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Volume 738
SELF AND NONSELF
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PREFACE

"It is an extraordinary fact that with many species, flowers fertilised with their own pollen are either absolutely or in some degree sterile; if fertilised with pollen from another flower on the same plant, they are sometimes, though rarely, a little more fertile; if fertilised with pollen from another individual or variety of the same species, they are fully fertile"

—Cross and Self-Fertilisation (Darwin, 1878)

In 1960 Sir Frank Macfarlane Burnet received the Noble Prize in Physiology and Medicine. He titled his Nobel Lecture "Immunological Recognition of Self" emphasizing the central argument of immunological tolerance in "How does the vertebrate organism recognize **self** from **nonself** in this the immunological sense—and how did the capacity evolve."

The concept of self is linked to the concept of biological self identity. All organisms, from bacteria to higher animals, possess recognition systems to defend themselves from nonself. Even in the context of the limited number of metazoan phyla that have been studied in detail, we can now describe many of the alternative mechanism of immune recognition that have emerged at varying points in phylogeny. Two different arms—the innate and adaptive immune system—have emerged at different moments in evolution, and they are conceptually different. The ultimate goals of immune biology include reconstructing the molecular networks underlying immune processes. This volume covers different aspects of the emergence of immune systems in the evolution of life.

The first part of the book focuses on the origin of the immune response during the development of multicellularity (Chapters 1-4). Bacteria have developed defense systems against viruses and conversely, viruses have devised escape mechanisms that allow infection. Most of the archaea and numerous bacteria possess an elaborate system of adaptive immunity known as the **CRISPR-Cas,** that confers resistance to mobile genetic elements. This continuous phage-host interaction is a strong selective pressure that triggers a rapid co-evolution of both entities. Nevertheless, the evolution of metazoans from their unicellular ancestors emerged as a novel self-identity that required mechanisms for cell adhesion and cell-to-cell communication. One of the most important cell adhesion

mechanisms for metazoan development is based on carbohydrate to carbohydrate self-assessment. The large variability of carbohydrates as the most exposed and dominant components of plasma membranes are involved in many cellular interactions essential for self-nonself recognition.

Since *cnidarians* are amongst the morphologically simplest metazoans, they are also the most suitable for studying the evolutionary origins of self-nonself recognition. A surprising characteristic is that they possess an exquisitely sophisticated histocompatibility system. When two allogeneic incompatible colonies come into direct contact, they develop inflammatory-like rejection lesions, called points of rejection (**POR**). The colonial ascidian *Botryllus schlosseri* manifests a unique allorecognition system that is controlled by a single histocompatibility **Fu/HC** locus, with a large number of expressed alleles, that also affects self-fertilization by sperm-egg incompatibility.

The second part of this volume covers immunity aspects of innate sensors (Chapters 5-11). Innate immunity is the dominant immune system found in plants, fungi, insects, and primitive multicellular organisms. In 1989, Janeway proposed that innate immune systems discriminate self and nonself through pathogen-associated molecular patterns (**PAMPs**). Some years later, Matzinger expanded Janeway's theory proposing the "danger signal theory", which states that the decision to respond or not to respond to a particular antigen depends on whether the antigen is "harmful or not" to our body.

Recognition is mainly based on a series of germ-line encoded pattern recognition receptors (**PRRs**) that have been selected during evolution to recognize nonself molecules present in microorganisms. Moreover, different types of intracellular sensors (Toll receptors) that recognize various forms of nucleic acids have been described in virus response. Similarly, plants utilize receptor-like proteins (kinases) as pattern recognition receptors which can detect conserved PAMPs. Charles Darwin made extensive observations of the pollination biology of a wide variety of plants. He carefully documented the consequences of self-pollination and described species that were self-sterile but that could easily be crossed with other plants of the same species. In fact, plants have evolved many complex mechanisms to prevent self-fertilization, and it is thought that this may partially explain the great success of the angiosperms. Self-incompatibility (**SI**) involves unique systems of cell-to-cell communication, cell-recognition and cell-to-cell rejection. Genetic studies show that a single polymorphic S-locus, encoding at least two components from both the pollen and pistil sides, controls the discrimination of self and nonself pollen.

Cell death is vital to the life of multicellular organisms, and it plays a role in the maintenance of population homeostasis of unicellular organisms. Apoptosis is the best known of these programs, and it has been suggested that it originated as part of a host defense mechanism. During apoptosis, cells maintain the integrity of their plasma membrane. In contrast, cell death by 'necrosis', which occurs in situations of uncontrolled tissue damage, is a 'passive' form of cell death which triggers inflammation. The component released during tissue injury, called damage-associated molecular patterns (**DAMPs**), also triggers innate immune response.

Recent evidence indicates that the cell homeostasis program is also triggered by inner sensors that intersect with the innate immune response. Here we also described how mechanisms such as the endoplasmic reticulum (**ER**) **stress** and **"autophagy**" are critical to restrict viral replication. Some viruses can also exploit these mechanisms. In

fact, regulating some aspects of these pathways, it is possible to favour viral replication and also inhibit the apoptotic machinery of infected cells.

