozoea (Ichthyosporea) (Glockling et al. 2013). Likewise, the Oomycota including *Saprolegnia spp.* and *Aphanomyces spp.* have been removed from the fungi as they are now considered water moulds, a separate clade to fungi (Derome et al. 2016). Unlike fungi, oomycetes do not have chitin-based cell walls; instead, their cell walls are composed of cellulosic compounds and glycan, and the nuclei within the filaments are diploid, rather than haploid as is the case in fungi (Fry and Grünwald 2010). Some oomycetes provoke an inflammatory response, which can develop into granulomas around the hyphae. *Aphanomyces* elicits a marked granulomatous response, which together with the large diameter of the hyphae (~10 μm) is pathognomonic (see Lilley and Roberts (1997) for a comparison of 58 fungal isolates including *Achlya* spp., *Saprolegnia* spp. and *Aphanomyces* spp.). Álvarez et al. (1995) found that the thymus of *Saprolegnia*-infected wild brown trout (*S. trutta*) showed loss of morphology, intercellular oedema, disappearance and pyknosis of thymocytes, hypertrophy and degeneration of epithelial cells with increased phagocytic activity.

Among the true fungi, ascomycetes such as *Phoma herbarum* cause systemic infections in coho salmon (*O. kisutch*) and Chinook salmon (*O. tschawytscha*) fry with fungal hyphae of 2–3 μm diameter. These infections are associated with haemorrhage and necrosis within the kidney (Wolke 1975). Brown septate fungal hyphae of *Exophiala* spp., 1–2 μm diameter, infect a wide variety of fishes (de Hoog et al. 2011) causing a granulomatous to granulocytic inflammatory response accompanied by giant cells; cell necrosis may be evident in internal organs, including kidney (Wolke 1975; Munchan et al. 2009; Derome et al. 2016).

Microsporidia were added to the fungi in 2007 (Hibbett et al. 2007) but have likewise been recently separated off as a sister group (Voigt et al. 2013). Microsporidia have the ability to invoke hypertrophic growth of infected cells, known as xenoma (Lom and Dykova 2005). Dykova and Lom (1980) described two main tissue reactions to Microsporidia. The first is a response to xenoma-inducing species where a weak initial inflammatory reaction is followed by formation of granulomas. The second type is due to *Pleistophora*-like species infecting muscles or oocytes, where an initial lymphocytic infiltration of myosepta is followed by phagocytosis of spores as the infected muscle fibre disintegrates, followed by formation of a wall of fibroblasts demarcating the parasitised muscle fibre mass. Microsporidian infections of the immune organs (other than MALT) are systemic infections because the parasite, while migrating, perhaps through infected leucocytes, has been trapped, often in MMCs, and has then proliferated.

18.5 Parasitic Infections

Some parasitic infections can cause pathology of immune organs. For example, the thymus of turbot experimentally infected with a myxozoan, *Enteromyxum scophthalmi,* showed decreased function, determined by gene expression and associated with inflammatory response, modulation of apoptosis and loss of cellularity (Ronza et al. 2020). Infections by large metazoans like trematodes and cestodes can replace a large percentage of anterior kidney, spleen or other immune organs. For example, *Euclinostomum heterostomum* metacercariae caused organ compression and atrophy of the kidney in infected *Channa striatus* and *Channa marulius* (Kaur et al. 2014).

Infection of trout by another myxozoan, *Tetracapsuloides bryosalmonae,* causes a chronic immunopathology characterised by a massive swelling of the kidney associated with lymphocytic hyperplasia, and hyperimmunoglobulinaemia (Clifton-Hadley et al. 1987; Hedrick et al. 1993; Okamura et al. 2011). The pathology includes infiltration with macrophages and lymphocytes as well as necrosis of interstitial tissue, and it depends on time and temperature (Bettge et al. 2009). This host response is due to a decrease in myeloid cells, potentially resulting from an inhibition of the innate immune response caused by the parasite and proliferation of lymphocytes in kidney (Chilmonczyk et al. 2002; Bailey et al. 2017). Dysregulation of Th and B lymphocytes, including polyclonal Ig activation most likely caused by the parasite, dilutes the specific B-cell response and results in ineffective antibody production (Abos et al. 2018). In rainbow trout (*O. mykiss*), inflammation remained for a long time after the clearance of the parasite (Bailey et al. 2020). This seems to differ from the response of hosts such as brown trout (*S. trutta*) that are well adapted to the parasite cycle. In this host, the infection causes macrophage infiltration, necrosis and presence of multiple thrombi composed of inflammatory cells and parasites (Fig. 18.4). During the infection, pathology can also be observed in other organs, including the anterior kidney, spleen and liver (Clifton-Hadley et al. 1987). A
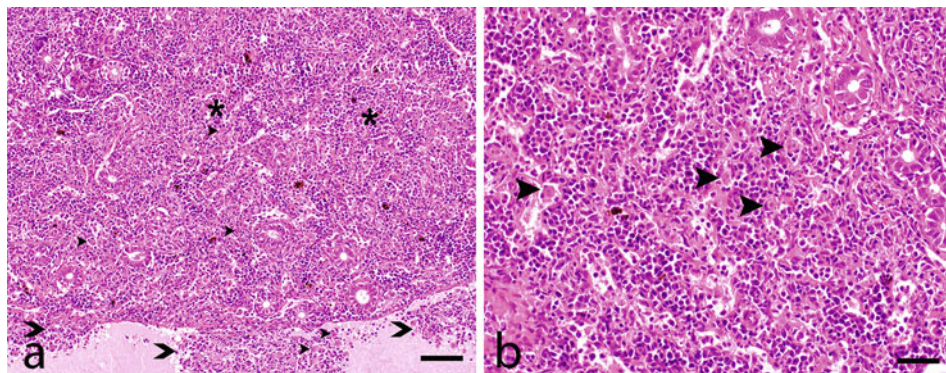
Fig. 18.4 Brown trout (*Salmo trutta*), granulomatous interstitial nephritis due to infection with *Tetracapsuloides bryosalmonae* inducing proliferative kidney disease (PKD), (**a**) interstitium is severely distended by macrophage infiltration, necrosis (asterisks) and parasites (closed arrowheads); multiple thrombi in the vessels (open arrowheads) composed of inflammatory cells and parasites (closed arrowheads); (**b**) higher magnification showing malacosporean parasites (closed arrowheads) surrounded by macrophages in vessels and interstitial tissue; HE stain, bars = (**a**) 50 μm, (**b**) 25 μm

reduction in MMCs has been reported in infected trout (Kent and Hedrick 1985; Clifton-Hadley et al. 1987).

An increase in MMC number or size is the most common response to parasitic infections. Gilthead sea bream (*Sparus aurata*) infected with *Enteromyxum leei* and turbot (*Scophthalmus maximus*) infected by *Enteromyxum scophthalmi* showed an increase in the number of splenic MMCs (Sitjà-Bobadilla et al. 2006; Ronza et al. 2013). A statistically significant increase in the percentage of splenic surface occupied by MMCs was present in the individuals of *S. auratus,* which were exposed to but remained uninfected by *E. leei* (see Estensoro et al. 2014). Significant positive correlations were identified between MMC area and numbers of eggs of the blood fluke *Cardicola* spp. in gills of southern bluefin tuna (*Thunnus maccoyii*). Similarly, MMC area was positively correlated with *C. forsteri* copy number ITS2 rDNA in hearts or gills and these relationships were significant for both splenic and renal MMCs (Widdicombe et al. 2020). Some of the hepatic and splenic MMCs contained blood fluke eggs (Widdicombe et al. 2020; Nowak et al. 2021). Melanomacrophages containing pigment positive for lipofuscin and hemosiderin were observed in one of the blisters in the heart of spotted seatrout, *Cynoscion nebulosus* (Sciaenidae) infected by *Cardicola laruei* (see McElroy et al. 2020). While this is not consistent for all host and blood fluke species, an increase in the numbers of MMCs with pigment positive for lipofuscin was observed in an infection of *Plectropomus leopardus* with *Pearseonellum corventum* (see Overstreet and Thulin 1989). Infection with *Euclinostomum heterostomum* metacercariae resulted in an increase in the number of MMCs in the kidney in *Channa marulius* (Kaur et al. 2014).

Changes to ILT were reported in Atlantic salmon affected by amoebic gill disease (Norte dos Santos et al. 2014) and included increased lymphocyte density at 7 days post-

infection followed by a reduction in lymphocyte density and an increase in surface area and length of ILT at 28 days post-infection. Cellular remodelling was particularly associated with the presence of *Neoparamoeba perurans* (see Norte dos Santos et al. 2014).

18.6 Nutritional Pathology

The link between fish nutrition and immune function has long been a focus of research, mainly in relation to vitamins and minerals. More recently, the effect of the incorporation of other proteins and oil sources to replace fish meal and fish oil in aquaculture diets has been investigated. Such research has been generated to develop cost-effective fish feeds, and the main focus has been sustainability of aquaculture, including fish growth and production costs. There has been much published on the effect of plant toxins and phytoestrogens on the structure and function of the gastrointestinal tract of salmonids driven in part by the significant dose-dependent enteritis associated with feeding soya bean or pea protein meals to carnivorous fish (Penn et al. 2011; Kononova et al. 2019). Diets rich in polyunsaturated fatty acids like n-3 highly unsaturated fatty acids can be associated with suppression of the immune system in rainbow trout (Kiron et al. 1995). However, a high docosahexaenoic acid/eicosapentaenoic acid ratio enhances phagocytic activity in leucocytes in *Epinephelus malabaricus* (Wu et al. 2003).

Interpretation of pathology resulting from the effects of nutrition seldom considers the combined effects of changes in temperature on structure and functioning of the immune response. For example, Urán et al. (2007) showed that intestinal disorders due to presence of plant meals in diets were greater in Atlantic salmon kept at 12 °C compared to those at 8 °C when both were fed 20% soya bean meal.

Most teleosts, including salmonids, are ectothermic so at lower environmental temperatures both fatty acid oxidation and cell membrane integrity are highly dependent on the fatty acid composition of the diet (Hazel 1979; Wahle 1983; Salte et al. 1988; Waagbø et al. 1993). Dietary lipids also influence the levels of circulating antibodies in fish and the bactericidal activity of macrophages (Kiron et al. 1995), presumably because of changes in the lipid membrane structure of the lymphocyte subpopulations, which can lead to observable membrane fragility (Schroit and Gallily 1979; Salte et al. 1988).

There have been few observations on the pathology of the thymus associated with nutrition. Malnutrition has been shown to induce atrophy-related alterations in the thymus of turbot (*Scophthalmus maximus*), consisting of "cell depletion, reduction of the cortex: medulla ratio and loss of corticomedullary limit distinction" (Ronza et al. 2020).

There appear to be very few published observations on the influence of nutrition on gill ILT, or indeed any teleost MALT. Zinc as a dietary supplement resulted a significant increase in area of the epidermis covered by mucous cells in the deeper layers after 2 weeks, while the main effect of dietary vitamin C on the skin of *S. salar* was seen on ability to repair damaged dermal fibres, on revascularisation and the re-establishment of normal dermal and muscle structure (Jensen et al. 2015).

MMCs within the spleen and kidney have long attracted research into their function, which is believed to represent sites of both immune system activation and phagocytosis, and they are a useful biomarker for environmental stress (see Steinel and Bolnick (2017) for review). Agius and Roberts (1981) established that starvation resulted in an increase in numbers of MMCs in the spleen and kidney of trout (*O. mykiss*) and other species, suggesting that their formation was in response to catabolic breakdown of tissues. Splenic MMC numbers significantly increase in starved captive *Diplodus annularis*, confounded by fluctuations in the control fish, leading the authors to conclude that factors other than starvation were involved (Micale and Perdichizzi 1990). Indeed, subsequent studies have shown that increases in MMC occur in farmed fish over wild-caught fish likely reflecting both stress and maladapted diet (Kurtović et al. 2008; Evans and Nowak 2016). High levels of gramine in the diet of rainbow trout (*O. mykiss*) can also cause an increase in the density of MMCs. Gramine has a strong anti-palatability effect, and the increase in density of MMCs is likely driven by starvation (Glencross et al. 2006). Chronic lysis of erythrocytes resulting from vitamin E deficiency also can cause splenomegaly and haemosiderosis (Noga 2006). An experimental diet producing a significant modulation of immune genes resulted in the reduction in the density of MMCs in anterior kidney of Atlantic salmon (Jalili et al. 2020), which was interpreted as a desirable change. However, an increase in splenic MMCs was reported in barramundi fed tuna hydrolysate, which showed increased resistance against *Streptococcus iniae* and improved growth performance (Siddik et al. 2018). This suggests that MMCs may show a range of responses, which can be also organ-specific.

18.7 Toxicologic Pathology

Toxicologic pathology in fish is a useful tool in the field of aquatic ecotoxicology, as it provides an insight into target organs and mechanisms of toxicity and it may be used as a promising alternative or a model for mammalian toxicology (Wester and Canton 1992; Wester and Vos 1994; Wester et al. 1994; Law 2003). Fish immune organs, such as kidney and spleen, can be exposed to toxicants via blood circulation as they function in filtration of blood (Muñoz et al. 2015). However, little information is available on the structural changes caused by toxic agents, including organochlorines, petroleum compounds, organophosphate, pesticides and heavy metals, on immune organs of the fish (see Table 18.1).

Some pathological effects of metal poisoning on immune organs, including copper (Cu), lead (Pb), mercury (Hg) and zinc (Zn), were observed in laboratory and field studies. For example, juvenile rainbow trout (*O. mykiss*) exposed to copper nanoparticles (Cu-NPs—one type of metal-containing nanomaterials) and copper sulphate ($CuSO_4$) under laboratory conditions was affected by necrosis of the haematopoietic tissue and elevation in the number of MMCs in the kidney (Al-Bairuty et al. 2013). The changes in the kidney of freshwater guppy (*Poecilia reticulata*) exposed to concentrations of methyl mercury chloride (MeHgCl) for 1 and 3 months (Table 18.1) included hyperplasia of the interstitial

Table 18.1 Examples of histopathological effects of toxic chemicals on immune organs of the fish

Toxicant	Fish species	Observed pathology in organs	Type of study	Temperature (°C)	Salinity (%)	Chemical concentration, exposure route and duration of study	References
Copper nanoparticles (Cu-NPs) and copper sulphate (CuSO₄)	Rainbow trout (*Oncorhynchus mykiss*)	Kidney: a few necrotic cells in the haematopoietic tissue, minor elevation in the number of melanomacrophage centres throughout the kidney	Laboratory	16.0 ± 0.01	NA	20 μgL^{-1} of dissolved cu (as CuSO₄) or 20 or 100 μgL^{-1} cu-NPs (measured primary particle size of 87 ± 27 nm) in a semi-static waterborne exposure regime for 10 days	Al-Bairuty et al. (2013)
Methyl mercury chloride (MeHgCl)	Guppy (*Poecilia reticulata*)	Kidney: Hyperplasia of interstitial haemopoietic tissue	Laboratory	NA	NA	Waterborne 0, 1.0, 1.8, 3.2, 5.6, or 10 mgL^{-1} aqueous methyl mercury chloride exposure in 2-L tanks for 1 and 3 months	Wester and Canton (1992)
Mercury (Hg)	Spotted seatrout (*Cynoscion nebulosus*)	Kidney: Renal interstitial inflammation	Field	NA	NA	NA	Adams et al. (2010)

Mercury (Hg)	Atlantic goliath grouper (*Epinephelus itajara*)	Kidney: Interstitial mononuclear infiltrates and multifocal MMCs	Field	NA	NA	NA	Adams and Sonne (2013)
Lead (Pb)	Silver catfish (*Rhamdia quelen*)	Spleen: Atrophy around blood vessels and an increased number of lymphocytes within splenic sinusoids and ellipsoids	Laboratory	19	NA	Waterborne Pb concentration (27 mgL^{-1}) corresponding to 25% of the LC50 for 96 h	Muñoz et al. (2015)
Dietary Pb(II)	Neotropical fish (*Hoplias malabaricus*)	Head kidney: Increased the number of MMCs in head kidney	Laboratory	21 ± 2	NA	Dietary 21 µg Pb (II) g^{-1} corresponding to 4 µg Pb(II) g^{-1} wet weight of exposed prey fish (for 5-day intervals, 14 doses).	Rabitto et al. (2005)
Zinc oxide (ZnO) particles and zinc chloride (ZnCl$_2$)	Gilthead seabream (*Sparus aurata*)	Spleen: Increased number and size of MMCs in spleen	Laboratory	NA	NA	Waterborne 1 mgL^{-1} of ZnCl$_2$ and ZnO particles (1.1, 1.2, and 1.4 µm) for 96 h.	Beegam et al. (2020)
Organotins: Bis (tri-*n*- butyltin) oxide (TBTO) and di-*n*-butyltindichloride (DBTC)	Guppy (*Poecilia reticulata*)	Thymus: Thymus atrophy Kidney: Hyperplasia of haemopoietic	Laboratory	23 ± 2	NA	Static renewal 0.01–32 µg TBTO L^{-1} over 1 and 3 months and 320–3200 µg	Wester and Canton (1987)

Table 18.1 (continued)

Toxicant	Fish species	Observed pathology in organs	Type of study	Temperature (°C)	Salinity (%)	Chemical concentration, exposure route and duration of study	References
		interstitial tissue of kidney				DBTC for 1 month	
Polychlorinated bi-phenyls (PCBs): 3,3′,4,4′,5-Pentachlorobiphenyl (PCB-126)	European flounder (*Platichthys flesus*)	Thymus: Induced thymus atrophy and CYP1A-positive cells in the haematopoietic tissue	Laboratory	19 ± 2	NA	Single oral administration of 0, 0.5, 5 or 50 mg PCB-126 per kg body weight for 16 days	Grinwis et al. (2001)
Hydrocarbon concentration (PHCs) in oil-contaminated sediment	Winter flounder (*Pleuronectes americanus*)	Spleen: MMCs contained hemosiderin in spleen	Laboratory	1–6	NA	Waterborne 100, 300, 600, 1000 and 2200 (adults only) µgg⁻¹ PHCs in crude oil-contaminated sediment in 300-L aquaria for 8 weeks	Khan (1995)
Polyaromatic hydrocarbons (PAHs): 2% naphthalene (PAH) in the crude oil	Alligator gar (*Atractosteus spatula*)	Spleen: Neutrophilic and eosinophilic infiltration next to blood vessel and congestion in spleen Anterior kidney:	Laboratory	NA	NA	0, 0.5 or 4.0 gL⁻¹ of crude oil in static tanks for 48 h	Omar-Ali et al. (2015)

β-Isomer of lindane: β-Hexachlorocyclohexane (β-HCH)	Guppy (*Poecilia reticulata*)	Eosinophilic infiltration in haematopoietic tissue. Kidney: Accumulation of hyaline eosinophilic PAS-positive droplets in the interstitial haemopoietic tissue	Laboratory	23 ± 2	NA	Waterborne 0, 0.0032, 0.01, 0.032, 0.1, 0.18, 0.32, 0.56 and 1.0 mg β-HCH/L in aquaria for 1 and 3 months.	Wester et al. (1985)
β-Isomer of lindane: β-Hexachlorocyclohexane (β-HCH)	Japanese rice fish (medaka, *Oryzias latipes*).	Spleen: Globular deposits of PAS-positive substance in spleen	Laboratory	24.5 ± 0.5	NA	Waterborne 0, 0.032, 0.056, 0.10, 0.18, 0.32, 0.56 and 1.0 mg β-HCH/l.	Wester and Canton (1986)
γ isomer of lindane: γ -hexachlorocyclohexane (γ -HCH)	Gilthead seabream (*Sparus aurata*)	Kidney: Loss of the haematopoietic tissue	Laboratory	19–20	33.5–35.0	16 μgL⁻¹ γ-HCH in containers for15 days	González De Canales et al. (2009)

NA not available

haemopoietic tissue and most cells contained a hyalinised, eosinophilic cytoplasm (Wester and Canton 1992). Acute exposure of silver catfish (*Rhamdia quelen*) to sublethal concentration of Pb (27 mgL^{-1} Pb) for 96 h resulted in lymphatic depletion around blood vessels and an increased number of lymphocytes within sinusoids and ellipsoids in the spleen, but no notable lesions in haemolymphopoietic tissue nor change in the number or size of MMCs were observed in the kidney (Muñoz et al. 2015). Necrosis and increased numbers of neutrophils and MMCs were present in the head kidney of a neotropical fish, *Hoplias malabaricus*, after dietary exposure to Pb(II) (for 5-day intervals, 14 doses, Table 18.1) (Rabitto et al. 2005). Histological examination of juvenile gilthead sea bream (*Sparus aurata*) exposed to ZnO particles revealed an increase in the number of MMCs in spleen (Beegam et al. 2020). Higher numbers of MMCs in exposed fish may be related to a higher content of cellular debris resulting from necrosis (Au 2004; Rabitto et al. 2005).