The third part of this volume is dedicated to the emergence of adaptive systems in metazoans (Chapters 12-17). During vertebrate evolution, transposable elements have repeatedly contributed with regulatory and coding sequences to the host, leading to the emergence of new lineage-specific genes. Human endogenous retroviruses (as **HERVs**), represent vestiges of ancient infections that resulted in stable integration of the viral genome. These have occurred during the first evolutionary stages of jawed vertebrates due of the acquisition of different gene-related systems (**Igs**, **MHC**, **TCR**), and the recombinatorial mechanisms of generation of antigenic diversity (RAG genes) and lymphoid organs. Recent work has shown that jawless vertebrates have lymphocytes that express somatically diversified antigen receptors that contain leucine-rich-repeats, termed variable lymphocyte receptors (**VLRs**), and that the type of VLR expressed is specific to the lymphocyte lineage. However, during the millions of years of co-evolution with their respective hosts, viruses have extensively captured cellular genes. Cytomegaloviruses (**CMVs**) constitute an outstanding example of the many and varied encoded proteins directed to modulate both innate and adaptive immune responses

The last part of the volume describes the emergence of the **M**ajor **H**istocompatibility **C**omplex (**MHC**). The MHC is a multigene family that has arisen through recurrent expansion and contraction of genes, and a continuum of the evolutionary process is observed in the teleost fishes. This system contains genes encoding proteins involved with antigen presentation, playing an important role in the adaptive immune system. The study of how the MHC appeared in vertebrates during evolution and how it is organized in different species can help us clarify what features are essential for self-nonself recognition. On the other hand, the recent sequencing and assembly of the genomes of different organisms have shown that almost all vertebrates studied have one or more clusters of genes encoding odorant receptors (**OR**) in close physical linkage to MHC. Social signalling associated to MHC has been identified in over 20 species of vertebrates and is likely the basis for a vertebrate-wide chemosensory communication system.

This book presents an integrated view of self and nonself recognition systems in the context of evolution. I hope it will contribute to the conceptual discussion of the emergence of immune systems in nature. I am extremely grateful to all authors for their excellent contributions.

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CHAPTER 1

THE ORIGIN OF THE BACTERIAL IMMUNE RESPONSE

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Abstract: Bacteriophages are probably the oldest viruses, having appeared early during bacterial evolution. Therefore, bacteria and bacteriophages have a long history of co-evolution in which bacteria have developed multiple resistance mechanisms against bacteriophages. These mechanisms, that are very diverse and are in constant evolution, allow the survival of the bacteria. Bacteriophages have adapted to bacterial defense systems, devised strategies to evade these anti-phage mechanisms and restored their infective capacity. In this chapter, we review the bacterial strategies that hinder the phage infection as well as the counter-defense mechanisms developed by the bacteriophages as an evolutionary response to the antiviral systems.

INTRODUCTION

The immune system has developed during evolution to defend our organism against nonself entities such as microorganisms, some inert injurious materials and tumour cells. This system in jawed vertebrates (like mammals) is very complex and contains an innate and an adaptive immune response. Such a complex organization of immune system probably provided survival advantages: These animals are also complex anatomically, generally need a long time to reach their reproductive maturity, possess a higher mobility and have a diversified diet. These characteristics generate a higher exposure to pathogens. Other types of animals also possess an immune system, although more primitive. Thus, all metazoans, including plants and the simplest multicellular organisms like the Porifera, need to distinguish self from nonself to maintain their integrity. The distinction between self

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Table 1. Summary of some of the phage defense mechanisms described in this chapter and their respective counter defense systems developed by phages

Bacteriophage Resistance Mechanisms	Counter Defense Mechanisms	Examples
Restriction and modification systems		
Type I	Reduction of recognition sites	Point mutations
	Modified bases	Hydroxymetiluracil
	Occlusion restriction sites	DarA and DarB proteins
	Depletion of cofactors	S-adenosy metionine hydrolase
	Inhibition R-M enzymes	OCR proteins
Type II	Reduction of recognition sites	
	Modified bases	
Type III	Reduction of recognition sites	
	Occlusion restriction sites	DarA and DarB proteins
	Inhibition R-M enzymes	OCR proteins
Type IV	Inhibition of R-M enzymes	IPI proteins for GmrS-GmrD system
CRISPR	Inhibition of recognition	Mutated proto-spacer sequences
Abortive infection systems		
Lit proteins	Reversion of Lit action	Reprocessing host $tRNA^{lys}$
Rex system	rII exclusion	rII gene

and nonself prevent them from being deceived by pathogens, which, without the existence of the immune system, eventually would invade the body and destroy the individual. In the simplest metazoans, this destruction is avoided by distinguishing their cells from the cells coming from other colonies and maintaining their integrity, as happens in sponges.

The immune system can be defined, in a broad way, as a system to distinguish self from nonself, whose function is to preserve the individual integrity both from an excessive competition for nutrients and from pathogenic assaults. The unicellular microorganisms, like bacteria, are able to perform this distinction. They can detect the presence of competitors that use the same nutrients and kill them by secreting anti-microbial substances. They are also able to detect and eliminate their own intracellular parasites.[1,2] This is, for example, the well-known function of the restriction enzymes, which evolved to destroy the invading bacteriophage genome while the bacterial DNA remained unharmed. In this chapter, we review the mechanisms used by bacteria to avoid a bacteriophage attack and destroy the phages once they enter inside the bacteria. The bacteriophages have an important role in bacterial evolution and have led to a great variety of defense mechanisms. Bacteriophages, for their part, have developed counter defense mechanisms to evade the bacterial antiviral mechanisms (Table 1, Fig. 1). We will also discuss how these defense mechanisms resemble those of more evolved forms of life.

BACTERIOPHAGE BIOLOGY

Most bacteria are infected by viruses called bacteriophages (also known as phages), which only have bacteria as a host (reviewed in ref. 3). Like other viruses, phages are

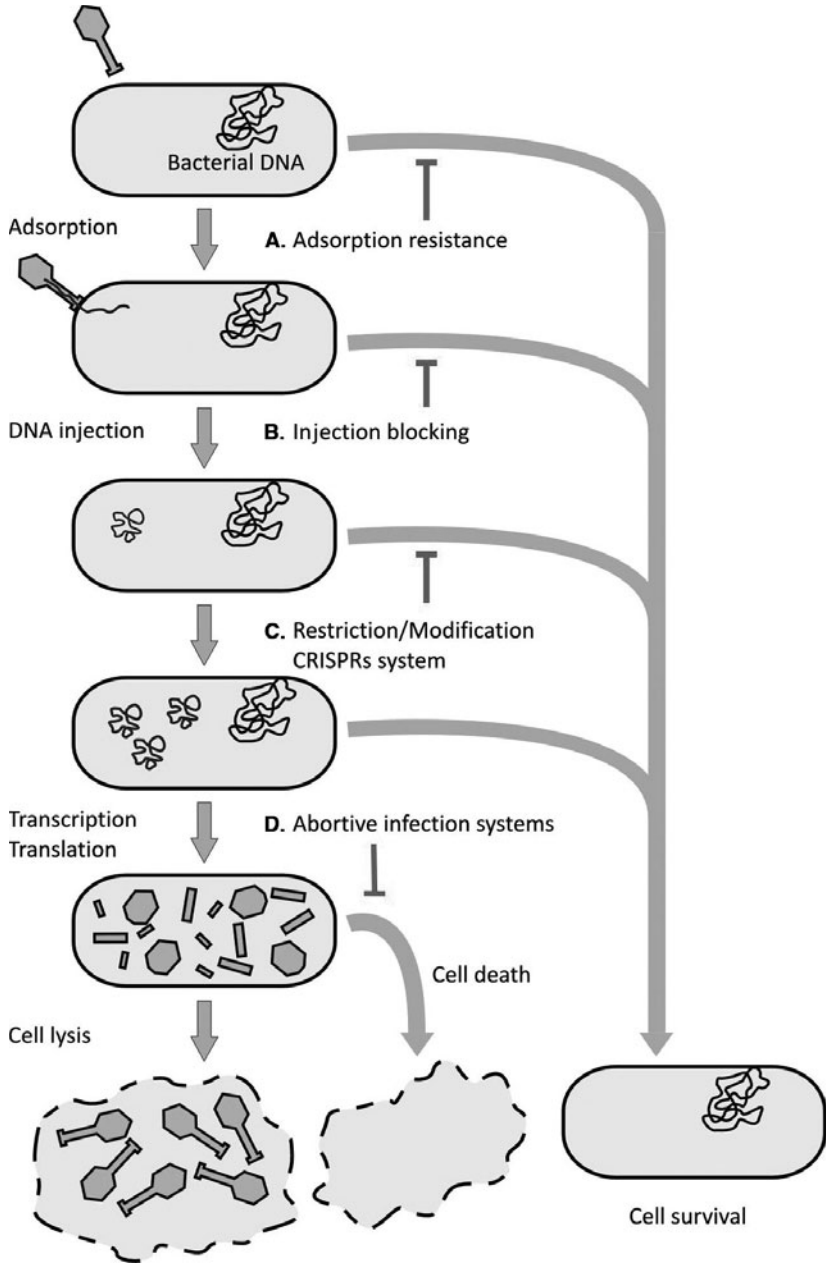

Figure 1. Phage infection stages and antiphage mechanism. Each stage of the phage cycle can be specifically inhibited by an antiphage mechanism. A) Absorption is blocked by several strategies that interfere with the recognition of a phage with its receptor. B) The DNA injection into the host also can by blocked. C) Restriction-modification system can detect and destroy foreign DNA while the host DNA remains undamaged. CRIPRS recognize phage DNA when the phage has infected previously the bacteria. D) Abortive infection systems affect the last stages of the phage cycle (replication, transcription or translation). These mechanisms lead to the death of the infected bacteria but protects the bacterial population.

parasites that only can live in their host cell. However, many phages can survive in the absence of the appropriate host for years and remain able to infect bacteria. Phages need an appropriate host that is generally a group of bacteria of one species, although some phages can infect several related species. Their host range is very broad and so, they can infect any bacteria group, including gram positive, gram negative and archaea, the latter being infected by specific viruses that are called archaephages. Phages are ubiquitously distributed; they can be found in all habitats where the bacteria or archaea can proliferate, including places with extremes temperature, pH, or salinity. Phages are probably the most abundant biological entities. They are perhaps the oldest viruses, having appeared before the split of the two bacterial kingdoms, bacteria and archaea. They are very diverse in structure, which indicates they have a polyphyletic origin.