In field studies, the specific causative factors, i.e. historical and analytical evidence, for an observed alteration of fish immune organs might be unknown. Histopathology of wild fish can still provide valuable information on adverse effects of toxic chemicals in the natural environment, and it can be used as an indicator of environmental exposure (Teh et al. 1997; Wester et al. 2002; Stentiford et al. 2003; Silva and Martinez 2007). Immuno-pathology reported from wild fish may be associated with pollution due to exposure of the fish to high levels of contaminants. For example, spotted seatrout (*Cynoscion nebulosus*) collected along the South Florida Atlantic coast showed lesions in kidney, including inflammation of interstitial tissue, potentially associated with the highest total Hg concentrations (0.44–1.12 μg/g wet weight) in kidney compared to other organs (Adams et al. 2010). Atlantic goliath grouper (*Epinephelus itajara*) collected from tropical and subtropical waters of the south-eastern USA had kidney pathology including interstitial mononuclear infiltrates and multifocal MMCs potentially related to Hg residues (Adams and Sonne 2013). These field study examples suggest the usefulness of immunopathology in wild fish species as indicators for assessing chemical-induced immunotoxicity associated with pollution in aquatic environments.

Non-metals, such as organic compounds used in agriculture and industry, including pesticides and herbicides, can be toxic to fish if their environmental concentration is high enough (Grinwis et al. 2000; Grinwis et al. 2009). There have been a few laboratory exposure experiments indicating immunopathology in fish related to the individual chemical used. For example, exposure to biocide bis(tri-n-butyltin) oxide (TBTO) and di-n-butyltindichloride (DBTC) caused thymus atrophy and hyperplasia of haemopoietic interstitial tissue in kidney of guppy (*P. reticulata*) (Wester and Canton 1987). Gilthead sea bream (*S. aurata*) exposed to 16 μg/L of the insecticide lindane (γ-hexachlorocyclohexane, γ-HCH) for 15 days (Table 18.1) showed loss of the renal haematopoietic tissue (González De Canales et al. 2009). Surface area of splenic MMCs increased in Nile tilapia (*Oreochromis niloticus*) after a 15-day exposure to 1 ppm of atrazine, but not after 7- or 15-day exposures to 2 ppm of atrazine (Oliveira et al. 2018).

Components of crude oil, such as polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), can affect fish immune organs (Danion et al. 2011).

For example, thymus atrophy was reported in European flounder (*Platichthys flesus*) exposed to 3,3′,4,4′,5-pentachlorobiphenyl (polychlorinated biphenyls; PCB-126) for 16 days (Table 18.1) under laboratory conditions (Grinwis et al. 2001). MMCs containing hemosiderin were mainly observed in spleen of winter flounder (*Pleuronectes americanus*) exposed to hydrocarbon concentration (PHCs) in crude oil-contaminated sediment for 8 weeks (Khan 1995). Alligator gar (*Atractosteus spatula*) exposed to crude oil naphthalene (PAH) for 48 h had lesions including neutrophilic and eosinophilic infiltration and congestion in spleen, as well as eosinophilic infiltration in haematopoietic tissue in anterior kidney (Omar-Ali et al. 2015). The lesions found in these laboratory exposed fish may be representative of immune organ changes and could facilitate the detection of immunotoxic effects of contaminants in further studies.

18.8   Neoplasms

Classification of fish tumours in general is based on the WHO classification, developed for human medicine (Swerdlow et al. 2016). However, in fish, classification is not always as clear as in mammalian medicine as there are some differences, such as rare occurrence of metastases in fish (Machotka et al. 1989). While they are now reported more commonly (Dale et al. 2009; Blazer et al. 2020), they are still not as common as in higher vertebrates. The reason for this less common incidence of metastases is still not clear (Ferguson 1989). One possible explanation might be the absence of lymph nodes in fish. In mammals, metastatic cells must overcome many hostile influences when entering the vessels, when travelling in the body fluids and when re-entering the new tissue. It appears plausible that the metastatic cells, unfiltered by lymph nodes, may be destroyed in another phase of their travel (Machotka et al. 1989). Many examples of fish neoplasia were collected in the 1980s and 1990s by the former US Registry of Tumors in lower animals at the National Museum of Natural History.

Neoplasms in lymphoid organs can affect all compartments present, such as lymphoid tissue and vascular tissue and for the thymus also the epithelioreticular compartments (Chilmonczyk 1992; Coffee et al. 2013).

The most common neoplasm is probably the lymphoma, which can arise in different lymphoid organs, like thymus, spleen and renal haematopoietic tissue (Dawe 1969; Meyers and Hendricks 1983). Lymphomas are a group of tumours that develop from neoplastic lymphoblast. Splenic lymphomas usually consist of a monomorphic population of neoplastic lymphoblasts together with a loss of normal architecture. In the thymus, the neoplasm usually consists of a solid mass of pleomorphic cells with a multifocal infiltration of residual thymocytes and lymphocytes (Fig. 18.5). In laboratory populations of Japanese medaka, *Oryzias latipes*, thymic lymphomas have been reported repeatedly (Battalora et al. 1990).

It can be difficult to distinguish this tumour type from leukaemia. In mammals, leukaemia is a neoplastic change in white blood cells in the bone marrow (Theilen et al.
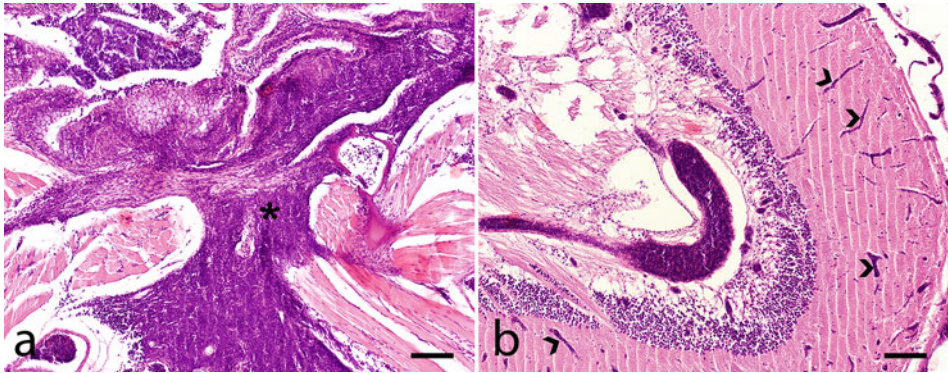
Fig. 18.5 Thymic lymphoma with a leukaemic phase in a stickleback (*Gasterosteus aculeatus*), (**a**) the normal thymus structure is completely replaced by a non-encapsulated, poorly demarcated mass of immature lymphoblasts (asterisk), infiltrating in surrounding tissues; (**b**) brain, vessels are filled with neoplastic cells (open arrowheads), HE stain, bars = (a,b) 50 μm

1968; https://www.cancer.gov/types/leukemia). The type of leukaemia depends on the blood cell type that becomes neoplastic and the progression (acute or chronic). In fish, the renal interstitial tissue is considered to be the bone marrow analogue. So far, there are no differentiation types of leukaemia described in fish. Leukaemia can occur in Chinook salmon (*O. tshawytscha*) with a probable viral aetiology (Kent et al. 1990). In addition, in koi carp (*Cyprinus rubrofuscus*), this neoplasm can be seen as an incidental finding (H. Schmidt-Posthaus, own observations). Leukaemia in fish presents as prominent hyperplasia of haematopoietic cells in the renal interstitium. These cells can invade the vascular system and are distributed in different internal organs, staying within the vascular system and showing high adherence to endothelial cells (Fig. 18.3). The mitotic rate is usually moderate to high.

Other tumour types arising in lymphoid organs are the haemangioma and haemangiosarcoma. These tumour types arise from endothelial cell types. Haemangioma consists of sheets of monomorphic, differentiated endothelial cells, with blood-filled spaces and compression of the surrounding tissue. Haemangiosarcoma is a rapidly growing, highly invasive neoplasm arising from pluripotent mesenchymal stem cells or endothelial cells. Haemangioma and sarcoma occur occasionally as spontaneous lesions in adult fish, including zebrafish and trout, and occur more frequently in carcinogenesis studies in zebrafish or medaka (Spitsbergen et al. 2012).

In the thymus, the epithelioreticular compartment can become neoplastic as well; this is called thymoma. In fish, this tumour type is rarely described (Romano and Marozzi 2004).

18.9 Conclusions/Outlook/Future Research

Fish immunopathology shows a lot of promise for a range of applications. Since the immune organs of fish have been revealed to be sensitive to the effects of xenobiotics, immunopathology can be one of valuable biomarkers to study effects of pollutants in the aquatic environment on the fish. However, there are some major gaps in our knowledge of fish immunopathology, further exacerbated by a large number of fish species, the impact of environmental conditions and immunopathology being largely descriptive. It is important to identify function(s) of pathogen virulence factors and effector molecules of toxicants contributing to pathology in immune organs (e.g. more studies with pathogen knockout mutants monitoring effects on host immune/inflammatory gene expression and immune organ tissue changes). There are relatively few papers describing histopathology of immune organs in teleosts other than changes associated with virus infections, which tend to target the immune organs, most likely due to limited sampling for diagnostic purposes. Furthermore, there are still gaps in the knowledge of the effects of different factors on immunopathology, for example the effects of starvation on fish immunopathology have not been fully addressed or changes associated with smoltification are poorly understood. Fish immunopathology is an important area for investigation as improved understanding of mechanisms of pathological changes in immune organs caused by infectious and non-infectious agents could significantly contribute to the development of improved strategies for avoidance, prophylaxis or disease control in fish farming.

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Current Use and Need for New Fish Vaccines

19

Paul J. Midtlyng

Abstract

Among disease control strategies in aquaculture, vaccination possesses the highest potential to prevent from clinical illness and mortality. Mass vaccination of fish became an aquaculture practice with the successful development of vaccines against furunculosis for the industrialized farming of Atlantic salmon, a high-value species. Oil-adjuvanted bacterins were developed to boost antibody responses and produce long-lasting protection and are now available for most farmed species in Europe. A second technology leap was the successful development of a plasmid DNA vaccine. In 2017, a vaccine against pancreas disease in Atlantic salmon was the first-ever DNA vaccine to be approved by the European Medicines Agency. The European use of vaccines for Atlantic salmon is estimated more than 700 million doses annually, in part being administered through fully automated machines. Mediterranean mariculture is estimated at 600 million vaccine doses or more. Adjustments to financial and regulatory means in support of new vaccine development are proposed. It is believed that innovative biotechnology start-ups will play an essential role in new vaccine development for fish and that both development time and costs will benefit from new technology platforms for antigen delivery that are being researched for the development of vaccines against COVID-19.

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Abbreviations

CMS   Cardiomyopathy syndrome
DNA   Deoxyribonucleic acid
ERM   Enteric red mouth disease
HSMI   Heart and skeletal muscle inflammation
IABS   International Alliance for Biological Standardization
IHN   Infectious hematopoietic necrosis
ISA   Infectious salmon anemia
PD   Pancreas disease
RNA   Ribonucleic acid
RTFS   Rainbow trout fry syndrome
VNN   Viral nervous necrosis
WHO   World Health Organization

▶ **Definitions**

Adjuvant: a helper substance in vaccine formulations to improve the protection conferred by the vaccine antigen.

Bacterin: a vaccine antigen prepared from an inactivated (mostly formalin killed) bacterial culture.

Cleaner fish: Wrasse (*Labrus*) species or lumpfish (*Cyclopterus lumpus*) that are held in salmon cages in order to forage on ectoparasites such as the salmon louse (*Lepeophtheirus salmonis*).

Cascade prescription: the legal opportunity to prescribe veterinary medicines for another target species or indication than licensed or to prescribe veterinary medicines that possess marketing authorization in another EU/EEA state if no national authorization exists.

Columnaris disease: cutaneous or systemic disease of freshwater-reared fish caused by *Flavobacterium columnare.*

Classical furunculosis: disease caused by systemic infection with *Aeromonas salmonicida* subspecies *salmonicida.*

Atypical furunculosis: ulcerative or systemic disease caused by infection by other subspecies of *Aeromonas salmonicida.*

19.1   Introduction

Vaccination is one of the main strategies for disease control in humans and animals including teleost fish. Compared to biosecurity measures, it has a higher potential to prevent from illness and mortality, and moreover, taking effect more rapidly than the other strategies (Midtlyng 2001). Another unique feature of vaccination is its potential to

provide protection even if minor fractions of a population remain unvaccinated—the so-called herd effect upon which much hope for future control of the current COVID-19 pandemic of the human population is built (WHO 2020). Vaccinology represents the applied side of immunology and differs from basic science in that the focus is placed on the clinical effects: the ability to protect from the clinical disease and mortality, and on concurrent adverse effects. In addition to fish immunology, the knowledge of epidemiology, of the strategies and tactics of disease control, and of the economics fish health (Pettersen et al. 2015) is needed for successful implementation of vaccine strategies in finfish aquaculture.

Even though vaccination predominantly is used to control endemic infections, it can be successfully employed in disease eradication programs and campaigns, as evident by the eradication of smallpox in humans, and of rinderpest in the veterinary sector (Ochmann and Behrens 2018). Strategic use of marker vaccines in order to minimize the risk for geographical spread and aiming for subsequent eradication in zones or regions is reality for livestock diseases like European swine fever (Blome et al. 2017). Vaccination as part of disease eradication programs—for example, in the zonal eradication of ISA—should also be considered for finfish aquaculture.

19.2 Major Leaps in Fish Vaccine Technology

Although several research reports on vaccination of fish species date earlier, the birth of finfish vaccine development must be ascribed to the salmon and rainbow trout culture of the North American Pacific Northwest (Evelyn 1997). However, the uptake of mass vaccination as an industrial practice came in the early 1990s, with the successful development of vaccines against classical furunculosis for the rapidly developing Atlantic salmon farming industry in Norway (Midtlyng et al. 1996). The formulation of inactivated bacterial antigens with oil adjuvants allowed the boosting of antibody responses to a level sufficient for effective and long-lasting protection. It also facilitated the inclusion of several antigens into multivalent vaccines to protect against most of the bacterial salmon diseases with only one injection. The oil adjuvant technology vaccine platform has since been expanded to nearly all industrialized aquaculture species, also in formulations containing antigens for viral diseases.

A second technology leap for fish vaccination was reached in 2005, through the development and licensing of a plasmid DNA vaccine against infectious hematopoietic necrosis (IHN) in Canada and USA (Garver et al. 2005). For 15 years, this vaccine has effectively prevented spillover of IHN from returning stocks of Pacific salmon to farmed salmon. The same plasmid DNA platform was later used for developing a vaccine against PD for European aquaculture (Thorarinsson et al. 2021). The safety evaluation performed by the European Food Safety Authority concluded that introducing genetic modifications by this vaccine was highly unlikely (EFSA (European Food Safety Authority) et al. 2017).

The DNA vaccine against PD was licensed in Norway in 2018. It is now increasingly used by the salmon farming industry.

19.3 Vaccines for European Salmon Aquaculture

Today, Europe leads the usage of licensed vaccines, with the Norwegian farming of Atlantic salmon the clearly largest segment (Table 19.1). Combination vaccines against five diseases (classical furunculosis, classical vibriosis, cold water vibriosis, winter ulcer disease, and infectious pancreatic necrosis) constitute approximately 90% of the doses used, with additional use of monovalent formulations containing PD antigen, and monovalent yersiniosis vaccines in many farms. Due to the rapidly increasing use of cleaner fish species for control of salmon louse (*Lepeophtheirus salmonis*) in marine salmon farming, bacterins against atypical furunculosis + classical vibriosis, and a number of further bacterial antigens found in these species have been developed and are being used as injectable, oil-adjuvanted, or immersion formulations. More than half of the annual production of lumpfish juveniles (in Norway alone more than 50 million) is apparently being immunized, predominantly using autogenous vaccines.

19.4 Vaccines for European Freshwater Aquaculture

The availability of licensed vaccines for European freshwater fish farming is still limited if not to say insufficient, despite the need for tools to control endemic infections (Table 19.2). Immersion vaccines conferring protection against ERM are widely used in freshwater trout farms. Some licensed vaccines are available for immersion (30 sec) of fish fry into a *Yersinia ruckeri* bacterin, a process that stimulates a series of innate and adaptive responses in rainbow trout. A single immunization immersion protects for 5–6 months, but a booster by immersion or injection extends the protective period (Chettri et al. 2013). With these few exceptions, licensed vaccines for use in European freshwater aquaculture are limited, likely also due to the fact that some of the relevant pathogens may shift antigenic profile (Desmukh et al. 2011). In this author's opinion, the described relative shortage is more due to regulatory and economic reasons than to the lack of scientific knowledge or technological capability. In face of the high costs for documentation and regulatory approval of veterinary vaccines, limited market size and low per fish value render new vaccine development unattractive for the large, well-established pharmaceutical companies. Unfortunately, European regulatory instruments for licensing of vaccines for minor uses and minor species (MUMS) have so far proven insufficient to stimulate new fish vaccines for the freshwater farming segment.