The phage virion consists of a capsid make up of proteins or lipoproteins that enclose the genetic material (the nucleocapsid). Their structure can be tailed, polyhedral, filamentous, or pleomorphic. Most of the phages described (95% of all phages) belong to the Order *Caudovirales* or tailed phages. The *Caudovirales* include the families *Myoviridae*, *Siphoviridae* and *Podoviridae*. Phages of these families are formed by an icosahedral head and a tail. The tail of the phage varies in length and can be contractile or not, depending on the family. These phages contain linear double-stranded DNA (dsDNA). The polyhedral, filamentous and pleomorphic phages comprise another ten families of tailless phages, with very few species described to date. A few species have not been assigned to any group yet. The phage capsid has different shapes and that include families with linear or circular dsDNA, two families with circular single-stranded DNA (ssDNA) and two families with RNA as genetic material, one of them with linear ssRNA and other with segmented dsRNA.[4]

The long period of time in contact between bacteria and phages could explain the lysogeny, a complex phenomenon that probably has required a long co-evolution of phages and their hosts.[3] Phages can be grouped into virulent and temperate phenotypes on the basis of their infection cycle.[5] After the infection, virulent phages have a lytic cycle: They immediately start the production of new viral particles using the bacterial molecular machinery and liberate new phages by lysing the host cell. Temperate phages have two alternative cycles when they infect the bacteria. They can follow a lytic cycle as aforementioned or alternatively, they can enter a lysogenic cycle: The phage remains in a quiescent state, their genetic material (known as a prophage) integrates into the bacterial genome or remains as a plasmid and replicates at the same time as the host. The phage stays in this state until a specific factor (dependent on the host's metabolic state) triggers the phage to leave this state and to enter a lytic cycle. Lytic phages affect all aspects of host metabolism: They modulate transcription and translation of proteins and alter the membrane and genomic integrity. On the contrary, the lysogenic cycle is not toxic to the host until it switches to the lytic cycle. The lysogenic cycle can be beneficial for the bacteria by preventing infection from other phages and providing resistance to antibiotics. As a consequence of this long contact between phages and their host, the majority of gram-positive and gram-negative bacteria contain prophages. In fact, prophages can constitute 3-10% of the genome of bacteria[6] and are the main contributor to genomic diversity in some species. These prophages become a stable part of the bacteria genome and they can be functional or defective.

We will discuss the mechanisms developed by the bacteria to protect themselves from these viruses. For this, it is interesting to consider that the phage infection involves several steps and at each step, the bacteria have developed resistance mechanisms to try to avoid or stop the infection. The steps in a phage lytic cycle are adsorption, genome injection, genome replication, phage transcription, translation, assembly and lysis.

In some cases, phage infection is possible only in a certain phase of the host's growth cycle. For example, the infectious cycle of *Bordetella*, a bacterium that causes respiratory infections, has two phases: The Bvg+ phase which expresses the virulence and colonization factors that are necessary for respiratory tract colonization and the Bvg- phase which expresses genes for ex vivo growth and survival but not genes for colonization. The bacteriophage BPP-1 can infect bacteria in the Bvg+ phase because it specifically expresses the adhesion protein pertactin, which is not expressed in the Bvg- phase. However, the BPP-1 phage has developed a mechanism to infect at different stages in the infectious cycles of *Bordetella*. The tropism in this phage is determined by the gene *mtd* (major tropism determinant), but *mtd* suffers the action of a reverse transcriptase enzyme that acts as a diversity-generating retroelement, since its only purpose appears to be to generate changes in the sequence of that gene. In fact, new phages have been found in which changes in the *mtd* gene have allowed the infection of the Bvg- phase *Bordetella*.[7,8]

PHASES OF THE IMMUNE RESPONSE

Bacteria, as the rest of organisms, are susceptible to being attacked by pathogens. Comparing the defense systems developed by the different organisms shows similarities between them, which indicates the existence of basic mechanisms that have appeared independently by convergent evolution both in higher muticellular organisms and in unicellular organisms. Considering the different aspects of the immune system, the self-nonself distinction is employed to maintain the host's integrity by detecting the presence of competitors that use the same nutrients (a defense mechanism present in unicellular organisms) or by detecting pathogens that try to colonize the organism (a defense mechanism present in both unicellular and multicellular organisms). Bearing in mind the latter aspect, when a pathogenic agent tries to invade an organism, the defense mechanisms act in two phases: (1) The first phase consists of trying to prevent or limit the injury when the microorganisms are outside the host and (2) a second phase in which the organism tries to destroy the internalized pathogens. Regarding the first mechanism, the antimicrobial peptides (defensins) and proteins (hemolysin, lysozyme) are some of the earliest forms of defense. Thus, defensins or defensin-like antimicrobial peptides have been found in animals (primitive vertebrates, arthropods, fishes, frogs, mammals, etc) and in plants. These types of peptides are also produced by bacteria, which use the peptides to thwart competing microorganisms or to avoid a phage attack. This first phase also includes the anatomical and physiological barriers that hinder the pathogen from penetrating the host body (skin or other membranes, mucus, etc). These barriers are present both in unicellular and pluricellular organisms and are a way to prohibit pathogens from gaining access to the inner tissues (or the cytoplasm) at the beginning of the infection. In jawed vertebrates the immune response has three phases. The first phase includes the non-induced (passive) and nonspecific response prior to the pathogen's entry into the body. The second phase is the innate response that occurs when the pathogen has been able to invade the organism. This innate response has a medium grade of specificity since it recognizes some specific structures that are shared by a broad range of pathogens. The third phase consists of the adaptive immune response that develops highly specific responses toward a specific pathogen. One of the characteristics of the adaptive immune response is the memory. Surprisingly, some bacterial defense mechanisms against phages possess this property.

Thus, as we describe below, in the Pgl and CRISPRs systems, the phage that infects a bacteria is recognized in the subsequent attacks, which allows a better response that avoid a new infection. In the following sections, we describe the different defense strategies used by the bacteria to respond to the biological agents that threaten them.

BACTERIAL IMMUNE RESPONSE BEFORE PHAGE ENTRY

As we mentioned above, microorganisms use antimicrobial peptides against a competing microorganism, especially under conditions of nutrient depletion. These peptides also play a role in the defense against bacteriophages in some cases. For example, the micromicin J25, a peptide secreted by Enterobacteria under conditions of nutrient depletion, are directed against related bacterial strains. The mechanism of action of Micromicin J25 involves the inhibition of RNA polymerase and altering the electric potential of cell membranes. This peptide has an additional function in the defense against phages: It affects FhuA, an *E. coli* outer membrane protein. FhuA is an iron transporter that serves as a receptor for the unrelated coliphages T1, T5 and φ80. FhuA is required for injection of phage DNA into the target bacteria. Micromicin J25 blocks phage infection by inhibiting the binding of the phages to PhuA and preventing phage adhesion.[9,10]

The outer membrane protein OmpA serves as a receptor for several T-even-like phages in *E. coli*. Some *E. coli* strains inhibit the injection of these phages by producing a protein in the outer membrane called Trat, which interacts with OmpA. The interaction of Tract with OmpA decreases the binding of phage and its injection into the bacteria.[11]

Bacteriophage super infection refers to the same bacteria cell being infection by more than one bacteriophage in a sequential manner. We have mentioned previously that there is an abundance of prophage or prophage-remnant sequences in the bacterial genome. Some phages have mechanisms to prevent a superinfection. Sfi21, a *Streptococcus thermophilus* temperate phage, encodes the superinfection exclusion gene *orf203*. This protection is effective against many virulent phages, but it does not affect their own infection.[12] The mechanism of sie_{2009} exhibits the same characteristics as *orf203*, is expressed in the temperate *Lactococcus lactic* bacteriophage Tuc2009 and encodes bacteriophage resistance mechanism that blocks bacteriophages from injecting their genome and capsid.[13]

Biofilms are aggregates of microbial cells encased in an external matrix secreted by the own microbes.[14] The matrix is composed of an exopolysaccharide that is the main macromolecular component, although it can also contain proteins and other components. However, water is the main constituent of the matrix. Biofilms are formed in response to certain external insults as a protection mechanism and require the bacterial community to produce signals that co-ordinate the production of the biofilm's components. When the complex has been formed, the bacterial cells are metabolically inactive, since the extracellular matrix only allows a slow diffusion of nutrients. There are relatively few studies on the effect of biofilms on the capacity of phage infection.[15] Extracellular polymers may prevent access of the phage to the cell surface in some cases.[16,17] However, some factors allow the phage access to the bacterial membranes in the biofilm. Thus, biofilms' structures contain some 'channels' that can be used by the phages. Furthermore, many prophages contain genes encoding biofilm-degrading enzymes, such as polysaccharases or polysaccharide lyases. These enzymes allow the phage to spread through the extracellular matrix and promote viral access to the bacterial surface and the infection. Biofim formation as well as other activities like expression of virulence factors, sporulation, or antibiotic

formation is controlled by quorum sensing (QS). Quorum sensing is a process of intercellular communication that enables the bacteria to detect their population cell density and the population density prescribes a co-ordinated gene expression throughout the population. Indirect evidence links QS and the regulation of the lytic/lysogenic switch.[18] Many pathologic bacteria use QS to escape host defense mechanisms.[18] It would be interesting to elucidate whether QS have also any relationship with the bacterial antiphage response.