Table 19.1 Estimated usage of vaccines for Atlantic salmon in Europe, compiled from diverse sources

Country	Number of doses per year
Norway	>600 mill (multivalent + monovalent vaccines)
Scotland	50 mill (multivalent, 85% including PD)
Faroe Islands	22 mill (multivalent including ISA)
Iceland	15 mill
Ireland	6 mill
Total Europe	~ 700 million doses

Table 19.2 Antigenic profile of vaccines for European freshwater farming of salmonids and cyprinids

Disease	Vaccination relevant for
Classical furunculosis	All of EU/EEA
Yersiniosis	All of EU/EEA
Lactococcosis	Mediterranean countries
Streptococcosis	Mediterranean countries
Flavobacterioses	All of EU/EEA

19.5   Vaccines for Mediterranean Mariculture

The dominant segment of Mediterranean aquaculture is the farming of sea bass and sea bream, each comprising close to 50% of the around 1.4 million fry annually being transferred to sea cages for final rearing. The role of other species (turbot, meagre) remains marginal. Vaccines are above all used for the farming of sea bass (*Dicentrarchus labrax*), both for immunization of fry by immersion, and as booster injections (Table 19.3). An injectable vaccine against viral nervous necrosis (VNN) caused by a nodavirus is also available. More than a dozen fish vaccine products are commercially available to protect the marine species being farmed in the Mediterranean (Miccoli et al. 2019).

19.6   Vaccine Administration Technologies

Due to the increase in production capacity per smolt farms, new and fully automated technology for the administration of injectable vaccines to pre-smolts is now in widespread use (Fig. 19.1). However, the feasibility of such technology is strongly dependent on the size of operation. Therefore, trained operators of semi-automated vaccination machines or manual injection teams, that can reach a capacity of 1000–2000 fish per man-hour, remain common for hatcheries producing up to three million smolts per year, taking advantage of greater logistical flexibility and low burden of investment. While vaccine administration by immersion or bath remains adequate wherever protection of fry or fingerlings is needed,

Table 19.3 Estimated vaccine usage in Mediterranean mariculture, compiled from diverse sources

Vaccines	Number of doses
Injectable vibriosis + pasteurellosis Immersion vaccines	Sea bass: >400 mill Fry: Unknown amount
Injectable, nodavirus	30–40 mill
Tenacibaculum maritimum	Injectable bacterin, unknown amount
Streptococcosis	Turbot, injectable bacterin, unknown amount
Total Mediterranean	> 600 million doses

Fig. 19.1 Fully automated vaccination machine used in large-scale salmon smolt farms. https://www.skalamaskon.no/aquakultur/vaksinering

administration of vaccines per oral delivery has not proven very successful in Atlantic salmon nor in the farming of most other European finfish species.

19.7 The Need for New Fish Vaccines in Europe

Due to the high number of individuals potentially in need for vaccination and high value per fish, vaccines to control endemic viral infections of the salmonid farming segment are among the strongest candidates for licensing in Europe. Marine salmon farming is facing serious challenges in controlling sea lice infestations, because resistance to chemotherapeutics leaves only non-medicinal methods for removing the lice from the fish, during extensive and stressful handling operations. Even though the cardiomyopathy syndrome CMS (caused by a totivirus termed piscine myocarditis virus) and heart and skeletal muscle inflammation HSMI (caused by a piscine orthoreovirus) may cause only low-to-moderate morbidity and mortality under undisturbed rearing conditions, repeated

anti-louse treatment of fish groups with inflammation in the cardiac musculature too often leads to high acute losses. Control of these viral infections through vaccination is thus urgently needed in order to reduce overall mortality in seawater farming of Atlantic salmon. Although successful report of vaccination against HSMI has been reported (Wessel et al. 2018), no vaccine against these viral infections has been licensed as yet.

In the farming of carp, effective vaccines to control severe disease caused by herpes virus (infection with CyHV-3) still remain to be licensed in Europe, despite attempts for vaccine development since more than 15 years (reviewed by Boutier et al. 2019).

Among the bacterial diseases, atypical furunculosis has proven an epizootic challenge to a variety of freshwater farmed species. The antigenic diversity of the causative aeromonads represents a challenge to commercial vaccine development, as cross-protection has so far proven limited, rendering multi-species vaccines unlikely. The use of autogenous vaccine concepts may offer a starting point from which the licensing of vaccines may eventually evolve (see below).

Last but not least, there is a need for vaccines to control infections with a range of flavobacterial infections in freshwater but also in seawater fish farms. Columnaris disease and fry syndrome of rainbow trout (RTFS) are examples from the salmonid farming segment, while ulcerative diseases caused by *Tenacibaculum maritimum* and related bacteria are frequently reported from the farming of marine species. Efficacy outcomes in experimental trials with vaccines against these infections have been variable (Frisch et al. 2018, Hoare et al. 2019).

19.8   How to Achieve Greater Diversity of Fish Vaccines in Europe?

Financial mechanisms tend to give priority to development of new vaccines for large and already profitable aquaculture segments that can sustain high costs per dose. The backside of that coin is that prophylactic tools that are needed to improve farm economics and support volume growth remain unaccessible.

For fish vaccines like for many other veterinary medicinal products, regulatory procedures and practices have gone with prolonged development time and costs. We can only hope that the achievements made during COVID-19 vaccine development will feed back into the regulatory systems, proving that not all vaccine developments need to take between 5 and 10 years when the need is urgent. In this author's opinion, a couple of further instruments can also be used in support of fish vaccine development and licensing:

- Proactive use of cascade prescriptions (within the EEA) or of time-limited, conditional authorization for vaccines licensed in other countries to satisfy currently unmet needs.
  - PD vaccines for salmon to control sleeping disease in rainbow trout.
  - Use of the DNA vaccine against IHN licensed in Canada to control IHN in Europe, including emergency vaccination to prevent between new zones or farms, and to support eradication efforts.

Such uses should be accompanied by building relevant safety and efficacy documentation alongside and during field use, eventually leading to a licensed extension of the species range and/or disease indications.

- There is no need to be afraid of autogenous vaccines, that should be seen as a potential pathway to achieve regularly licensed products:
  - For the fish farming value chain, "autogenous" should be defined as antigen strains that are shared along the entire production chain (hatchery–smolt farm–finishing farm) or by natural distribution mechanisms (rivers, currents).
  - There should be no need for renewing isolates for manufacturing every 6 months, provided antigen monitoring within the value chain can show stable strain identity.

    Except for very particular situations, the use of autogenous vaccines should allow building relevant safety and efficacy documentation during field use, with the aim to support regular licensing for the most voluminous uses and segments over time.

19.9   Who Will Develop and License the New Fish Vaccines?

A commonly known experience in medicinal product development is that "Big Pharma" will only start development of vaccines or medicines that promise high profit and appear a low-risk investment. Early development of innovative medicines is left to start-ups and SMEs. They may eventually be acquired by larger corporations if they succeed. For fish vaccines, a possible exemption to this general picture is veterinary vaccine SMEs wishing to expand into the aquaculture market for strategic reasons.

It is therefore more likely that new fish vaccines will build on research and early development performed in innovative start-up companies, addressing specific (but initially volume limited) user needs. In many aspects, the BioNTech example (having an established technology basis from anticancer vaccine research, swiftly recognizing new user needs and commercial opportunities) provides an example that is relevant also for the veterinary vaccine sector in future.

The ability and willingness of the aquaculture industry—for example, the Norwegian Seafood Research Fund and—to join forces with public funding bodies in the support of independent experimental and field evaluations of fish vaccine prototypes will co-determine if or how fast urgently needed fish vaccines can proceed to licensing. With the general attitude that "applied research is for the supplier industry to fund on their own," a change in the current pace appears unlikely.

In this author's opinion, another key factor for the future developments is the opportunity and ability to generate incomes quite early in the development process of new fish immunologicals. It is believed that the increased use of so-called platform technologies (delivery systems such as vectors, carrier molecules, RNA, or DNA) will facilitate cost reductions and time to market during novel vaccine development and licensing (IABS 2021[16]). Together with a proactive use of the cascade and of time-limited authorizations

(see above), this new development can support a more diverse and timely supply of fish vaccines for European aquaculture.

19.10 Conclusions and Outlook

The current use of fish vaccines is massive and remarkably successful, especially in controlling systemic bacterial diseases and in the highly industrialized segments of aquaculture. Advanced immunology research is being carried out, and new delivery platform technologies (DNA vaccination) are already being implemented. Making vaccines commercially available for viral infections that are endemic in regions or farming segments and for bacterial diseases in conventional, less industrialized freshwater farming remains important tasks for the near future. If additional efforts can be mobilized to transfer more of the knowledge from immunology and the other basic science disciplines to real-life immunoprophylaxis, it will support the diversity of food fish production in European waters.

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Stress and Immunity in Fish

20

Lluis Tort and Joan Carles Balasch

Abstract

Fish inhabit microbial- and parasite-enriched environments that constrict the adaptive choices and evolutionary pathways of regulatory physiological systems. Teleost immune architecture reflects those selective pressures, and the modulation of immune performance by glucocorticoids and other mediators of perceived stress have sculpted the type, intensity and scope of stress-related immunity in fish. In this chapter, the immune response to stress in teleosts and the role of the principal mediators such as hormones, cytokines and neurotransmitters are reviewed. The interactions between the immune, endocrine and neural regulatory systems and the influence of the local environment in the response are also considered.

Keywords

Stress · Innate immunity · Adaptive immunity · Teleosts · Neuro-immune axis · Environmental stressors

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Abbreviations

Ag	antigen
AID	activation-induced deaminase enzyme
AMP	antimicrobial protein
APC	antigen-presenting cells
APP	acute-phase protein
APR	acute-phase response
BMR	basal metabolic rate
CSR	class switch recombination
DAMPs	danger/damage-associated molecular patterns
E2	17β-oestradiol
EE2	ethinyloestradiol
FDC	follicular dendritic cell
GR	glucocorticoid receptor
HIRECs	human-induced rapid environmental changes
HPG	hypothalamic–pituitary–gonadal axis
HPI	hypothalamic–pituitary–interrenal axis
HPT	hypothalamic–pituitary–thyroid axis
IFN	interferon
IGF	growth hormone (GH)/insulin-like growth factor axis
LLPC	long-lived plasma cell
LPS	bacterial lipopolysaccharide
MHC	major histocompatibility complex
MMC	melanomacrophage centre
MR	mineralocorticoid receptor
NK	natural killer cells
PAMP	pathogen-associated molecular pattern
PKD	proliferative kidney disease
PRR	pattern recognition receptor
RAG	recombination-activating gene
ROS	reactive oxygen species
SC	sympatho-chromaffin axis
SHM	somatic hypermutation
TCR	T-cell receptor
TLR	Toll-like receptor
VLRs	variable lymphocyte receptors

20.1   Introduction

Environmental changes, unpredictable or expected, persistent or serendipitous, biotic or abiotic, mellifluous or obnoxious, constitute the fuel upon which selective processes boost normal or abnormal stress responses (Romero et al. 2009). A majority of these changes may elicit harmless adjustments in the physiological constraints that define the tolerance limits of core temperature, osmolyte levels or acid–base balances. Others, unexpected, rare and intense changes may force stressful, allostatic adjustments, enhancing the activation of neuroendocrinological and immune cascades, in an effort to reverse unsafe levels of endogenous variables. In addition, a minority of those variations, mainly induced by human-related industrial and productive activities, may severely compromise the health of organisms in a species-specific manner, mostly in local scenarios in which the defining parameters of niche minutiae may change abruptly (Halpern et al. 2008). A classic example is the impact in coastal fish of endocrine disruptors, whose concentration may change dramatically due to aquaculture or industrial run-offs, fluvial outflows and seasonal weather episodes, in the reproductive, tiroidal and stress neurohormonal cascades (Gaw et al. 2014; Szwejser et al. 2017). Confronted with such human-induced rapid environmental changes (HIRECs), stressed individuals skew their energetic budgets, dedicated to metabolism, reproduction and growth, in favour of physiological trade-offs, usually weighted by the species-specific scope and versatility of the evolved adaptive behavioural repertories that allow for coping with environmental stressors, but also by the efficiency and unfolding capabilities of the immune response (Balasch and Tort 2019). Accordingly, the stress response becomes a result of interactive processes between nervous, immune and endocrine systems, devoted to overcome the challenge caused by the stressor and to compensate for its effects, a connection already observed some decades ago when several works demonstrated that not only hormones altered immune functions but also that the immune mediators influenced the neuroendocrine system (Chiappelli et al. 1993). As we discuss below, both stress-related and immune physiological systems share common components and pathways, but a precise description of neuroimmunoendocrine crosstalk is still lacking for fish species. In this sense, the molecular, cellular and systemic scaffolding of stress and immune responses may be considered a common mechanism of defences that share the burden of limited metabolic budgets in what can be described as a collaborative network of mutual influences.

Any particular stressor (or combinations of stressors) may generate an evolutionarily conserved *adaptive* stress response or, alternatively, maladaptive ones if the intensity, persistence and resilience values of the stressor depart from the "classical" selective pressures, such as depredation, parasitism, or intraspecific and interspecific competition for resources and sex partners, in a given ecological niche. Considering fish species, the evolutionary road to immune sense—i.e. the meaningful detection of self- and non-self-antigens—and sensitivity—measured as the minimum defensive response threshold to foreign microorganisms or xenobiotics—is paved with hardwired anticipatory stress

responses constructed during the convoluted evolutionary history of survival and extinction in microbial-rich environments.

20.2 The Aquatic Constraints on Fish Immunity: Navigating Stressful Seas

Fish inhabit aquatic environments prone to pathogen spillover and xenobiotic spreading that stress their immune systems, usually less complex, in terms of both diversity of cellular phenotypes and specificity of antigen recognition components, than those of mammals (Flajnik 2018). Ocean microbiota abundances range from estimates of 10^4 to 10^6 cells mL^{-1}, or, in the case of virus (dominated by phages), up to 10^8 viruses mL^{-1} in open waters and 10^{31} in the sub-seafloor (Whitman et al. 1998; Middelboe and Brussaard 2017; Cai et al. 2019). In open oceans, the taxonomic and functional composition, distribution and stratification of microbial communities correlate strongly with temperature and dissolved oxygen (Brum et al. 2015; Sunagawa et al. 2015), with salinity, nutrient content and currents seemingly playing minor roles. Microbial communities in aquatic dwellings are characterized by (1) differential local and global clustering of viral and bacterial communities, influenced by host–pathogen interactions, hydrogeographic features or biogeographical regimes (Brum et al. 2015), (2) complex interactions between bacteria and virus (Breitbart 2011; Orsi 2018) that influence biogeochemical cycling, bacterial gene expression (due to horizontal gene transfer) and pathogenicity (extensive to marine invertebrates and vertebrates), (3) high persistence (thousands of years) of viral and bacterial communities in the energy-starved seafloor (Lomstein et al. 2012; Cai et al. 2019) and (4) huge genetic diversity reservoirs, especially in deep-sea sediments crowded by viral phenotypes, which may fuel evolutionary changes (Jousset et al. 2017). Virus and transposable elements have been postulated as the origin of early immune pathways in marine basal vertebrates (Broecker and Moelling 2019), and fish house large numbers of viruses, prone to jump between hosts (Shi et al. 2018; Geoghegan et al. 2018). In fact, the microorganism richness of aquatic realms continues to impact the co-evolution of complex host–pathogen interactions (Zhang et al. 2018), and maybe even influenced the faster rate of genetic evolution in teleost fish compared to mammals (Takezaki 2018). This, in turn, may have facilitated their bewildering morphological and physiological diversity. Fish show extremely diverse lifestyles, having colonized abyssal wastelands (Swann et al. 2020), submerged murky caves (Maldonado et al. 2020), polar waters (Farrell and Franklin 2016), hypoxic sulphidic mangroves (Rossi et al. 2019) and desiccated ponds (Wright and Turko 2016) due to their remarkable phenotypic plasticity. Coupling the expression of fish genomes to changing environmental constraints allows for the expression of plastic phenotypes, but the vast majority of studies of developmental or behavioural plasticity in fish have been focused more in the metabolic adjustments of energy reservoirs, osmoregulation, coupled acid–base respiratory trade-offs and growth in heated, cooled, hypoxic or

contaminated dwellings, and less in the effects on immune responses to such environmental insults.

Parasites also contribute to energy and nutrient flows in aquatic ecosystems, modulating host population dynamics and trophic networks, interfering with reproductive seasonality and influencing coevolving morphologies and complex lifecycles involving multiple hosts for retaliating immune strategies of antigen sensing in the host (Marcogliese 2002; Rohde 2002; Rohlenová et al. 2011). The extent of parasite biomass and communal structure is not yet well characterized, but some studies have highlighted that the effect of temperature in virulence and speciation rates in ectoparasites, or endoparasites in ectotherm species, may depend upon differences between freshwater and marine environments (Scharsack et al. 2016; Poulin 2016; da Costa and Val 2020). Temperature may also have a relevant influence in the still unaccounted importance of cryptic parasites, i.e. species genetically distinct but with very similar morphologies (Poulin 2011). Pulse warming events, short-term temperature fluctuations derived from seasonal anomalies and extreme weather related to global warming, has been shown to affect fish immune responses, habitat use and migratory adjustments due to the expansion of parasite ranges, altered life stages and increased virulence in the host–parasite trade-offs (Claar and Wood 2020).

The impact of human-induced rapid environmental changes (HIRECs) on fish immune performance is still in its infancy, and it will require an appropriate conceptual framework that may encompass the multilevel, transgenerational and overlapping influences on immune outcomes of anthropogenic and "natural" (as opposed to human-dominated), changes in aquatic landscapes (Sievers et al. 2018; Donelan et al. 2020). The effects of HIRECs on fish diversity and performance depend largely on habitat distribution, with coastal environments more prone to suffer from human-derived disturbances (Halpern et al. 2008). Human-induced alterations in aquatic ecosystems include, but are not restricted to, climate change, pollution, eutrophication, exploitation, habitat degradation, invasive species and hatchery production, inducing local, ecological and also commercial extinction of the most consumed species (McCauley et al. 2015). Coastal estuarine areas may suffer from acidification due to eutrophication, aquaculture practices, freshwater run-offs and tidal exchanges, besides the continuous increase in atmospheric P_{CO2} (Duarte et al. 2013). The impairment of connectivity networks between habitats may affect food webs and species abundance and dispersion, altering the host–pathogen cycles (Duarte et al. 2020). Albeit the modelling of the changes in climate patterns is as good as the quality of the data that validate and support the forecasting, it is beyond doubt that, for ectotherms, climate change may influence distribution patterns, species richness, genetic variation along biogeographical clines, colonization and diversification rates and immune responses to pathogen outbursts (Stanley et al. 2018; Clucas et al. 2019; Manel et al. 2020). These and other ecological constrictions limit the scope, performance and diversity of fish immune systems, in what has been increasingly recognized as a complex system of mutual interaction between the needs of immune surveillance and response, and the physical and biotic intricacies of aquatic realms. An ecological immunology approach to analyse the

evolutionary road to fish immune constructs is much needed as it may help to prevent and anticipate the changes in aquatic communities due to expanding human activities.