BACTERIAL IMMUNE RESPONSE AFTER PHAGE ENTRY

Restriction and Modification Enzymes

The restriction and modification (R-M) systems were discovered in the 1950s during investigations into the ability of certain bacteria to avoid the propagation of viruses that were able to infect other strains of bacteria. Thus, restriction enzymes were one of the first mechanisms involved in the microbial immune system to be discovered and appeared to be exclusive to unicellular organisms.[19] The protection against invading DNA is probably the main function of these systems although they could also participate in other processes such as DNA repair.[20] There is no clear evidence of these alternate functions. On the other hand, R-M systems are non-essentials for bacteria since strains deficient for this system are defective only in the susceptibility to phage infection. R-M systems consist of two activities performed generally by separate enzymes: Restriction endonuclease (REase) and methyltransferase (MTase). The nonself DNA is recognized by the endonucleases that interact with specific DNA sequences and trigger the cleavage of the nonself DNA. Their own bacterial DNA is protected by the MTases that methylate the adenine or cytosine within the specific sequence recognized by the restriction enzyme. As the methylation generally confers protection from cleavage, only the foreign DNA is recognized by the endonuclease. The four groups of R-M systems (Type I-Type IV) differ in enzyme activity, cofactor requirements, recognition sequences and cleavage sites.[21,22] Type I enzymes recognize unmethylated substrates, require ATP, S-adenosyl methionine (SAM) and Mg^{2+}. They cleave the DNA at variable locations away from the recognition site. Type II enzymes are the most numerous. They recognize specific DNA sequences and cleave it at a constant position, generally at the recognition site. They require Mg^{2+} as a cofactor. Type III enzymes need ATP, SAM and Mg^{2+}. They cleave at a specific distance away from the recognition site. Type IV enzymes require Mg^{2+} and recognize only modified DNA (methylated, hydroxymethylated and glucosyl-hydroxymethylated), cleaving both DNA strands twice and excising the recognition site.

The R-M systems are common in bacteria from all taxonomic groups, which indicates the importance of this defensive system. However, phages have developed anti-restriction strategies to avoid cleavage of their DNA.[1] The bacteriophage's most simple approach is to avoid the endonuclease recognition. Phages have evolved by modifying their sequence and accumulating point mutations, which reduce the number of recognition sites.[23] The genomes of some phages contain unusual bases like 5-hydroxymethyluracil instead of thymine or hydroxmethylcytosine (HMC) instead of cytosine and also glucosylated HMC. These bases, which are absent from the host genome, protect the phage genome against restriction enzymes since R-M systems are generally unable to recognize sequences containing this modified bases. However, bacteria have evolved to be able to recognize these modified phage DNA using the modification-dependent systems (MDS), which are also a nuclease-based

host defense mechanism directed towards modified bases. Although only a few DMS systems are described, the glucose-modified restriction (Gmr) S-GmrD system, a Type IV R-M systems, only recognize glucosylated hydroxymethylcytosine-modified DNA.[24,25] In this case, the host DNA is not recognized and there is no need for host DNA protection. The co-evolution of the attack and defense mechanisms has spurred the development of the internal protein I (IPI) which also inhibits the GmrS-GmrD system. When the bacteriophage T4 infects a *E. coli* strain encoding the *gmrs*/*gmrd* genes, its genome is degraded. However, some T4 bacteriophages possess the *ip1* gene in their genome which encodes the IPI protein. In this case the *Ip1*-containing phage is able to successfully infect the bacteria. The gmrS/gmrD genes encode two proteins, GmrS and GmrD that form a complex and degrade the glucosylated hydroxymethylcytosine-modified DNA. The IPI protein binds to the GmrS—GmrD complex, inhibits its activity and prevents the digestion of the T4 DNA.

Another phage resistance system is the phase-variable, phage-growth limitation (Pgl) system, which is an unusual phage resistance mechanism present in *Streptomyces coelicolor*.[26] This phase variation mechanism is a method used by bacteria for adapting promptly to new environments. The Pgl system consists of reversible variations of protein expressions. *S. coelicolor* A3(2) frequently varies from Pgl^+ to Pgl^- and vice versa. This variation mechanism allows changes in the phenotype much more quickly that those produced by mutational changes in the genome and is associated generally with bacterial immune evasion, especially against infection by phages. The phage φC31 can infect *S. coelicolor* (Pgl-) and produce viable progeny. In contrast, infection of a Pgl^+ strain produced phages that are severely attenuated in a subsequent infection. The mechanism of this resistance system is not completely understood, but has been proposed that Pgl^+ strains modify phage and this modification is recognized in a second infection by Pgl^+ hosts but not Pgl- hosts.[27]

Additional resistance mechanisms affect the enzymatic activity of the R-M systems. One of these mechanisms is the depletion of intracellular cofactors that are necessary for enzyme activity. For example, Type I and Type III R-M enzymes require SAM for their activity. Phage T3 encodes a SAM hydrolase which eliminates intracellular SAM pools, inhibits the enzyme activity and allows phage survival.[28] Another strategy is the production of proteins that interfere directly with the enzyme activity. For example, phage T3 and T4 encode the overcome classical restriction (Ocr) proteins, which bind specifically to Type I R-M enzymes and inhibit their activity. Because dimeric Ocr protein mimics approximately 20 bp of B-form DNA in the shape and charge distribution, Ocr acts as an anti-restriction protein by binding to Type I DNA restriction enzymes. The binding of Ocr to the Type I restriction enzymes prevents their binding to their DNA target and competitively inhibiting the action of the enzymes.[29] These resistance mechanisms are only effective when expressed soon after the entry of the phage bacteria into the host and thus, Ocr and SAM hydrolase are some of the first proteins to be expressed by phage T7 and T3, respectively.

CRISPRs

Many prokaryotes can acquire heritable immunity to phages by incorporating viral DNA into their own genome. This mechanism of anti-viral defense is known by the acronym CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats). The phage

or plasmid DNA sequences are integrated between repeated sequences in the CRISPR locus of the genome of prokaryote. This integrated DNA provides further interference for the exogenous genetic elements in a manner analogous to RNA interference (RNAi) in eukaryotic organisms. CRISPRs system may evolve rapidly, by acquiring new phage sequences to adapt to highly dynamic viral population.[30,31] Nevertheless, CRISPR system imposes a strong selective pressure on phages and has led to rapid mutation of viral genomes. CRISPR provides one explanation for the high evolutionary rates observed in phages. This primitive system of immune defense was discovered by comparative genome analysis in 1987 in the bacterium *E.coli by* Ishino and colleagues.[32] They found 14 repeats of 29 base pairs that were interspersed by 32-33 base pairs nonrepeating DNA sequences that were adjacent to the isozyme-converting alkaline phosphatase gene in *E.coli*. Computational analyses later revealed that the CRISPR system is present in the genomes of approximately 40% of bacteria and 90% of archaea.[33,34]

CRISPR systems are composed by multiple short DNA repeats that are separated by similarly sized non-repetitive DNA sequences termed "spacers". Each cluster is flanked by a varying number of genes called *CAS* (CRISPR-associated) genes.[32] Although many prokaryote genomes contain a single CRISPR locus, *Mathanocaldococcus jannaschii* has 18 loci, totalling more than 1% of the genome.[35] DNA repeats are composed of 24 to 47 base pairs.[36] Despite being divergent between species, the number of repeats per array varies from 2 to 249. Some groups of repeats contain a short palindrome (5-7 base pairs), hence the name palindrome in the CRISPR acronym. These palindromes likely contribute to RNA stem-loop secondary structure. Many repeats also have a conserved 3′terminus GAAA (C/G). Both structures are suggested to act as a binding site for cas proteins.[37] DNA repeats are interspaced by non repetitive spacers of DNA sequence of 26 to 72 base pairs.[35] The spacers are usually unique in a genome; a few exceptions, which are thought to have resulted from duplications, have been found to match sequences in phage genomes.[38] These spacers can be acquired from phages and subsequently help to protect the cell from infection. Removal or addition of particular spacers modified the phage-resistance phenotype of the cell.[30] CRISPR systems also comprise a leader A/T-rich, noncoding sequence, which is located immediately upstream of the first repeat and likely acts as the promoter for the transcription of the repeat-spacer array into a CRISPR transcript, the precrRNA.[39,40]

Cas genes are present in genomes of prokaryotes containing CRISPRs, but are absent from genomes that lack CRISPRs. More than forty different cas protein families have been described.[41] Particular combinations of *Cas* genes are found together, along with characteristic subclasses of CRISPR repeat sequences. These combinations appear to represent distinct CRISPR/Cas subtypes. Several different subtypes may occur in a single genome. Some Cas proteins are involved in the acquisition of novel spacers; others provide CRISPR-encoded phage resistance and interfere with invasive genetic elements. CRISPR-associated gene 1 (cas1) encodes the only universally conserved protein component of CRISPR systems.[42] Cas1 appears to be a double-stranded DNA endonuclease that produces double-stranded DNA fragments of approximately 80 base pairs in length. Its endonuclease activity suggests that it is part of the machinery for processing foreign nucleic acids. CRISPR1-associated *cas7* gene is involved in the integration of novel spacers after phage exposure.[30] Cas2 may act as a sequence-specific endoribonuclease that cleaves uracil-rich single-stranded RNAs (ssRNAs).[43]

The exact mechanism of the anti-phage or anti-plasmid activity of the CRISPR system is not fully characterized (Fig. 2). However, exogenous DNA is apparently processed