20.3   The Fish Approach to Immune Defence

The pathogenic load, together with the spatial and temporal heterogeneity and resilience to unpredictable changes in aquatic habitats, affects all levels of endogenous responses, from major neurosecretory and hormonal regulatory systems, to plastic behavioural phenotypes and moving population assemblages (Egerton et al. 2018; Flajnik 2018; Broecker and Moelling 2019; Guo et al. 2019). In this sense, the majority of efforts traditionally invested so far in the analysis of immune responsiveness in fish have been dedicated to describe the components of immunity in model or commercial teleost species subjected to pathogenic challenges, sometimes coupled with physiological responses to variable temperature or oxygen levels or common handling procedures in aquaculture practices. Current approaches rely on the analysis of immune genome repertoires and the development of transgenic models to elucidate host–pathogen crosstalk in immunocompromised species. However, the influence of past evolutionary events on species-specific immunity is still almost absent in these analyses (Solbakken et al. 2017). Fish are basal roots in the phylogenetic vertebrate branching and have endured a long evolutionary history of diversification and extinction cycles (Near et al. 2012) and several rounds of genome duplications (Glasauer and Neuhauss 2014). They also show extreme differences in morphology and life stories, colonization of extreme, nutrient-scarce habitats (Priede and Froese 2013; Crawford et al. 2020; Maldonado et al. 2020) or adaptive radiations in the aftermath of environmental upside downs and evolutionary transitions (Smithwick and Stubbs 2018; Ribeiro et al. 2018). All these upheavals have coalesced in a panoply of extremely diverse lifestyles, varied reproductive strategies and immune trade-offs that prevent a unified definition of common patterns of defensive responses in fish. In addition, the vast majority of fish retain a hardwired ectothermy that constricts immune function, which is why some species seem to try to become endotherms by means of metabolic and behavioural adjustments (Dickson and Graham 2004).

Broadly speaking, fish immunity encompasses three adaptive strategies evolved to cope with a microbial world, namely the approaches observed in "agnathans" (jawless fish), "chondrichthyes" (cartilaginous fish) and "osteichthyes" (bony, ray-finned fish, Actinopterygii, and lobe-finned fish, Sarcopterygii, including coelacanth and lungfishes). A detailed description of the immune intricacies of jawless and cartilaginous fish is beyond the scope of this review, and the readers are referred to several excellent reviews (Flajnik 2018; Smith et al. 2019). Nevertheless, a very brief sketch of immune features in jawless fish and sharks may be useful to illustrate the high variability of immune components described for marine and freshwater fish. Both cartilaginous and bony fish display the following traits: (1) dedicated organs (thymus, spleen) for the maturation of immune cells (generalist phagocytes, specialized B cells and T cells), (2) production of antimicrobial

proteins and mediators of immune responses (cytokines, immunoglobulins with variable domains), (3) the achievement of a reasonable high level of diversity for antigen-recognizing membrane receptors (TCRs) and immunoglobulins due to enzymatic rear-rangement of immune genes and (4) the capability of internalization, processing and use of exogenous antigens using products of polymorphic gene sequences (MHCs, see below) as a means to coordinate the mutual activation and regulation of the distinct immune cellular subtypes. The more ancient jawless fish, lampreys and hagfish, lack specialized immune organs, except for the thymoid, an equivalent of thymus. They have B-cell and T-cell-mediated responses, and probably other cellular components of immune responses. They may have cytokines but lack immunoglobulins, and instead of MHC-regulated arrangements of immune receptors, they have evolved exclusive variable lymphocyte receptors (VLRs) as a means to diversify antigen recognition. Genomic analysis uncovers immune genes shared for all fish groups on a regularly basis, and a high degree of convergence is expected regarding common characteristics of cellular phenotypes and molecular mediators between agnathan and gnathostomata fish species. Additionally, at least three rounds of genome duplications have defined the expansion of the fish immune repertoire (Kuraku et al. 2009), two of them before the agnathan/gnathostomata divergence, around 500–800 million years ago (Mya), and the most recent after the split of the teleost lineage, 325–350 Mya, probably helping in their successful adaptive radiation (Glasauer and Neuhauss 2014). A fourth genome duplication event 88–90 Mya has equipped salmonids with several paralogous and duplicate genes still retained in extant species (Christensen and Davidson 2017), and, more recently, around 12 Mya, in carps (Xu et al. 2019).

Teleostei house the vast majority of extant fish (Nelson et al. 2016) and may be considered the most advanced ray-finned fish (Actinopterygii) in terms of alternate functional physiology frameworks, but it is not possible to extrapolate the immune features of teleosts to all fish. In fact, the fuzzy definition of "fishes", a term used to refer to a multiplicity of morphologies, species and taxonomic phylogenies, is still controversial, even in the phylogenomic era (Hughes et al. 2018), and confuses the description of common fish immune responses. An ever-growing number of fish genomes have been sequenced in non-model species (Buonocore and Gerdol 2016; Wcisel et al. 2017; Hara et al. 2018; Ravi and Venkatesh 2018; Smith et al. 2018), but Actinopterygii have been the object of the vast majority of functional immune studies to date. Therefore, here we will focus on teleost models to discuss the effects of environmental stressors in immune performance.

20.3.1 Teleostean Immunophysiology (I): Common Vertebrate Features

In infected or immunocompromised vertebrates, as it may happen after stress episodes, the return to original homeostatic grounds starts with the innate toolbox of local inflammatory responses. Broadly speaking, this includes a repertoire of several key overlapping

processes (Medzhitov 2008), among others: (1) on-site release of PAMPs and DAMPs (pathogen- or tissue-damaged danger-associated molecular patterns, respectively), (2) complement-mediated opsonization cascades (see Chap. 9), (3) degranulation of resident granulocytes, (4) release of antimicrobial proteins (AMPs) and pro-inflammatory/chemotactic cytokines (see Chap. 10), (5) recruitment of circulating phagocytes and cytotoxic natural killer (NK) cells to the site of infection or damage, (6) activation of hepatic acute-phase proteins and (7) antigen (Ag) recognition by dendritic cells, macrophages and other antigen-presenting cells (APCs).

The response to bacterial, viral of fungal PAMPs (see Chap. 2), is orchestrated by an array of germline-encoded soluble, membrane-bound and intracellular pattern recognition receptors (PRRs) (Takeuchi and Akira 2010), such as the highly diverse Toll-like receptors (TLR). PRRs are expressed primarily by immune cells, but also by tissue-specific epithelial/endothelial cells. Interferons (IFN) are the main mediators of antiviral responses (Secombes and Zou 2017). Pathogens can subvert the PRR recognition masking themselves with endogenous antigens, delocalizing or modifying the structure of PRRs to prevent proper bonding to antigens, changing their phosphorylation/ubiquitination features, thus targeting them for degradation, and inhibiting downstream signalling components (Majzoub et al. 2019). Exogenous and endogenous antigens are processed, fragmented in manageable peptides and presented to immune cells through highly polymorphic major histocompatibility complex (MHC) proteins (Kotsias et al. 2019). Widespread exposure to self or altered cytosolic peptides bound with MHC class I molecules in almost all cell surfaces activates cellular crosstalking and polarization of immune phenotypes, fully unrolling the inflammatory process by activation of membrane receptors of $CD8^+T_C$ (cytotoxic) cells. If the MHC I complex is impaired by viral assault, a "missing self" signal ensues and the infected cell is destroyed by NK cells. Other non-classical MHC I molecules are induced in stressed cells and contribute to the clearance by NK cells upon recognition by their fast-evolving variable receptors, a trademark of these cytotoxic cells (Parham and Moffett 2013). Extracellular antigens, once endocytosed, fragmented and bound to MHC class II molecules constitutively expressed in immune cells (mainly APCs), stimulate $CD4^+T_H$ (helper) cells that coordinate the nascent immune response. In non-APCs, such as epithelial cells and fibroblasts, interferons (IFN-γ), TLRs, TGF-β and other signalling molecules can stimulate MHC II expression during inflammatory processes, rendering the global network of interactions more complex (Neefjes et al. 2011). Several non-classical MHC molecules also bind small metabolites and participate in the editing of antigen-derived peptides, and activation of T cells and NK cells (D'Souza et al. 2019).

If the containment measures of innate immunity are ineffective, the immune response broadens, and the adaptive, highly specific immune modules, dominated by B- and T-cell subtypes, are brought into play (see Chap 4). Haematopoiesis increases, and lymphocytes with variable surface receptors begin to be mass-produced and selected for improved antigen affinity in specialized lymphoid organs, such as lymph nodes and spleen. To ensure the diversity of antigen-recognizing receptors, at earlier stages of B- and T-cell

differentiation, RAG1-RAG2 endonuclease complex helps rearrange the gene segments that encode the different Igs and TCRs, enabling the ulterior clonal selection of high-affinity receptors capable of recognizing a vast array of antigens (Ru et al. 2018).

The vast majority of T cells consist of $\alpha\beta$T cells, which participate in the overall adaptive response to antigens, whereas the less abundant, and still less studied, $\gamma\delta$T cells have also been implicated in antigen recognition in innate-like immunity (Rampoldi et al. 2020). Immature B cells express IgM/IgD surface receptors, and in mammals, but not in fish (see Chap. 8), if stimulated by antigens and cognate T cells, change the isotype to IgG, IgA and IgE. Once exposed to antigen, B cells also endure several cycles of somatic hypermutation (SHM) of immunoglobulin gene segments, facilitating the cloning and expansion of Ag-specific cellular populations. Once diversified, these cellular populations are confronted by follicular dendritic cells (FDCs), APCs and T follicular helper (T_{FH}) cells in the germinal centres of lymph nodes (absent in fish) and other secondary lymphoid organs. These processes mature the affinity of the differential surface receptors for the antigen in the precursors of Ig-producing B cells. The mutagenic changes in Ig genes elicited by activation-induced deaminase enzyme (AID) rearrange gene segments and, ultimately, favour SHM and immunoglobulin isotype class switch recombination (CSR), which, in turn, refine and enforce the antigen–antibody (Ag-Ab) bond, dramatically improving the overall immune response (Methot and Di Noia 2017). Once matured, B cells produce specific antibodies, whereas $CD4^+$ T_H and regulatory (T_{REG}) cells orchestrate the immune crosstalk and reactivity, and $CD8^+$ T_C cells neutralize infected cells and collaborate to the overall cytokine signalling. If effective, the adaptive countermeasures end with (1) the controlled and apoptotic-regulated clearance of activated immune cells, (2) the nesting of quiescent populations of long-lived plasma cells (LLPCs), true memory B cells and T cells and probably NK memory cells (Beaulieu 2018) that guarantee rapid responses upon re-exposure to the same antigen and (3) the clean-up of immune debris and tissue healing, mainly by specialized macrophage M2 phenotypes (see Chap. 6) and other tissue-healing cells (Yunna et al. 2020).

After immune challenge or stress, the complex intricacies of coordinated networking between all the participants at each stage of immune responses result in high time-dependent malleability of cellular phenotypic changes as defensive actions progress. All these cellular immune phenotypes, innate or adaptive, either recirculating or anchored to specific tissues, come in several forms, developmental stages and polarized functions that usually coexist spatially and functionally and can contribute to blur the distinction between innate reactivity and adaptive reactivity (Van Kaer et al. 2019). Differentiated tissue-resident macrophages, for example, are a heterogeneous composite of embryonic, steady state and adult monocyte-derived active cells that adds up to the complexity of local, tissue-specific inflammatory responses (Ginhoux and Guilliams 2016). On the adaptive side, naïve T_H cells adopt several phenotypes, T_H1, T_H2, T_H17 or T_{REG}, depending on the timely production of specific cytokines during inflammation. This differential cellular polarization may bias T_H responses towards general cytotoxicity against cancer cells or intracellular pathogens (T_H1 cells), immunosuppressive processes (T_H2 cells), intestinal microbiota

homeostasis (T_H17/T_{REG}), regulation of immune responses against self-antigens (T_{REG}) and protection against extracellular bacteria and fungi (T_H17), although there could be some functional overlap between the different T_H phenotypes (Yamaguchi et al. 2015). Both innate myeloid and lymphoid cells may display a short-term memory, antigen-independent, that enhances reactivity to repeated exposure to the same antigen. Once active, monocytes, macrophages and NK cells undergo epigenetic reprogramming that change their chromatin profiles, enhance glycolytic metabolism and increase cellular responsiveness after being immunologically "trained" (Netea et al. 2016). Overall, this suggests that it is not possible to divide the vertebrate immune responses between rapid, unspecific, early-responding innate components, and long-lasting latecomers, highly specific ones, capable of establishing a memory of recent infections. As has been recognized in the last decades, many participants of adaptive immunity in vertebrates educate, and are triggered by, the concerted action of innate immune components (Iwasaki and Medzhitov 2015) in what is now considered a continuum of progressive defensive responses, and, more importantly, a source of immune plasticity when confronted with environmental insults.

20.3.2 Teleostean Immunophysiology (II): Particularities and Drawbacks

Teleosts differ from mammalian counterparts in the organography, components, onset, duration, memory and functional effectiveness of immune responses (Salinas 2015; Geven and Klaren 2017; Secombes and Zou 2017; Flajnik 2018; Reverter et al. 2018).

The main regulatory hub and primary lymphoid tissue of both stress and immune responses in fish is the head kidney (Fig. 20.1), where mixing of neuroendocrine and haematopoietic/immune cell populations occurs, resulting in a constant molecular crosstalk, and can be considered a functional analogue of mammalian bone marrow and the adrenal gland (Geven and Klaren 2017). Fish tend to diminish the barriers between capsulated and structured organs devoted to a single function and, instead, possess multifunctional integrative organs (see Chap. 1). In this sense, in teleosts, the head kidney coordinates the neuroimmunoendocrine crosstalk derived from the innervation of lymphoid organs (Balasch and Tort 2019). Head kidney cells produce and secrete the two most relevant stress hormones after stressful stimuli: adrenaline by chromaffin cells and cortisol by interrenal cells (Fig. 20.1). Moreover, the co-localization of these two cell types enables paracrine interactions between these cells, thus enabling cross-influences (Rotllant et al. 2006). Cortisol and catecholamines are important inducers of cytokines from macrophages, as these cells have both α-adrenergic and β-adrenergic receptors (Maciuszek et al. 2019) and glucocorticoid receptors. Moreover, together with cytokines, glucocorticoids evoke a strong synergistic enhancement for most acute-phase proteins (APPs) (Bayne and Gerwick 2001). The presence of adrenergic and hormonal receptors in immune cells modulates cytokine secretion that, in turn, docks in immune receptors in neuroendocrine cells, closing the homeostatic circle (see Verburg-van Kemenade et al. 2017 for a thorough review).
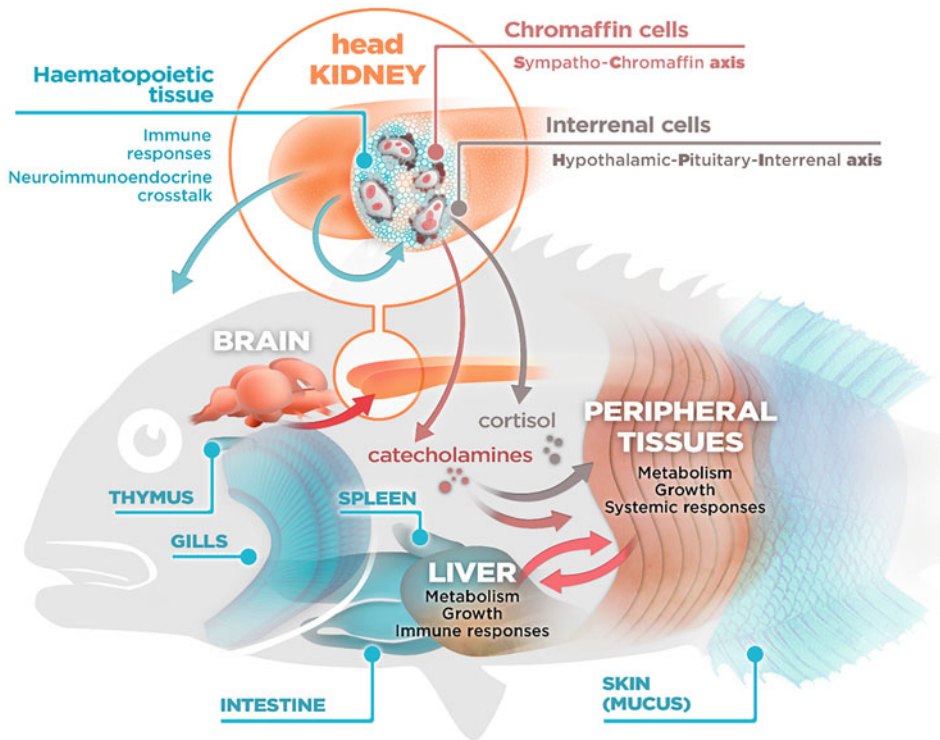
Fig. 20.1 Stress axis and immune dedicated tissues and associated organs in teleosts. Head kidney and spleen are considered the main lymphoid organs, facilitating cell trafficking and harbouring melanomacrophage centres (MMCs), involved in antigen trapping and processing. Mucosa-associated lymphoid tissues in gills, intestine and skin endure parasite attachment and ever-growing microbial communities that modify and educate host immunity. Liver produces acute-phase response antimicrobial peptides and innate complement factors. The influence of immune responses reaches the overall metabolic trade-offs and affect and is affected by the activation of several main regulatory axes, among them the catecholamine-producing sympatho-chromaffin (SC) axis, and the cortisol-producing hypothalamic–pituitary–interrenal (HPI) axis. Under stressful environmental changes, metabolic trade-offs influenced by temperature changes, parasite load and other environmental stressors may impair growth and overall allostatic adjustments

The thymus acts also as a primary lymphoid organ dedicated to the development of T cells and, as described for mammals, regresses with age, but only in some species (Bowden et al. 2005). The spleen and the lymphoid infiltrates in mucosal tissues are considered secondary lymphoid organs in an immune system that not only harbours less immune cell phenotypes and molecular effectors than mammals (Flajnik 2018) but also lacks mammalian-like highly structured intestinal lymphoid nodules, using melanomacrophage centres as surrogates of lymph nodes (Stosik et al. 2019).

Upon detection by dendritic cells, macrophages, granulocytes and IgT/Z+ B cells in mucosal tissues (see Chap. 12), clearance of undesired antigens or malfunctioning cells in

fish requires the coordinated participation of the head kidney's immune axis (Fig. 20.1), catecholamine-producing sympatho-chromaffin (SC) axis and cortisol-producing hypothalamic–pituitary–interrenal (HPI) axis (Khansari et al. 2018; Balasch and Tort 2019). Inconclusive immune outcomes require additional recruiting of cellular and molecular components of the overall defensive responses, including the hypothalamic–pituitary–thyroid (HPT) axis (Geven and Klaren 2017) or hypothalamic–pituitary–gonadal (HPG) axis and growth hormone (GH)/insulin-like growth factor (IGF) axis, especially in migratory species such as salmonids (Ueda 2019).

Overall, cellular and humoral responses in teleosts seem to follow the same pattern as in mammals (Rebl and Goldammer 2018; Smith et al. 2019). However, several mammalian-like and fish-specific TLRs have been described, being highly variable between species (Palti 2011). An illustrative example of the alternative, non-mammalian ways of pathogen sensing in fish is TLR4, an essential PRR for the recognition of bacterial lipopolysaccharide (LPS). Many teleost fish lack a functional TLR4 framework and have greater tolerance to LPS doses than mice or humans in endotoxaemic models (Swain et al. 2008). Alternative sensing pathways to LPS in transcriptomic models of *Schizothorax prenanti*, a cultured fish species in China, challenged with *Aeromonas hydrophila* and *Escherichia coli* LPS include expression of regulatory cytokines (*IL-1β, IL-10* and *IL-8*) and PRR (*TLR5, TLR25, PTX3* and *C1q*) transcripts that have been suggested to mediate the LPS responses in this teleost species (Li et al. 2020). However, the higher tolerance to LPS in teleosts remains intriguing, and, again, the microbial-rich characteristics of dwelling in an aquatic environment seem to be involved (Lieschke and Currie 2007).

All T-cell subsets (T_H, T_{REG} and T_C) seem to be present in teleosts (see Chaps. 3 and 4), but the morphological and functional description of cellular immune phenotypes in fish is still far for being complete, especially for innate lymphoid cells. Thymic homing from haematopoietic tissues and RAG-mediated selection for non-self-reactive T-cell phenotypes are believed to be similar in teleosts and mammals, but little is known about the commitment of αβ and γδ T-cell categories in teleosts (Bajoghli et al. 2019). B cells develop and mature in the head kidney and migrate to secondary lymphoid organs such as the spleen and posterior kidney, where the antigen-mediated stimulation takes place (Saunders et al. 2010). Once matured, the final plasma cell phenotype recirculates to the head kidney (Ye et al. 2011). Unlike mammals, teleosts produce only three types of immunoglobulins (see Chap. 7), IgM, the main circulating Ig, IgD and IgT, the latter more abundant in mucosal tissues, similar to IgA in mammals, and involved in the mutual regulation of complex host–microbe interaction (Zhang et al. 2010). IgD is still poorly studied, but the presence of IgD^+IgM^- precursor B cells in mucosal surfaces suggests that, as in mammals, IgD participates in pattern recognition (Gutzeit et al. 2018; Perdiguero et al. 2019). Notwithstanding the lack of germinal centres and FDCs in fish, affinity maturation seems to be present in teleosts. Melanomacrophage centres (MMCs), clusters of melanomacrophages (MMs) enriched in melanin, lipofuscin and hemosiderin that are present in lymphoid tissues, are postulated to be the analogues of germinal centres in fish. These are sites where antigen trapping and presentation to activated IgM^+B cells may

enhance Ag-Ab bonding, coordinated by MMs acting as functional analogues of FDCs (Stosik et al. 2019). However, the main function of MMCs is to accumulate and destroy metabolites and discarded cells. Indeed, the highly interspecific variability and temperature sensitivity of the activation-induced deaminase (AID) enzymatic activity suggest that the maturation of affinity may not reach the levels described in mammals, where up to 1000-fold higher affinity for the antigen is seen in plasma cells relative to that of the original mature naïve B cell, compared to a 100-fold increase described for ectotherms (Magor 2015). Affinity maturation has been described recently for long-lived plasma cells (LLPCs), primarily in the head kidney but also in the spleen of channel catfish (*Ictalurus punctatus*). Upon immunization, LLPCs appear in the head kidney 4 weeks post-immunization, after activation in the spleen and other peripheral lymphoid tissues, and secrete high-affinity antibodies, with serum titres that reach a peak 6 weeks post-immunization (Wu et al. 2019). It has been suggested that secreted teleost IgM tetramers undergo variable disulphide polymerization that may also contribute to affinity maturation of antigen recognition (Ye et al. 2010). Class switch recombination (CSR) in activated B cells, a change in the constant region of the Ig heavy chain that leaves the variable region untouched but expands the immune interaction and recognition capabilities by producing different Ig isotypes, has not been described in fish (Patel et al. 2018). Overall, antigen recognition and Ig-mediated responses in teleosts lack the efficacy and diversity observed in endotherm vertebrates.

It is relevant to note that erythrocytes are nucleated cells in fish, and hence can perform gene transcription and within their transcriptome are immune-related genes. For example, RNA-Seq analysis after poly (I:C) treatment shows diverse cohorts of mRNA transcripts related to immune function in erythrocytes. Moreover, erythrocytes can express a type 1 IFN response (Morera et al. 2011) and in vitro can endocytose TNF-α and VHSV G-protein fragments (Puente-Marin et al. 2019). Therefore, erythrocytes play a role in the immune response of fish. It is not yet clear whether this is a primary or a subsidiary or complementary role, particularly under stress situations, but it should be noted that there is a huge quantity of red blood cells compared to leucocytes, so even a small individual role will have an impact in the overall immune response (Morera and MacKenzie 2011; Anderson et al. 2018).

Surprisingly, some species, including Gadiformes, anglerfish and pipefish, have lost the MHC II. The evolution and maintenance of MHC diversity in teleosts (see Chap. 11), and therefore the competence to recognize self- and non-self-antigens effectively, depend on the delicate interplay between the unavoidable requirements of physiological trade-offs in specific niches. This is guaranteed by the ecological relevance of parasite load and their intrusive abilities when confronted with mucosal barriers, and the great plasticity of immune responses in fish. The loss of MHC II pathway components in pipefishes (*Syngnathus*), for example, has been attributed to their sex role reversed parental care strategy. *Syngnathus* feature male pregnancy, housing fertilized eggs in inverted tail brood pouches, feeding the developing embryos through placenta-like organs that allow for both the transmission of oxygen and also immune components. Gene expression studies of

Vibrio spp. and *Tenacibaculum maritimum*-challenged pipefish suggested differential parental immune priming of F1 and even F2 offspring, with parent males influencing strongly on offspring innate immunity and complement system components (Beemelmanns and Roth 2016; Beemelmanns and Roth 2017). Interestingly, sex-specific methylation patterns of immune genes were also observed, which are influenced by sex in other fish (Caballero-Huertas et al. 2020) and may be related to predictable, and stable parasitic load pressures over time. As described for Gadiformes (see below), in addition to the loss of MHC II, and CD4 components, diversification of MHC I pathway genes was also found in pipefish. However, MHC I transcripts and pro-inflammatory- and adaptive-related immune genes were found to be downregulated (Roth et al. 2020). This indicates, as the authors suggest, a convergent strategy to minimize the perils of embryo rejection as described for pregnant mammals, in which T_{REG}-mediated suppression or downregulation of MHC I and MHC II genes in the placental barrier ensures no immune reactivity to the antigens of the other parent (Prabhu Das et al. 2015).

At early stages, the development of primary and secondary lymphoid organs, together with the production of associated B/T cellular phenotypes, depends on environmental variables (such as temperature and food abundance) and varies greatly on a species-specific basis. Small eggs correlate with shorter yolk sac stages and fast hatching, but spleen, thymus and the haematopoietic/immune cellular subtypes produced in the head kidney appear several days after hatching, during the fast growth period characteristic of teleost larvae (Falk-Petersen 2005; Zapata et al. 2006). During the yolk stage, maternal transference of immunoglobulins, components of the complement system and other anti-microbial proteins (AMPs) help the developing embryo to keep pathogens at bay (Swain and Nayak 2009; Roth et al. 2018). This transgenerational immune priming is a strategy shared also by several invertebrate taxa (Tetreau et al. 2019). In addition, as suggested by transcriptomic analyses of immune gene expression across several stages post-hatching in the common sole (*Solea solea*), PPR sensing is also enhanced at hatch and first feeding, whereas increased abundance of MHC and TCRs transcripts correlates with the maturation of lymphoid organs during the metamorphosis (Ferraresso et al. 2016). The same study failed to detect RAG1 or CD4$^+$ and CD8$^+$ markers, suggesting that at the end of metamorphosis T-cell differentiation is still incomplete in this species. In fact, the maturation of immune organs constricts the efficacy of temperature-dependent antiviral and pro-inflammatory responses during larval stages, as demonstrated for zebrafish (*Danio rerio*) and European eel (*Anguilla anguilla*). This would impair the immune response to pathogens (Dios et al. 2010; Miest et al. 2019), even in anadromous and catadromous species undergoing the metamorphosis-like rearrangements prior to migration (McMenamin and Parichy 2013; Yada et al. 2018). Juvenile Atlantic salmon (*Salmo salar*) enduring the osmoregulatory and metabolic strengthening from the smoltification process prior to the downstream migration to open sea usually are affected by immunosuppression (Johansson et al. 2016). Hence, thermal stress may modify the timing of migratory behaviour, biasing metabolic, growth and immune trade-offs, ultimately changing the structure and composition of local age classes in the population (Cline et al. 2019). The

reliance on innate/unspecific immunity during the first stages of development renders fish larvae more prone to high mortalities, not only during the yolk phase, when high mortalities are expected, but also during the juvenile and early adult stages, that are more sensitive to stressful changes in environmental variables due to the incomplete maturation of adaptive defence mechanisms.

Overall, ecological factors limit the development and the functional performance of mature immune systems in fish, determining the extent to which individuals may cope with complex environmental stressors. In aquatic dwellings, immunity serves as a high-demanding metabolic sieve to screen for opportunist and virulent pathogens that crave for mucosal epithelia to attach in a seasonal, cyclic way, influenced by rises in temperature in a system dominated by ectotherms.

20.4   The Scalability of Stress Responses in Fish: From Stressed Cells to Systemic Immunomodulation

The processing of relevant information by the components of the immune system requires metabolically expensive parallel and distributed complex computation (Cohen and Efroni 2019) to solve the related problems of identifying cellular and humoral friends and foes, inflammation management, tissue repair, cancer control and, especially, symbiont housing, leaving sticky notes of the immune progression in the form of specialized receptors in neuroendocrine cells. In this computational sense, the overall performance of immune activation resembles those of the neuroendocrine machinery in terms of complexity and scope, and can be considered as an emergent feature based upon a plethora of underlying local cellular and molecular interactions and cross-recognitions between modular (i.e. classical innate and adaptive) components (Sotiropoulos and Tsihrintzis 2017). All vertebrate immune systems can be modelled as problem-solving algorithms dedicated to live, learn and remember the stressful changes in the pathogenic and xenobiotic environment (Fig. 20.2).

Fish may endure resilient human-induced environmental changes and thrive throughout sustained global pathogenic blooms, marine heating or acidification in part because of in-built plastic neural and behavioural adaptive mechanisms (Ebbesson and Braithwaite 2012; Maruska et al. 2019). However, these capabilities tend to be highly species- and niche-specific, and strongly constricted by each particular evolutionary history of physiological regulatory systems (Lee 2006; Solbakken et al. 2017). In this sense, higher lifestyle diversity and niche occupation may be beneficial in altered ecosystems, but the particular idiosyncrasies of each species or individuals constitute a major drawback for establishing a common unified framework to describe, evaluate and palliate the effects of compromised immune performances in the context of neuroendocrine responses (Balasch and Tort 2019; Taborsky et al. 2021).

Although a precise definition of stress is difficult due to the number of aspects associated with this concept, there is a general agreement in considering stress as an altered situation
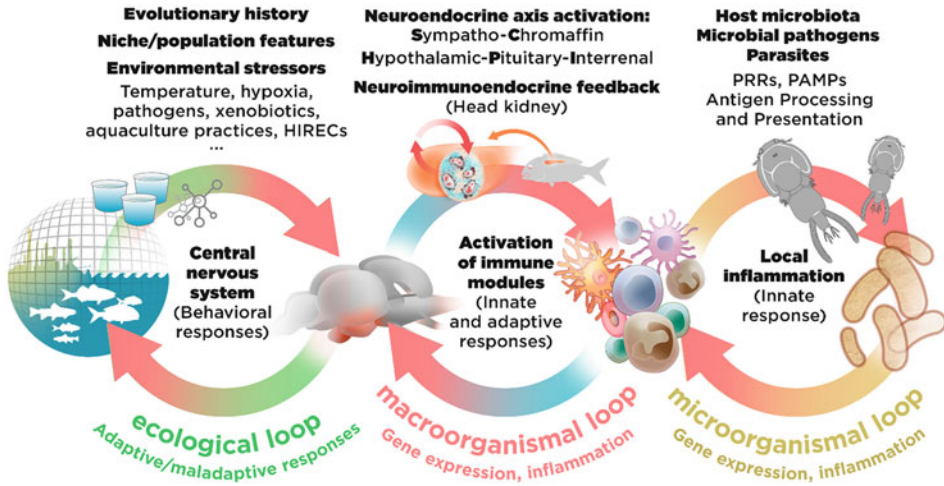
Fig. 20.2 Nested loops of neuroimmune control in fish. A series of nested loops participate in the mutual regulation of neural and immune responses (Irwin and Cole 2011). In fish, environmental stressors activate the hypothalamic–pituitary–interrenal axis (HP), the sympatho-chromaffin (SC) axis, and the haematopoietic/immune tissue in the head kidney (HK). The crosstalk between chromaffin, interrenal and haematopoietic cell populations in the HK guarantees a constant neuroimmunoendocrine local regulatory feedback that helps in the activation of immune modules. The transcriptional outcome of the inflammatory processes depends on the intensity of the environmental insults, and the species-specific determinants of the allostatic processes in the organism (extrinsic control of the immune response), as well as the local recognition of soluble or membrane-bound evolutionary conserved pathogen-associated molecular patterns (PAMPs) by means of pathogen recognition receptors (PRRs) in host cells (intrinsic control of immune local inflammation). Once started, inflammation may progress to systemic immune responses, recruiting cells and tissues to mount an adaptive immune response that, in turn, may alter the behavioural phenotype of stressed organisms. Several subloops (not shown in the figure), such as the crosstalk between innate and adaptive immune components, or the delicate equilibrium between host symbionts and mucosal immune surfaces of gills, gut, skin and nasopharyngeal spaces contribute to the fine regulation of neuroimmunoendocrine circuitry in fish

of the organism from its normal physiological scope as a result of a real or symbolic challenge. The stressor is defined as the challenging agent causing such a response. Stress is a universal phenomenon, and thus, any living organism may experience situations that cause these reactive or compensatory physiological responses, the so-called, stress response. It should be remembered that, since all organisms are subjected to challenges, all organisms experience stress responses and it is therefore observed that some of the basic components of the stress response have been maintained in all living organisms, and consequently, different taxa show similar molecular components and pathway mechanisms. In most cases, one can identify a specific number of molecules mediating the stress response that are common among animals. For instance, a group of these molecules, namely corticosteroids, adrenaline or cytokines, do exert the same networking

function in the stress response in all groups of vertebrates (Hau et al. 2016; Miles et al. 2019). Thus, although the stress concept was defined for mammals by Hans Selye in the last century, it is currently applied to all types of animals, plants and even to different levels of organizations (cell stress, organ stress and whole organism).

Stressed cells share common activation pathways to limit molecular damage and preserve macromolecular structure (and, hence functionality) by maintaining a robust proteostasis network of chaperones, components of the proteasome complex and other mediators that help to synthesize, fold, traffic and degrade proteins, guaranteeing a low level of misfolded or aggregated molecules in the proteome (Sala et al. 2017). Among pre-transcriptional responses, chromatin remodelling involves control over transcription, as there are a number of phenomena that will modify the translation of messenger RNA, such as alternative splicing, or gene silencing by miRNA. Regarding the post-translational response, the ubiquitination system uses small proteins that covalently modify other proteins in cells with consequences in transcription, proliferation, DNA repair, protein degradation and nuclear localization (Mazzucotelli et al. 2008). Under stress conditions, altered or wounded cells, ubiquitins upregulate the NKG2D (natural killer group 2, member D)-activating receptor in mammals, through high levels of MIC ligands (MHC class I polypeptide-related) and activating NK cells and T cells that, ultimately, eliminate altered cells (Stern-Ginossar et al. 2008). The evolution of metazoan *bauplans* requires the coordinated participation of several cell lineages across different tissue and systemic regulatory networks, which in turn rely on paracrine crosstalk to transfer the cellular distress between nervous, endocrine and immune systems. Unsurprisingly, some components of the proteostasis network, and also several intracellular pathways that allow for their activation, are shared between stress and immune main activation axis in vertebrates (Miles et al. 2019). At the cellular level, the response of the organisms to any type of tissue damage and infection will involve immediate-specific reactions in order to repair the damage, promote wound healing, protect against invading organisms and contribute to host defence mechanisms, in particular the innate immune response. These rapid reactions involve a number of specific proteins, the acute-phase proteins involved in the so-called acute-phase response, APR, mainly produced in the liver that may enhance or depress inflammatory responses (Black 2003). Some of these molecules involved in cell stress responses are well conserved through evolution and have been used as molecular indicators of any kind of stress including immune stress. Therefore, such proteins would be involved in the regulation of both cell immunity, cell damage or altered metabolism. Among the specific proteins associated with immunity and stress is NF-kβ, a highly conserved family of related protein complexes in metazoans (Ghosh et al. 1998) that, once activated by bacterial or viral antigens, activates or represses hundreds of genes in stress-related immune responses (Zhang et al. 2017), including inflammation, cell migration, cell repair and metabolic activation. Map kinases (MAPKs) are signal transduction mediators that are central for the correct function of many aspects of cell physiology, including cell defence against pathogens. MAPKs also activate SAPKS or JNK after alterations in cell osmolality, stress stimuli, inflammatory signals, variations in the level

of reactive oxygen and other signs of cell stress associated with defence and immunity (Kyriakis and Avruch 2012). Another kinase, AMPK, is activated when there is a low level of intracellular ATP, cell growth arrest and biosynthetic reduction. Its activation induces glucose and fatty acid uptake and oxidation to recover cell energetics in a subset of lymphocytes, T regulatory cells, that, together with M2 macrophages, the alternative anti-inflammatory phenotype of classically activated M1 macrophages (Locati et al. 2020), regulate the termination of inflammation (Michalek et al. 2011).

Among stress proteins, one group that has been widely associated with stress is the heat shock protein group (HSP), a superfamily of proteins that have been well conserved through evolution, thus present in bacteria, fungi, plants and animals (Feder and Hofmann 1999; Timperio et al. 2008). Although they were discovered after heat shock treatments, they are induced by a huge variety of environmental stressors. HSP and other stress-related proteins work as chaperones, responsible for maintaining the integrity of the molecules in the cell that are transcribed in response to stressful stimuli and they are conserved in all domains of life, but they can also act as DAMPs, damage-associated molecular patterns that initiate inflammatory processes (Schaefer 2014). In mammals, several HSP families have been described to activate immune responses, participate in the pro-inflammatory and apoptotic responses to viral infection, usually enhancing the immune reactivity, but also helping to replicate or assembly the viral particles during dysregulation episodes in HSP function (Wan et al. 2020). The production of hepatic C-reactive protein (CRP), an AP protein, stimulated by an inflammatory cytokine, interleukin 6 (IL-6), has also been associated, in addition to immune responses, with fatty acid metabolism in muscle, feeding, regulation of adipose tissue and overall metabolic maintenance (Del Giudice and Gangestad 2018), processes that may interlock in part during stressful outcomes and suggest a multifactorial role of immune mediators in stress-related metabolic trade-offs. For example, in mammals, chronic stress enhances several inflammatory cytokines, including IL-6 and CRP, probably by means of desensitization of immune cells to the effects of glucocorticoid inhibition (Cohen et al. 2012).

20.4.1 The Central Role of Glucocorticoids

As chemical messengers secreted to blood mainly from the pituitary and head kidney, fish hormones are principal mediators of the systemic stress response but also influence the immune system. Their effects normally take some time because hormones have to reach the receptors in the target tissues that subsequently regulate processes like gene expression, morphological changes or protein synthesis. Nonetheless, it has also been shown that some hormones can be produced locally, which enables them to act acutely through a paracrine process, and therefore have no delay due to transit time by the bloodstream (Calisi and Saldanha 2015). Among the many hormones activated after stress, glucocorticoids or corticosteroids are crucial for modulating the response of the immune system, in addition to also modulating many other functional processes, including metabolism, development or

behaviour. Thus, following stress challenges, glucocorticoids will affect multiple physiological, behavioural (Kelly and Vitousek 2017) and life-history (Crespi et al. 2013) elements as components of the homeostatic response. Hence, circulating glucocorticoids represent a plastic phenotype coming from a single genotype that allows individuals to release different concentrations of these circulating messengers depending on the varying environmental conditions and individual perception (Romero and Gormally 2019). One of the sites where endogenous glucocorticoids exert powerful effects is on inflammatory and immune processes. Nevertheless, as pointed out by evolutionary and comparative biologists, in terms of the overall understanding of the glucocorticoid effects, it becomes paramount to quantify the individual variation of these mediators, as this would help to evaluate the current variability in immunity, behaviour, physiology and overall response performance (Guindre-Parker 2020). As a consequence, the recognition of such variability has shown that analysis of cortisol alone is insufficient when used as a single bioindicator to measure the overall stress response and its consequences (Romero and Gormally 2019; Telemeco and Gangloff 2020).

Cortisol modulation entirely depends on the presence of glucocorticoid receptors in the cells. The pleiotropic effects of glucocorticoids will then largely depend on the extension of binding to the different glucocorticoid receptors found in fish tissues (Landys et al. 2006). Thus, it is relevant to point out several features that are important regarding cortisol receptors in fish. First, fish present two glucocorticoid receptors (GR1 and GR2) and one mineralocorticoid receptor (MR) (Faught and Vijayan 2018). Second, alternative splicing variants of glucocorticoid receptors have been found under stress conditions (Romero et al. 2020). Third, receptors are ubiquitously expressed in all tissues including immune tissues. Therefore, the receptor-mediated response is complex in fish and for other vertebrate species.

Altogether, thanks to their ubiquity and complexity, cortisol receptors bring a high plasticity in terms of regulation of physiological and immune responses after stress. For example, high-affinity and low-capacity mineralocorticoid receptors are sensitive to relatively low concentrations of circulating cortisol that will be helpful in regulating metabolism and energetics, whereas high cortisol concentrations in the blood will initiate acute alarm responses including immune signalling (Landys et al. 2006). Receptor type, receptor density, time course and transcriptional effects of circulating glucocorticoids in the blood will involve differential effects across tissues and environmental contexts, thus modulating the action of cortisol and therefore the influence on the immune response (Breuner et al. 2013; Guindre-Parker 2020). Two mechanisms of action have been described after activation of glucocorticoid receptors. First is the genomic signalling, which takes hours to days to express the corresponding effects (Nicolaides et al. 2010). Second, the non-genomic signalling, a rapid mechanism taking only seconds to minutes, that activates plasma membrane proteins using second messengers such as cytoplasmic free calcium (Panettieri et al. 2019; Johnstone et al. 2019; Faught and Vijayan 2019). These signals will modulate the transcription of key genes of the pathways concerned with the generation of immune responses.

Once glucocorticoid receptor signalling is activated, some pathways of innate immunity are enhanced, but most pathways of adaptive immunity are suppressed (Cain and Cidlowski 2017). Low concentrations of endogenous corticosteroids upregulate PRRs, thus sensitizing the innate immune system through cytokine receptors and complement factors, thus allowing the induction of acute responses to danger signals (Cain and Cidlowski 2017). High concentrations of glucocorticoids, by contrast, suppress signals that are mediated by PRRs and cytokine receptors, thereby preventing excessive and/or prolonged immune responses. The inhibition of the transcription of immune response genes is mediated by three mechanisms. First, suppression of the glucocorticoid receptor to gene promoter sequences, second, induction of glucocorticoid receptor-mediated transcription of anti-inflammatory genes (such as NF-κβ-IA) and, third, non-genomic antagonism of pro-inflammatory transcription factors (such as NF-κβ) or via protein–protein interaction (Verburg-Van Kemenade et al. 2009). It should also be noted that the reverse signalling pathway has been observed, whereby GR expression is affected by immune stimulation. Thus, injection of LPS increases expression of GR in the spleen of gilthead sea bream (Acerete et al. 2007), and in common carp, GR1 expression in peritoneal leucocytes increased after 1 and 2 days of zymosan treatment (Stolte et al. 2009).

In addition to the modulation of inflammation, not only the effects of glucocorticoids on the immune system are pleiotropic and varied, but also the effects depend on the specific cell or tissue. They mediate antiviral responses including signals that are induced by affected host cells, such as necrosis or apoptosis to generate an enhanced immune gene transcription response. In addition, environmental information reaching the brain allows initiation of immune response programmes via hormones and neurotransmitters in order to mitigate autotoxic risks associated with excessive signalling through enhanced cytokine activation (Verburg-van Kemenade et al. 2011). Thus, it has been shown that glucocorticoid treatments at low concentrations or concentrations that do not involve a significant systemic challenge can enhance inflammatory responses. At higher concentrations, glucocorticoids result in suppressive effects through cytokine signalling, for instance, inhibiting the production of B cells and T cells (Cain and Cidlowski 2017).

The suppressive effects of glucocorticoids have also been shown under in vitro experiments: cortisol in vitro has been shown to reduce phagocytosis in common carp, tilapia and silver sea bream head kidney leucocytes (Law et al. 2001). It also inhibits respiratory burst activity, phagocytosis and chemotaxis dose-dependently in a goldfish macrophage cell line (Wang and Belosevic 1995). Cortisol also decreases respiratory burst activity in sea bass (Vizzini et al. 2007) and sea bream leucocytes (Esteban et al. 2004). Glucocorticoids upregulate IL-10, IL-4 and TGF-β production but downregulate IL-12, TNF-α, and IFN-γ (Verburg-van Kemenade et al. 2011). In vitro cortisol treatment of head kidney leucocytes significantly depresses phagocytosis, chemotaxis and respiratory burst activity in carp, tilapia and silver sea bream (Wang and Belosevic 1995; Verburg-Van Kemenade et al. 2009). Decreased respiratory burst activity has also been observed in sea bream head kidney leucocytes (Esteban et al. 2004). Additionally, cortisol inhibits the LPS-induced expression of serum amyloid protein (Fast et al. 2002; Stolte et al. 2006),

inhibits the proliferation of monocytes/macrophages (Pagniello 2002) and induces apoptosis in silver sea bream and Atlantic salmon macrophages isolated from stressed fish, which also show decreased survival when exposed to *Aeromonas salmonicida* (Fast et al. 2008).

Recently, it has been shown that glucocorticoids also appear to be involved in the polarization of macrophages in fish (Maciuszek et al. 2019), which would explain the contribution of cortisol in the regulation of the macrophage after stress. After pathogen challenge, LPS treatment or, after stress, macrophages polarize inducing an increase in specific cytokines such as TNF-α, which will regulate the resolution of inflammation. Thus, stimulated monocytes produce 11β-HSD1 that converts inert internal cortisol to active internal cortisol, which plays a key role in regulating polarization by promoting the transition from M1- to M2-type macrophages (Thieringer et al. 2001; Maciuszek et al. 2019). Macrophages initiate the immune reaction for pathogen eradication as polarized M1 macrophages under the influence of IFN-γ and/or LPS, but for the modulation of this reaction and for tissue regeneration/wound healing, the polarized M2 macrophages are needed, which appear upon IL-4 or cortisol stimulation.

Although less studied, immune modulation has also been found in the adaptive arm of the immune system after stress. Corticosteroids regulate adaptive immunity by inhibiting lymphocyte activation and promoting lymphocyte apoptosis. In carp, in vitro stimulation with cortisol reduced IgM secretion by head kidney cells, spleen and blood lymphocytes (Saha et al. 2004). In vivo, thermal stress treatments induced decreased antibody responses after immunization (Verburg-van kemenade et al. 1999), inhibition of proliferation and induction of lymphocyte apoptosis (Saha et al. 2004).

Nevertheless, not all effects are suppressive. Interestingly, for instance, neutrophilic granulocytes remain protected and they do not show decreased numbers after acute stress or glucocorticoid treatment: while a significant lymphopenia is observed after stress treatments, a significant increase in promyelocytes and myelocytes as well as metamyelocytes and mature polymorphonuclear neutrophilic granulocytes takes place (Wojtaszek et al. 2002; Engelsma 2003). Therefore, a dual-cell response has been observed depending on the leucocyte type. As neutrophilic granulocytes are of great importance to the first line of defence, in particular for the phagocytic response, it would be dangerous to leave the whole immune system without a fast active response. Therefore, granulocytes would be beneficial in situations when pathogens may attack an animal subjected to acute stress or injury, having the other responses in a depressed status.

20.4.2 The Neuro-Immune Circuitry Under Stress

In fish under inflammatory episodes, interaction between the neuroendocrine and immune system is always taking place and glucocorticoids are one of the regulators of such inflammatory responses through programming of macrophages. As a consequence, a regulatory balance between pro-inflammatory and anti-inflammatory actions mediated by glucocorticoids is customary. Inflammatory reactions have to be carefully controlled, as

high concentration of pro-inflammatory cytokines or the prolonged induction of these cytokines, reactive oxygen species or nitric oxide may be detrimental for host tissues. Glucocorticoids can modulate an excessive inflammatory reaction. Conditions that are associated with acute or chronic stress may either suppress or potentiate control pathways leading to disease progression through modulation of systemic or local pro/anti-inflammatory cytokines and mediators and, consequently, the Th1/Th2 (T helper cells) balance (Verburg-Van Kemenade et al. 2009). Thus, during the activation of the systemic stress response, the immune and inflammatory mechanisms are modulated towards induction of a Th2 shift. In this way, the system protects the organism from excessive activation of Th1 pro-inflammatory cytokines (Calcagni and Elenkov 2006) that could end up with a cytokine storm. Corticosteroids are also able to inhibit inflammation by decreasing signal transduction downstream of PRRs, cytokine receptors and high-affinity IgE receptors (Fce receptors).

In addition to the global modulatory effect of glucocorticoids, a second pathway mediated by the sympathetic nervous system also contributes to the modulation of pro-inflammatory and anti-pathogen programmes of the immune response (Fig. 20.1). Therefore, the regulation of the immune response becomes more complex since activation or deactivation of immune response genes is conditioned by both external influences driven by neural sensors and brain pathways, and internal factors such as cell damage or pathogenic alterations. Furthermore, it should be remembered that the interaction is reciprocal; i.e. regulation of neural activity is modulated by immune response activity. Other important factors such as biological rhythms, previous experience or time course are also processed primarily through the brain and neural circuits, thus influencing the immune response. As the regulation of immune defence in complex organisms such as vertebrates including fish is exerted through leucocytes, such cells will integrate the influences coming from intrinsic immune-related inputs associated with the interaction with microorganism invasion, and the extrinsic inputs derived from the coordinating role of the brain. Hence, in both mammals and fish, catecholamines directly affect immune responses.

Activation of the sympathetic nervous system has been found to alter the production, release and circulation of leucocytes, through upregulation of myelopoiesis, activation of haematopoietic stem cells, NK cells and release of neutrophils and monocytes from the spleen. One example of this crosstalk is the brain-mediated suppression of the sickness and depressed status or the immobilizing behaviours following inflammation, as a way to facilitate other urgent responses such as fight-or-flight behaviour in order to avoid predation or aggression. So far, only a limited number of studies concerning the effects of catecholamines on the expression of inflammatory mediators have been performed compared to studies on glucocorticoids. In carp, secretion of adrenaline reduced the percentage of monocytes/macrophages at the site of inflammation after induced peritonitis while maintaining the total number of leucocytes, which may be related to elevated apoptotic activity and a reduction in mature monocyte/macrophage populations in the peritoneum (Kepka et al. 2012). Stress-related hormones, and particularly adrenaline, modulated the expression of pro-inflammatory and anti-inflammatory cytokines in cultured sea bream

leucocytes (Castillo et al. 2008). Whereas cortisol reduced the expression of the assessed cytokines, the effects of adrenaline were not general, less evident and time-dependent. Noradrenaline via stimulation of β-adrenergic receptors modulated gene expression of leucocytes, associated with the signalling cascade of second messengers such as adenylyl cyclase, cyclic AMP and protein kinases. After β-adrenergic signalling, the transcription of IL-4 and IL-5 (Th2 cytokine genes) is stimulated in lymphocytes, whereas the expression of IFN-γ and IL-1β (T1 genes) is suppressed. Thus, biogenic amines not only modulate the adaptive immune response but also can affect innate response programmes, such as suppression of antiviral type I IFN-mediated responses and upregulation of pro-inflammatory cytokine genes, such as IL-1β, IL-6 and TNF (Verburg-Van Kemenade et al. 2009). In addition, it should be noted that catecholamines may act as glucocorticoid secretagogues in the head kidney interrenal tissue. As demonstrated in sea bass (Rotllant et al. 2006), an intracellular adenylate kinase pathway after β-adrenoreceptor activation leads to increased cortisol production. Thus, additional regulation of immune function by adrenaline through cortisol increase can be induced.

In fish, adrenergic agonists decreased phagocytosis of fish macrophages (Roy and Rai 2008). Furthermore, adrenaline and isoproterenol (adrenaline receptor agonist) reduced the production of reactive oxygen species (ROS) in rainbow trout (*Oncorhynchus mykiss*) pronephric phagocytes (Flory and Bayne 1991), while noradrenaline promoted the respiratory burst in *O. mykiss* and *Channa punctata* leucocytes. Both ligands generated this effect via adrenergic receptors. In vitro administration of adrenaline reduced the synthesis of ROS and nitric oxide, while enhancing arginase activity in carp phagocytes. Furthermore, in vitro adrenaline inhibited the expression of pro-inflammatory cytokines, chemokines and their receptors. It was therefore hypothesized that adrenaline will downregulate phagocyte skewing towards innate polarization (Kepka et al. 2012). Innervation of lymphoid tissue has been found in the spleen of Coho salmon (*Oncorhynchus kisutch*), where nerve fibres are associated with the vasculature and the MMCs. Moreover, immune cells express receptors for neurotransmitters, including adrenergic receptors. So far, adrenergic receptors have been sequenced in zebrafish, goldfish, trout and catfish leukocytes (Kepka et al. 2012).

Regarding brain structures, the blood–brain barrier is similar in fish compared to mammals, showing both molecular and functional similarities, as observed in studies on *D. rerio* (Jeong et al. 2008). So, the fish brain would be accessible or sensitive to cytokines or other mediator molecules induced by cytokines. However, although the cytokine receptor IL-1RI mRNA was found in the preoptic area of carp brain (Metz et al. 2006) it is not clear whether IL-1β or other important cytokines from outside the brain interact at the brain level, since there are large and hydrophilic molecules that are unlikely to pass through the blood–brain barrier by passive diffusion. Prostaglandins could be the mediators, as they are smaller, lipophilic and neuroactive (Maier 2003).

Evidence suggests that signalling immune mechanisms to the brain in fish may also present high similarities to mammals. It has been proposed that the vagus nerve could serve as a cytokine-to-brain communication route (Maier 2003). Nonetheless, as stressors can

induce the increase in peripheral cytokines by the activation of systems such as the noradrenergic pathway, these in turn may lead to increased brain IL-1β via one of the immune to brain routes. Again, few studies have investigated the signalling routes from peripheral blood molecules to the brain, and so, the role of cytokines in the central regulation of the stress reaction and HPI and sympatho-chromaffin (SC) axes activation in fish is not well defined. Cytokine modulation has been shown by adrenaline in fish (Khansari et al. 2017a, b), which could induce the activation of signalling pathways to the brain. Within the brain, pro-inflammatory cytokines reduce the activity of neurotransmitters such as noradrenaline, dopamine and serotonin, which will significantly determine the modulation of behavioural patterns (Weber et al. 2015). Moreover, cytokines activate several other signalling pathways into the brain to integrate both peripheral pro-inflammatory and antigenic signals with physiological and behavioural responses, such as fever, aggression or social interaction. All these mechanisms will work together under stress episodes to integrate the physiological and behavioural response with the necessary protective reaction of immune cells.

Finally, other types of indirect regulation can occur in fish, for instance associated with the connection between the gut and the brain. It has been shown both in mammals and in fish that information by chemical signals is taking place between both organs. Different strains of Gram-negative bacteria are particularly responsive to catecholamines (Butt and Volkoff 2019). Many other hormones are concerned regarding stress episodes and endocrine influences. Therefore, functions like reproduction or growth will be also affected since the neuro-immuno-endocrine interaction is effectively triggered. A clear case study is the Sockeye salmon (*Oncorhynchus nerka*) in which degeneration of a number of glands and organs has been observed associated with very high levels of steroids and the loss of immunocompetence, particularly innate responses, during the migration period (Dolan et al. 2016). Cortisol and other reproductive steroids divert energy from several biological processes including immune functions to mobilize energy that ensures the fish are able to spawn. Sex steroids have also been found to modulate cortisol production in the interrenal of salmonids (McQuillan et al. 2003). Other hormones are released to the circulation after stress, although they do not show the ample consequences of corticosteroids and catecholamines (Yada and Tort 2016). For instance, the teleost CRF hormone family is tightly associated with immune responses against microbial pathogens. In goldfish (*Carassius auratus*) and tilapia (*Oreochromis mossambicus*), brain CRF expression is increased following immune stimulation (Volkoff and Peter 2004; Pepels and Balm 2004). Urocortin, a neuropeptide belonging to the CRF family of hypothalamic hormones, induces changes in many peripheral tissues such as heart, kidney, skin, muscles, spleen and immune cells including macrophages, fibroblasts, lymphocytes and mastocytes in which its receptors CRF1R and CRF2R are highly expressed (Choy et al. 2020). Urocortin has also been shown to help in the regulation of inflammation, interacting with IL-6 and cytokine production, promoting macrophage phagocytosis and bacterial killing, and inducing anti-microbial activity against Gram-positive and Gram-negative bacteria in mammals (Campos-Salinas et al. 2013), thus reducing bacterial burden. In addition, peripheral

secretions of urocortin are involved in modulating the peripheral immune response by acting on specific receptors in different populations of immune cells to produce a wide range of effects (Dermitzaki et al. 2018).

20.4.3 Metabolic Trade-Off in Stress-Related Immune Responses

A relevant component of the stress response in animals including fish is the energetic component. Several authors have previously described such an energetic approach by defining the concept of allostatic load, i.e. the fact that alterations in the homeostasis of the animal will produce a cost in energetic terms that will have to be paid back in order to maintain the metabolic and physiological equilibrium, either in the short term or in the longer term (Romero et al. 2009). Thus, any surplus in energy expenditure devoted to overcome the stress situation will generate compensations associated with disposal of energetic resources, otherwise used for other purposes. This compensation may affect the efficacy of some functions, in particular immune induction and cell proliferation, which are highly energy-demanding: in mammals, under hypoxic (1–4% oxygen) conditions, T cells cease to proliferate, cytokines are not produced, and even granulocytes that depend on anaerobic glycolysis may be impaired (Ohta 2018). Resting immune cells, such as circulating monocytes, memory T cells, plasma B cells and their naive phenotypes, tend to rely on minimal metabolism fuelled by oxidative phosphorylation, low-level glycolysis and fatty acid oxidation, but once activated during inflammation, switch to metabolic reprogramming that translates in enhanced glucose uptake, aerobic glycolysis and synthesis of fatty acids (Gaber et al. 2017). This sustains proliferation, clonal expansion, chemokine and cytokine release, and regulation of lymphoid lineages during stress-derived pro-inflammatory processes, controlled by the activation of the hypoxia-inducible factor 1α (HIF-1α) and mTOR pathways, whereas AMP activation results in anti-inflammatory onsets that inhibit mTOR signalling and reduce cell responses (O'Neill and Hardie 2013). Overall, mounting a sustained inflammatory response increases the basal metabolic rate (BMR) by an estimate of 30–50% in homoeothermic vertebrates, with half of the energetic requirements dedicated to produce the hepatic acute-phase response (Lochmiller and Deerenberg 2000; Straub et al. 2010). In fish, vaccinated rainbow trout may endure a 20% increase in BMR over the course of 1 month (Ackerman et al. 2000; Skinner et al. 2010), whereas *E. coli* lipopolysaccharide (LPS) elicited a transient (48 h) increase in BMR in mosquitofish (*Gambusia holbrooki*) to overcompensate for the inflammatory response (Bonneaud et al. 2016). Interestingly, a recent study about the impact in juvenile *O. mykiss* of several stressors failed to show a strong correlation between metabolic and immune trade-offs (Wernicke von Siebenthal et al. 2018). The authors assessed the effects on resource allocation in trout enduring limited food availability (thus mimicking the seasonally scarcity of resources in the original habitat), parasitic infection by *Tetracapsuloides bryosalmonae*, a myxozoan parasite that causes proliferative kidney disease (PKD) in salmonids (Sudhagar et al. 2019), and exposure to ethinyloestradiol (EE2), a common

endocrine disruptor (Aris et al. 2014). The compensatory responses favoured the production of immune mediators in infected fish, but also the growth of immune organs in the resource-depleted experimental group, albeit with little metabolic trade-offs between EE2-induced vitellogenesis, infection and growth. These and other results indicate the importance of metabolic plasticity in acute vs chronic inflammation, but also the complex intricacies, still unresolved, of the species-specific combined effects of the type and dosage of the stressor in fish, even if the ecological niche or population specificities are considered.

20.5   Immune Futures: A Glimpse of the Complexities of Environmental Influences in Stress-Related Immune Responses

Searching for a conceptualization of the unavoidable scalability of stress-related immune responses and mechanisms across cells, tissues, organs, individuals and ecological assemblages, several authors have proposed theoretical approaches to the whole concept of biological stress, such as the control theory model (Del Giudice et al. 2018) based on the regulatory mechanisms to recover physiological balance, or the damage fitness model (Wada and Heidinger 2019; Breuner and Berk 2019), based on the mechanisms to avoid or recover from damage and maintain the overall health (Romero 2004). However, the mismatch between the multiple dimensions of the physiological response and the individual tools and indicators used to quantify and evaluate such responses makes it difficult to obtain fine-grain resolution of such multilevel theoretical approaches required for analysing the complexity of real-world scenarios (Telemeco and Gangloff 2020). Moreover, even if the effects of stress-related molecular mediators on the susceptibility to diseases may be similar in metazoans (Costantini et al. 2011), the secretory patterns of the same molecules that are the trademark of stress overtaking organism homeostasis in vertebrates, such as corticosteroids, are highly variable between sexes, populations, species, life-history strategies, lifecycles, social status and biotopes (Breuner et al. 2013; Hau et al. 2016), impairing the formulation of a cohesive conceptualization of the coupling of stress responses to environmental changes. Defining the factors that constrict the immune features and capabilities of fish enduring unexpected and persistent stressors in ever-changing environments demands a multilevel approach based on the evolutionary history of each species (Fig. 20.2). As described elsewhere, such *stressotopes*, adaptive scenarios with variable but measurable selective pressures over multiscale immune responses, niche-specific and capable of generating allostatic loads (Balasch and Tort 2019), may enable, together with conceptualizations of stress and immune responses based upon evolutionary roadmaps (Becker et al. 2020; Taborsky et al. 2021), a precise framework to analyse probable outcomes of immune response in stressful environments.

It is expected that, confronted with human-induced abrupt changes, fish may rely on developmental plasticity mechanisms to cope with stressful environments (Petitjean et al. 2019), but to what extent altered phenotypes may influence survival and unexpected

changes in environmental variables is still unknown. An example may help to outline the difficulties of analysing a common stressor in the context of the fish immune response. Fish (with the exception of a few pelagic species with high metabolic basal rates) are ectothermic and their immune response diminishes in cold waters (Tong and Li 2020). Therefore, temperature changes are the main driver behind the evolution of immune repertories in fish, with pathogenic as an undesired direct effect of rising temperatures that strongly modulates host–pathogen interactions.

Any attempt to describe the effects of temperature on teleost immunity ends, as often happens with interspecies studies in fish, with a hard to summarize list of enhancements and impairments of temperature-regulated ups and downs of immune functionality. This is due to different experimental approaches, variable effects of optimal temperature-dependent virulence factors in pathogenic bacteria, several differences in the thermopreferendum of hosts and combined stressors (such as low oxygen levels, xenobiotics, emergent contaminants or hormone-mimicking pharmaceuticals) and the fact that temperature effects on teleost defensive responses are strongly seasonal, highly variable between species, populations with different life histories and even between individuals and sexes (Butler et al. 2013; Guijarro et al. 2015; Abram et al. 2017; Stewart et al. 2018; Marchand et al. 2019; Petitjean et al. 2020). Although no specific rules seem to apply to what is clearly a multifactorial sum of effects, recent surveys suggest that low temperatures may impair adaptive immune responses (especially T-cell-mediated immunity) and potentiate innate, unspecific defensive mechanisms, especially when several stressors are combined (Wentworth et al. 2018; Defo et al. 2019; Ignatz et al. 2020; Sun et al. 2020). In this sense, it has been suggested that infected or immunocompromised fish tend to actively search for hot spots to increase body temperature (Gräns et al. 2012; Boltaña et al. 2013). Inducing such behavioural fever (Covert and Reynolds 1977) contributes to enhance immune dynamics, mimicking the immune performance, both innate and adaptive, showed by temperate teleost species in spring (Buchtíková et al. 2011; Brown et al. 2016). Seasonal oscillations in temperature affect immunocompetence directly, by enhancing the circannual gene expression of immune-related genes (Gracey et al. 2004; Boltaña et al. 2013; Stewart et al. 2018; Abolfathi et al. 2020), but also indirectly, by interaction with the programmed onset of the reproductive hormonal axis, and seasonal cycles of pathogen abundance (Baekelandt et al. 2020). Long-term acclimation to non-optimal temperature regimes may improve an otherwise sluggish immune response at low temperatures, and accelerate phenotypic plasticity of immune-related gene expression responses, even in high-temperature scenarios (Scott and Johnston 2012; Abram et al. 2017; Tong and Li 2020).

To further complicate matters, the seasonality of immune function is multifactorial (Fig. 20.2), and the effects of temperature can be overridden by exogenous and endogenous variables, usually related to the onset of the reproductive hypothalamic–pituitary–gonadal (HPG) axis during the mating season, which affects immunocompetence. For example, male three-spine sticklebacks (*Gasterosteus aculeatus*) parasitized by *Schistocephalus solidus* and exposed to 17β-oestradiol (E2) suffered greater parasite load than females

(Macnab et al. 2016; Ling et al. 2020), indicating sex-specific (and also host-specific) effects on the interaction between the HPG and immune axis. Similarly, the effects of altered temperature regimes, combined with acute gill net entanglement and air exposure, two common stressors in aquaculture practices, suppressed female maturation and enhanced immune responses, increasing the parasite burden in a sex-dependent manner (Teffer et al. 2019). As mentioned before, shared receptors between immune and endocrine cells (Fig. 20.1), together with the intimate housing of the HPI and HPG axis in the teleost head kidney, facilitate neuroimmunoendocrine crosstalk, and estrogens have been deemed the culprit of immune modulations in fish (Szwejser et al. 2017; Cabas et al. 2018; Chaves-Pozo et al. 2018). The seasonal surges of E2 in teleost mature breeding females may affect immune responses, decreasing IgM levels and impairing cytokine networks (Cuesta et al. 2007; Seemann et al. 2016), altering the expression of Foxp3, a transcription factor for the development of T_{REG} cells (Wei et al. 2013). However, these and other immunosuppressive effects are markedly species-specific and still not well understood.

In addition, the link between temperature changes and pathogen distribution and virulence is expected to alter the immune plasticity of fishes (Claar and Wood 2020). In an ocean of microbes, temperature-driven pathogen outbreaks may act as an adaptive cue to boost metabolism, substitute behavioural fever for genuine endothermic fever, and abandon ectothermic metabolism in favour of a more efficient, memory-based, adaptive immunity, less dependent on low temperatures. In ectotherms, it has been argued that an immunity with increasingly cell consuming efficient affinity maturation of Ig in well-structured lymphoid organs cannot be realized due to an unaffordable metabolic cost by most ectotherms with slow metabolism (Lee 2006; Sandmeier and Tracy 2014). Besides, investing in increasingly efficient pathogen detectors may set the basis for an arm's race that selects for strategies of immune evasion and rapid changes in virulence from pathogens (Hedrick 2004; Guo et al. 2019), which would throw both host and pathogen to unending Red Queen evolutionary dynamics (Liow et al. 2011) of continuous adaptations simply to remain unharmed. These processes may have precluded an evolutionary urge for the development of fully functional endothermy in fish. Clearly, the origins of endothermy are far more complex and more species-specific than to be reduced only to immune solutions to compromised or stressful habitats (Nespolo et al. 2017; van de Pol et al. 2017), but the very few cases of functional endothermy found to date in fish (Soyano and Mushirobira 2018) seem to suggest that fish have adopted an immune strategy of resistance to pathogens, which appears somewhat defective compared with the mammalian-type hyper-specialized systems (such as immune receptor affinity maturation, multiple niche- and organ-specific cellular phenotypes, Ig isotype diversification and switching, and long-lasting memory). Between tolerance to pathogens (Råberg and Stjernman 2012), as showed by some aquatic invertebrates, or resistance, teleosts seem to have opted by fitting ectothermic metabolism to already highly plastic immune capabilities, suggesting that, in teleosts, the evolvability of the immune system has contributed to their evolutionary success, high diversity of lifestyles and widespread adaptive radiations (Solbakken et al. 2017).

Overall, even a common stressor of fish, namely temperature changes, requires an evolutionary background to delineate the foreground characteristics of stress-related immune performance. The consequences of the extreme diversity of fish lifecycles on stress-related immune function are overwhelmingly complex, but immensely rewarding for testing evolutionary models of immune system development coupled with ecological parameters. To date, few multiscale functional studies have addressed the intricacies of host–pathogen interaction in teleosts beyond the snapshot-like analysis of transcriptomes, proteomic surveys and metabolomic assessments of biological pathways. Genomic data are incredibly useful to systematize fish immune diversity and have rapidly becoming the by-default approach to evaluate environmental insults in fish physiology. However, integrated multiscale efforts are needed to discriminate between evolutionary changes in phenotypic plasticity in order to clarify the impact of combined stressors in host-pathogen immune negotiations in changing ecological contexts. Life story trade-offs often rest upon tortuous evolutionary paths across multiple interactions between overlapping species, competitive scenarios and unexpected environmental changes, sometimes ill-suited to human-induced extensive remodelling of contemporary environments. Combined analysis of environmental stressors in species-specific preferred niches may help to elucidate direct or indirect immune-related effects of environmental variables, which, to date, seem to affect immune responses in an indirect and elusive way. Ocean acidification, for example, seems to affect mainly acid–base homeostasis, branchial osmoregulation and overall metabolic scope, resulting in behavioural alterations, again in a species-specific manner (Esbaugh 2018). However, some results are controversial (Clark et al. 2020), and immune function under rising CO_2 partial pressures is poorly studied. Transcriptional analysis of acidification effects in gill tissues of barramundi (*Lates calcarifer*) suggests that acidified seawater modulates the expression of transcripts related to antigen processing and presentation, mucosal Ig and NK-mediated cytotoxicity (Ma et al. 2020). Similarly, Senegalese Sole (*Solea senegalensis*) enduring severe aquatic hypercapnia showed a short-term (up to 24 h) increase in plasma total bactericidal and antiprotease activities in mucosal lymphoid branchial tissues (Machado et al. 2020). However, chronic exposure (4 weeks) enhanced the expression of a limited repertoire of cytokine (IL-1β, IL-10) and cyclooxygenase 2 (COX2) transcripts related to inflammatory responses. Even if these results indicate an activated immune response in branchial tissues, the systemic effects of acidification are far from being resolved, and may be restricted mainly to local inflammation in gill mucosae or a result of impaired metabolic trade-offs that prime other physiological systems. In any case, as demonstrated by the evolutionary intricacies of amphibious fishes (Wright and Turko 2016), phenotypic and developmental plasticity must be included in the short- to long-term response to rapid environmental changes in aquatic CO_2 levels in fish. In this sense, resolving the fish immune tree of life will necessarily require a compilation of branching ecological influences on host lifetime, together with hologenomic approaches and niche-specific preferences.

20.6   Conclusion

Vertebrate immune systems suffer from acute horror vacui. Confronted with heavily microorganism-loaded environments, and constantly changing ornamental antigens anchored to pathogen surfaces, aquatic animals respond with functional redundancy of defensive components, multifunctional organs, constant production of unrequested naive lymphocytes and iterative cellular selection processes that completely fill the physiological tapestry of organisms. From an ecological perspective, fish immune phenotypes change over evolutionary time resembling developmentally and evolutionarily labile dose–response frameworks (Hedrick 2004; Grimholt 2018; Ravi and Venkatesh 2018). They are nurtured by the constant confrontation with stressors that question self-, non-self- and altered-self-antigenic components, but also by abrupt changes in environmental shelters, for instance, human-induced disturbances in preferred niches, or reproductive and metabolic adjustments to harsh habitats in the heart of a microbial-enriched environment (McCauley et al. 2015; Kelly and Salinas 2017; Ling et al. 2020). Fish possess the unspecific immune response to foreign molecules characteristic of invertebrates, reinforced by the canonical adaptive, specific and energetically expensive inflammatory reactivity of vertebrates (Palti 2011; Flajnik 2018; Patel et al. 2018; Rebl and Goldammer 2018). However, as discussed, a handful of exclusive characteristics and drawbacks differentiate fish immunity from that found in endothermic (i.e. birds and mammals) vertebrates. Several factors account for these differences, the most preeminent being the (1) physical, microbiological and human-influenced characteristics of aquatic dwellings, (2) long-vanished palaeoclimatic events (Solbakken et al. 2017) and (3) the high species specificity of immune responses, partly ameliorated by phenotypic and developmental plasticity to environmental changes (Farrell and Franklin 2016).

Overall, the immune reactivity of fish to environmental insults is influenced by the type and intensity of exogenous stressors (alone or combined), may be modulated by the phenotypic plasticity of behavioural responses, depends on sex-, population- species-specific and niche-related differences, and suffers the lags in the maturation of adaptive immunocompetence in larvae and adults. Even though stress-related neuroendocrine interactions have been and continue to be thoroughly analysed in teleosts, and several studies addressed neuroimmunoendocrine influences in stressed fish (Verburg-van Kemenade et al. 2017; Das et al. 2018; Shepherd et al. 2018; Hou et al. 2019), the impact and crosstalk of immune altered states in the activation of stress-related neurohormonal frameworks are still not fully characterized in fish. This is partly due to the model-centric methodological approach that favours some laboratory-friendly, novel translational research-related or simply commercial teleost species, at the expense of less known but more abundant and diversified fish (Schartl 2014). In an attempt to describe a more global picture of allostatic load effects in stressed fish, the focus now is on multibiomarker approaches that may render accurate descriptions of fish immunoreactivy in realistic multifactorial scenarios, where several combined parameters (temperature, pathogen load, multiplicity of stressors, behavioural phenotypes, etc.) can be analysed

simultaneously in an integrated framework (Khansari et al. 2018; Silva et al. 2018; Vargas et al. 2018; Dallarés et al. 2020). Unfortunately, we are still far from the development of an analytical framework, such as *stressotopes* (Balasch and Tort 2019) that may allow for the comprehensive multilevel description of evolved adaptive interactions between an individual's physiology and their preferred niche. However, a promising step may be the hologenomic approach (Limborg et al. 2018), in which the diverse methodologies for the expression analysis of fish host genomes, together with commensal and pathogenic mucosal microbiota, coalesce to integrate molecular and functional data in the light of evolutionary choices under environmental pressures.

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Genetic Breeding, Disease Resistance and Immunity

21

Kurt Buchmann

Abstract

Natural susceptibility or resistance of fish towards various pathogens (viral, bacterial, parasitic, fungal) may involve a range of anatomical, physiological and immunological elements. The mere chemical composition and the anatomy of the external fish surfaces are likely to affect pathogen adherence and may prevent entrance of pathogens. Certain conformations of membrane proteins may act as an external shield and determine if a virus is able to attach to and infect a host cell. Innate and adaptive immune mechanisms are nonetheless always activated and play a central role when the pathogens reach the host mucosa in order to enter the organism. These traits are all inherited and thereby available for selection and selective breeding. Classical breeding programmes, aiming at increasing the natural resistance of fish towards various diseases, span over decades due to the long generation time of the fish. They have shown success in a number of systems, but due to the increasing amount of pathogens challenging aquacultured fish, the fish farm industry may benefit from improved and faster genetic breeding methods. The use of genome-wide association studies (GWAS), recording of markers such as microsatellites and single nucleotide polymorphisms (SNP), makes marker-selected breeding possible. Several studies have proven these methods are able to describe quantitative trait loci (QTL) and pinpoint individual fish with a high natural resistance towards diseases. Following experimental exposure of a fish population to a certain pathogen, the most susceptible fish will exhibit clinical symptoms whereafter they are immediately euthanized and DNA-typed. Surviving fish showing no disease signs will

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subsequently be sampled and correspondingly analysed. By comparing the SNP composition among surviving and dead fish, or by focusing on time to death upon exposure, the specific markers associated with genetic resistance are revealed and their localization in the host genome determined with high precision. This information can then be applied by selection of DNA-typed parent fish, whereby a new generation of fish with increased resistance can be produced within months. In addition, further analyses of the genomic regions with relevant markers and concomitant transcriptome analyses allow identification of immune genes (related to innate and/or adaptive responses) associated with disease resistance.

Keywords

SNP · QTL · GWAS · Selective breeding · Fish · Innate immunity · Adaptive immunity

Abbreviations

AMP	Antimicrobial peptide
BCWD	Bacterial coldwater disease
C3	Complement factor C3
GWAS	Genome-wide association study
IFN	Interferon
Ig	Immunoglobulin
IPNV	Infectious pancreatic necrosis virus
MHC	Major histocompatibility complex
NFκB	Nuclear factor-kappa B transcription factor
QTL	Quantitative trait loci
RTFS	Rainbow trout fry mortality syndrome
SAA	Serum amyloid protein A
SAV	Salmonid alphavirus
SNP	Single nucleotide polymorphisms
TLR	Toll-like receptor
TNF	Tumour necrosis factor
VHSV	Viral haemorrhagic septicaemia

21.1 Introduction

With the expansion of aquaculture activities worldwide, it has been increasingly clear that fish disease programmes are central for securing a sustainable development of this protein producing industry. Viral, bacterial and parasitic pathogens represent the most serious

challenges for fish welfare and the external environment. Major achievements have been reached within the field of vaccinology, and protective vaccines are available for a range of fish species against viral and bacterial pathogens (see Chap. 19). Unfortunately, the same success has not been seen for anti-parasitic vaccines. Several prototypes of vaccines targeting both protozoan and metazoan parasites have been developed, but their usage is still at the experimental level. In addition, vaccination programmes are costly. Expenses related to vaccine products, labour and machinery challenge farm economy, with production loss connected to starving of fish before, during and after vaccination procedures (Karami et al. 2020). Another approach is to look into classical selection programmes, which have been working for decades with a series of eminent results. Following exposure of a fish population to a certain pathogen surviving fish can be used as breeders for coming generations. However, due to the long generation time of certain fish species the resulting fish strains with elevated resistance may take years to establish (Gjedrem and Baranski 2009). During the latest decades, novel strategies have shown value as replacements of classical breeding approaches. DNA markers in the fish genome can be applied to identify individual fish with traits for disease resistance (Fraslin et al. 2020). Markers ranging from microsatellites, MHC alleles and single nucleotide polymorphisms (SNP) have been widely used in genomic studies (Yang et al. 2010). Genome-wide association studies have then proven effective to describe quantitative trait loci (QTL) and pinpointing individual fish with a high natural resistance towards diseases. An important major achievement for rainbow trout breeding was the production of an array employing 57,501 single nucleotide polymorphisms (SNP) and their location on the 29 chromosomes of this fish species (Palti et al. 2015). Corresponding arrays are available for other fish species, and this molecular tool is now highly applicable for revealing QTL identifying fish with a particular status of susceptibility or resistance towards a certain pathogen. The QTL can then be applied for selection of relevant breeders and fast production of disease-resistant fish. The potential benefit for the industry for improving fish welfare, fish health and economy is indisputable.

21.2 Specific Diseases Targeted by Breeding Studies

The main diseases in salmonid aquaculture have attracted considerable research activity resulting in definition of QTL associated with disease resistance.

21.2.1 Viral Diseases

The viral disease viral haemorrhagic septicaemia (Egtved disease) caused by the rhabdovirus VHSV is responsible for high mortality in both farmed and wild salmonids and existence of trout strains with different susceptibility has been described, with particular emphasis on differential immune responses (Karami et al. 2018). Markers have been found

for resistance in rainbow trout against this rhabdovirus giving the potential for production of farmed fish less prone to infection (Verrier et al. 2013). In addition, one of the most economically challenging viral diseases in farming of Atlantic salmon (*Salmo salar*) is caused by the infectious pancreatic necrosis virus (IPNV). Application of a thorough screening programme allowed description of QTL associated with IPNV resistance (Houston et al. 2008; Moen et al. 2009, 2015), and a practical application of the results has been its implementation in Norwegian breeding programmes that resulted in a significant disease reduction from 2009 to 2018. QTL for resistance of salmon to infection with the salmonid alphavirus (SAV), another viral disease causing severe economic losses in mariculture, were described by Aslam et al. (2020). Using a 54 k SNP array for Atlantic salmon, the authors pinpointed chromosome 3 as the location of the QTL. The most significant SNPs occurred in regions with genes encoding antiviral effector molecules and immunoglobulin heavy chains. Cyprinid fishes, such as common carp (*Cyprinus carpio*), suffer from koi herpesvirus infections, and thorough analyses have pointed a significant QTL for resistance to this infection (Palaiokostas et al. 2018b). Similar approaches showed a comparable result in sea bass (*Dicentrarchus labrax*), towards viral nervous necrosis virus (Palaiokostas et al. 2018a).

21.2.2 Bacterial Diseases

Vibrio anguillarum is an important bacterial pathogen in maricultured fish, and some protection is afforded by various forms of vaccination (Viele et al. 1980). Due to costs associated with vaccination programmes, it is worthwhile to explore possibilities to establish breeding systems based on one or more markers associated with resistance to vibriosis. Following a major *V. anguillarum* exposure study using rainbow trout (*Oncorhynchus mykiss*) as host, and DNA typing using the 57 k array, Karami et al. (2020) identified a major QTL for resistance against vibriosis. Chromosome 21 appeared to carry genes associated with resistance, and the major SNPs were located in genomic regions highly equipped with genes encoding light-chain antibody segments. The genes responsible for natural protection may be elucidated by extensive screening techniques, and immune-related genes have been in focus. Chistiakov et al. (2010) pointed to the effect of certain variants of the pro-inflammatory cytokine gene IL-1β for resistance against this bacterial pathogen. Thus, in this case immune-related genes seem to play a role and this was further supported by studies in other host species. *V. anguillarum* resistance in Japanese flounder (*Paralichthys olivaceus*) (Wang et al. 2014; Shao et al. 2015), turbot (*Scophthalmus maximus*) (Zhang et al. 2019) and tonguefish (*Cynoglossus semilaevis*) (Tang et al. 2016) was shown to involve at least 12 immune-related genes, all associated with the major histocompatibility complex (MHC) class I and class II (Shao et al. 2015; Du et al. 2011). Further, based on SNP markers and gene expression studies Zhang et al. (2019) identified genes associated with MHC and transcription factor NF-kB pathways involved in initiation of inflammation and to be central for protection against

V. anguillarum in turbot. Infection of maricultured salmonids caused by *Piscirickettsia salmonis* eliciting salmonid rickettsial septicaemia (SRS) is regarded as the main challenge of mariculture. Observations that certain Pacific salmon families exhibit a lower susceptibility to infection (Dettleff et al. 2015) make search for markers connected to resistance, a highly relevant topic, as pursued by Barria et al. (2018) for Coho salmon (*Oncorhynchus kisutch*) and rainbow trout (Barria et al. 2019). Studies in another host fish resulted in identification of two QTL for SRS resistance in Atlantic salmon (Correa et al. 2015). Furunculosis, caused by *Aeromonas salmonicida,* is a major threat to salmonid fish farming. Vaccination programmes have been in place since the early 1990s and have contributed significantly to control of the disease (Midtlyng et al. 1996), but improved genetic composition of the fish is a major goal, which has been targeted by a series of studies (Gjedrem and Gjøen 1995; Gjøen et al. 1997). Improved survival rates of Atlantic salmon were found associated with specific MHC alleles (Grimholt et al. 2003; Langefors et al. 2001). Further investigations applying genetic tools aimed at finding QTL for *A. salmonicida* resistance were therefore implemented, and QTL for this trait were described in turbot by Rodriguez-Ramilo et al. (2011). Subsequent studies have identified a number of SNPs in the rainbow trout genome associated with disease resistance. This host is highly susceptible to *A. salmonicida,* and following a bath challenge, juvenile trout experience a mortality exceeding 80% (Marana et al. 2021). When comparing the SNP composition in susceptible and resistant trout, certain genomic regions appeared to play a major role in natural resistance. The genome-wide association study (GWAS) revealed two SNPs on chromosome 16, explaining 17% and 14% of the genetic variance, respectively. It is the case, however, that not all bacteria are naturally controlled by simple mechanisms and genes at well-defined genomic locations. Development of marker-assisted selection of production strains is of particular importance and relevance for disease in very young fry with incompletely developed immune systems. Rainbow trout fry mortality syndrome (RTFS) is caused by *Flavobacterium psychrophilum,* which also elicits bacterial coldwater disease (BCWD) even in older fish. However, the immunocompetent fish can be immunized and obtain protection, whereas fry still exhibit a high mortality rate following exposure at farm settings. Selective breeding of naturally resistant fish strains will secure a lower mortality rate and a higher fish welfare level and improve the farm economy. Several studies have shown a QTL on rainbow trout chromosome 19 (Wiens et al. 2013; Vallejo et al. 2014), and selective breeding programmes have been evaluated at the farm level (Liu et al. 2018). In contrast, experimental exposure of rainbow trout to *Yersinia ruckeri* showed a polygenic resistance pattern (Zuo et al. 2020). Although no QTL was identified, it was noted that central immune reactions are associated with resistance. Genes encoding IFN-γ, TNF-α, antimicrobial peptides (AMPs), C3 and lysozyme were upregulated in naturally resistant fish exposed to this bacterial pathogen (Zuo et al. 2020).

21.2.3 Parasitic Diseases

Inheritable resistance traits towards parasitic diseases have been reported and demonstrated for several host–parasite systems. Some resistance traits in Atlantic salmon towards amoebic gill disease (AGD) were described by Robledo et al. (2018) and Boison et al. (2019). In addition, Jaafar et al. (2020) demonstrated QTL for resistance in rainbow trout against parasitic ciliates. Exposure of rainbow to the parasitic ciliate *Ichthyophthirius multifiliis* may lead to more than 90% mortality in the host population if left untreated. During the course of infection in a confined environment, such as a fish pond, the infection pressure increases continuously and exponentially. Each trophont leaving the fish epidermis may within a few days give rise to several hundreds of infective theronts. It is evident that fish resisting the course of the infection have a markedly higher resistance towards the parasite compared to the fish developing disease at the earliest time points. Comparative analyses of the SNPs in susceptible versus resistant fish showed a significant location of SNPs associated with resistance in rainbow trout chromosomes 16 and 17 (Jaafar et al. 2020). Immune genes responsible for the increased protection were not pinpointed, but it was noteworthy that surviving fish, still exposed to infective theronts, exhibited significant upregulation of genes encoding effector molecules such as IgM, AMPs, complement factor C3, serum amyloid protein A and lysozyme. This suggests that both innate and adaptive factors play a role in natural resistance to this parasite (Jaafar et al. 2020). The scuticociliate *Philasterides dicentrachi* is currently creating disease problems in turbot farming, and application of at least partly resistant production fish would improve economy and fish welfare in the production systems. By use of microsatellite recording in turbot, exposed to this parasitic ciliate, it was found that at least two QTL were available (Rodríguez-Ramilo et al. 2013). Both amoebae and ciliates are unicellular microparasites, and responses are likely to differ from metazoan parasite responses in fish (see Chap. 17). The multicellular parasites, comprising myxozoans, monogeneans, digeneans, cestodes, nematodes, acanthocephalans and crustaceans, represent a similar threat to fish health. Susceptible and resistant strains of rainbow trout towards the myxozoan *Myxobolus cerebralis* were reported by Hedrick et al. (2003) and Baerwald et al. (2011). Correspondingly, Gilbey et al. (2006) found genetic markers for resistance in Atlantic salmon towards the freshwater monogenean *Gyrodactylus salaris,* which has been responsible for dramatic mortality events in wild Norwegian salmon populations. QTL for resistance in the marine yellowtail (*Seriola quinqueradiata*) against a major pathogenic capsalid monogenean *Benedenia seriolae* were reported by Ozaki et al. (2013). Natural resistance in salmon against the relatively large crustacean ectoparasitic *Lepeophtheirus salmonis* is likely to include many aspects of the fish host physiology and immune response armament. The louse is covered by a chitinous exoskeleton and can probably resist the action of soluble immune factors released by the host skin. The question was investigated by Gharbi et al. (2009) and Robledo et al. (2019) providing clues for the existence of QTL for salmon louse resistance. All of these parasites induce marked immune reactions in the host, and it is worthwhile to supplement genetic analyses with extensive gene expression studies, which eventually

could link genetic markers with immune genes conferring specific or non-specific immunity to parasites.

21.3 Expression of Decisive Genes

21.3.1 Approaches

Extensive gene expression studies (transcriptomic analyses) may supplement the marker selection studies adding to knowledge on genes directly associated with resistance (Fraslin et al. 2020). Comparative approaches of different host organs sampled throughout the course of infection (susceptible diseased fish, resistant surviving fish) are likely to indicate some genes, which may be associated with innate and adaptive immunity. When performing experimental challenges of fish, included in genome-wide association studies, it is therefore of relevance to perform additional comparative analyses of the immune gene expression in exposed fish showing disease signs, exposed fish without symptoms and non-exposed fish. Full transcriptomic analyses would clearly highlight more genes being actively transcribed during exposure to a certain pathogen. This would supplement genomic analyses of chromosomes carrying markers, such as SNPs, significantly involved in protection (Aslam et al. 2020). Due to the constitutive expression of some genes, which may be involved in natural resistance, this approach is not sufficient in all cases. However, it may provide clues to improve our understanding of the immunological pathways involved in resistance. It is also relevant to perform expression analyses to detect the pathogen level in various compartments and organs of the exposed host. This will show whether the pathogen in question has denied access to the host or actually is present in various organs of the host without developing disease. Useful pathogen specific primers and probes applicable for bacterial (Zuo et al. 2020; Karami et al. 2020) and parasitic (Jaafar et al. 2020) pathogens are available.

21.3.2 Immune Genes

As suggested by Moen et al. (2015), surface proteins like cadherin may determine the susceptibility of Atlantic salmon to a virus such as IPNV. Viral entry into the host may be prevented simply by certain conformations of the surface molecule. This suggests that the genetic background for resistance towards diseases may not (at least in certain cases) be directly related to central immune genes and their action. However, it is worthwhile to describe the involvement of central immune genes in fish with and without clinical signs as these observations may coincide with genes associated with significant markers. This approach is promising, but the results should be critically evaluated. All pathogens invading external and internal compartments of the fish organism will elicit immune reactions—in both susceptible and partly resistant fish. This has been established by

numerous investigations involving viral, bacterial and parasitic pathogens in a wide range of fish hosts. Therefore, strict qualitative and quantitative analyses must be performed to disclose the possible decisive candidate genes. For example, various forms of brown trout (*Salmo trutta*) establish a broad but differential range of responses when infected by VHSV (Karami et al. 2018), and Atlantic salmon react similarly towards SAV (Aslam et al. 2020) allowing candidate genes to be selected for further scrutinization. Gene expression studies have improved our understanding of host reactions in both highly and less susceptible trout towards ciliate parasites such as *I. multifiliis* (Olsen et al. 2011; Jaafar et al. 2020), but the immune genes responsible for protection are not yet determined. Atlantic salmon and rainbow trout exposed to the crustacean ectoparasite *L. salmonis* establish a marked skin response (Dalvin et al. 2020; Holm et al. 2015). The response differs between susceptible and resistant strains of the host species (Holm et al. 2015). A critical comparison, omitting the effect of parasite load on the expression profile, should be performed, and the difference may be associated with Th2 skewing of the response (Braden et al. 2015). A number of fish species exposed to, e.g. *V. anguillarum,* generally activate a range of immune genes. Atlantic cod (*Gadus morhua*) (Caipang et al. 2008; Seppola et al. 2008), sea bream (*Sparus aurata*) (Lopez-Castejon et al. 2007) and sea bass (*Dicentrarchus labrax*) (Meloni et al. 2015) initially express pro-inflammatory cytokine genes whereafter other humoral processes (such as IgM production) (Khansari et al. 2019) become increasingly involved. Cytokine genes become activated concomitant with a clear involvement of AMPs, and the host organ affected is determined by where the pathogen attacks. Other effector molecules are upregulated, and especially, immunoglobulin genes, cathelicidins (AMPs) and acute-phase reactants such as SAA and lysozyme are clearly important elements during the initial pathogen attack and possible rejection. Differences are found between internal organs and gills (Zuo et al. 2020; Karami et al. 2020; Marana et al. 2021), and it may be worthwhile to focus on the ratio between external and interior responses when judging the severity of an infection. In all cases, the comparison between the transcriptomic data and marker positions must be treated with caution. When exploring QTL for resistance towards salmonid alphavirus in Atlantic salmon, Aslam et al. (2020) showed that the SNP of the highest importance co-occurred with antiviral effector molecules. In addition, genes encoding the immunoglobulin heavy chain mapped to clusters with other SNPs. The importance of immunoglobulins in rainbow trout resistance was also indicated by the early upregulation of Igs following *V. anguillarum* exposure. For example, the consistent activation of IgD genes (membrane-bound and secreted forms) associated with *V. anguillarum* clearance is noteworthy. It is therefore relevant to explore the QTL for *V. anguillarum* resistance on rainbow trout chromosome 21 and the significant SNPs recorded (Karami et al. 2020). This task is huge as this chromosome (at present) carries more than 1600 annotated genes. However, with regard to immunoglobulins and their antigen-binding sites, it is noteworthy that sequences for kappa and lambda light-chain immunoglobulin-like proteins are present and mapped with a SNP of high significance on chromosome 21 (Karami et al. 2020). In addition, this chromosome contains a series of immune-relevant genes such as those encoding macrophage receptors, natural killer cell

receptors, pentraxins, leucine-rich repeats, perforin and mucin. The connection between resistance and any of these immune factors may then be investigated and finally established by extensive screening using gene knock-out studies. Systematic elimination of specific gene functions followed by controlled challenge trials will validate if one or more of the genes are responsible for the observed resistance towards a specific pathogen. The practical application of description of QTL in a certain fish species is the generation of new generations of fish with the beneficial traits. When combining these trials with thorough transcriptomic analyses, it should be expected that a better overview of the field can be obtained.

21.4   Conclusions

All organisms—spanning from primitive protozoans to developed vertebrates—possess a series of genes conveying a degree of protection against potentially invasive microorganisms. Some are directly encoded factors related to innate and/or adaptive immune mechanisms. Others are simply encoded molecules presented by the host to the environment as cell surface molecules. Alternatively, the molecules may act as exported repellents, which interfere with parasite invasion. Recent investigations have shown that different fish species are fully or partly protected against many pathogens (viral, bacterial, parasitic, fungal) and that the protective traits are encoded by QTL. Based on novel developments within host genotyping, it is possible to localize the markers (associated with disease resistance) on specific host fish chromosomes (and specific chromosome regions), enabling knowledge about the mechanisms determining susceptibility/resistance towards a certain pathogen to be obtained. This has demonstrated that innate and adaptive immune mechanisms often play a central role when the pathogens are kept from entering the host organism. In some cases, evidence has been presented that specific conformations of surface molecules prevent viral adherence and cell invasion. Other studies show that antibodies are likely candidates. In all cases, these traits are inherited and thereby subject to selection and selective breeding. The use of marker-selected breeding is superior to classical breeding programmes, which have been in use for many decades. Their application has indeed increased the natural resistance of aquacultured fish towards various diseases and thereby increased their health status. However, due to the long generation time of fish (for salmonids often 3–4 years) this classical approach becomes outdated when detection of markers is at hand. The present chapter has presented a series of results from genome-wide association studies conducted over the last two decades, to demonstrate how results can be applied to increase the health of farmed fish. Thus, examples were given on the application of genome-wide association studies (GWAS) providing information about which parent fish, which should be selected for production of new generations of fish with elevated natural resistance to selected diseases. The associated analyses have also provided tools for further inspection of the genomic localization of genes associated with disease resistance. This has allowed description of genes or pathways responsible for protection

and, importantly, has pointed out that the protective mechanisms, even in the same host species, differ with regard to viral, bacterial and parasitic pathogens. However, published studies have, based on the economical importance of the fish hosts, focused on the main fish species used for aquaculture. It is therefore important to expand future studies to other species as well. Such an approach is likely to provide us with a much deeper insight into genes encoding the basic immune factors and their interaction, which give fish improved immunity to infection.

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