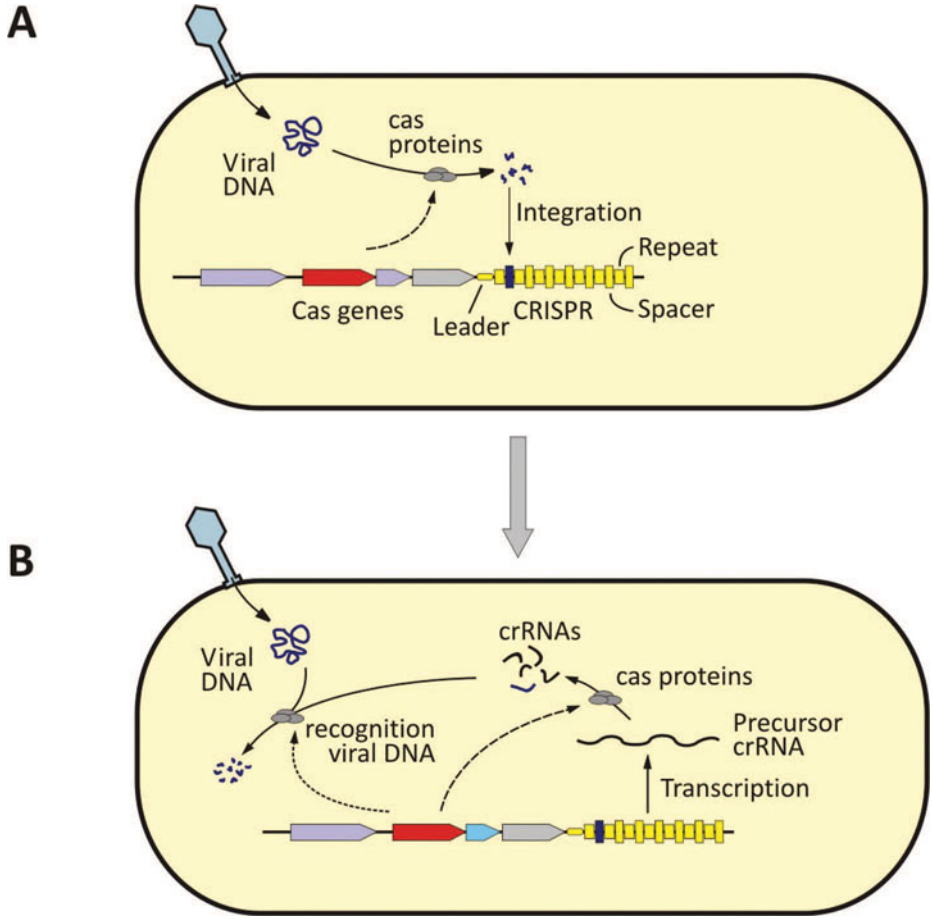

Figure 2. CRIPRS mode of action. A) Viral infection starts with the injection of phage DNA. Cas protein complex recognizes viral DNA and generates small DNA fragments using an unknown mechanism. Some of the small DNA fragments generated from the virus can be incorporated in the CRISPR locus as a new spacer, having then acquired the bacterial immunity against that virus. B) CRISPR repeat and spacer cluster is transcribed into a precrRNA that is processed by the cas protein complex into crRNAs, which are composed of a spacer and two half repeats. A new infecting phage is recognized when there is a crRNAs complementary to it. In this case, the cas protein complex, along with the respective crRNA recognize and destroy the invading DNA by an unknown mechanism.

by proteins encoded by some of the cas proteins into small elements (of about 30 base pairs in length), which are then inserted into the CRISPR locus near the leader sequence. The repeat-spacer array is constitutively transcribed into a full-length precrRNA and subsequently processed into specific small RNA molecules that correspond to a spacer flanked by two partial repeats.[40,44,45] The crRNAs seem to specifically guide the cas interference machinery toward foreign nucleic acid molecules that match its sequence, which leads ultimately to degradation of the invading element.[44] It has been proposed that they may act in a manner analogous to RNAi in eukaryotic organism. However, in spite of many similarities between CRISPR systems and eukaryotic system, key differences exist.

First, the enzymatic machinery differs between RNAi and CRISPR system.[46] Second, the crRNAs are larger than the short RNA duplexes generated by eukoryotic organisms (typically 21 to 28 nucleotides in length) because the CRISPR spacer (23 to 47 nucleotides) is flanked by partial repeats. Finally, RNAi involves RNA-dependent transcription, generation of double stranded RNA and use of the cleaved target RNA, in contrast to the CRISPR systems.

Although CRISPRs represents an effector element of a very primitive immune system, prokaryotes have the same dilemma as eukaryotic organisms. They also have to discriminate between self and nonself to avoid autoimmune disease. CRISPR systems have to target foreign extra-chromosomal material, but they have to avoid targeting their own spacer DNA. The mechanism is not fully understood, nevertheless, it has been proposed that in *Staphylococcus epidermidis,* target crRNA mismatches at specific positions outside of the spacer sequence leads to interference, but extensive pairing between crRNA and CRISPR DNA repeats prevents interference-targeting of their own prokaryote DNA and autoimmunity.[47]

ABORTIVE INFECTION (ABI) SYSTEMS

Abi sytems avoid phage infection in the remaining steps (replication, transcription and translation). They are peculiar in comparison with other resistance mechanisms as eventually they result in the death of the host bacteria. The Abi systems exhibit more specific nonself recognition than the aforementioned systems, such as the R-M systems. However, the immune response preserves the individual but the Abi systems destroys not only the phage but eventually also the host. Contrarily to other defense mechanisms, the Abi system protects the population, but not the individual. Many types of Abi systems have been described, especially in lactic bacteria, in which the phage-resistance mechanism has been studied at length due to the bacteria's economic importance. The mechanisms of these systems appear to be variable and details remain unclear. One example of this system in *E. coli* is the Rex system: Abi acts as a phage sensor and induces cell death by producing the loss of membrane potential. Another Abi system involves the PrrC protein, a ribonuclease specific for the host $tRNA^{lys}$: PrrC becomes active after T4 infection and produces cell death. The Lit protein is a metalloprotease that is also activated by T4. Lit specifically but relatively slowly cleaves the host's elongation factor (EF)-TU, inhibits protein synthesis and induces bacterial death (reviewed in ref. 48).

As with the rest of the resistance mechanisms, phages also have developed methods to circumvent the Abi systems. The *rII* gene allows phage T4 to survive the action of the Rex system.[49] The action of the Lit protein is reversed in some T4 phage by repairing the host $tRNA^{lys}$ with RNA ligase activity.[50]

CONCLUSION AND FUTURE PROSPECTS

The presence of an immune response is probably a characteristic of all living beings since all organisms can be attacked by pathogens. Regardless of the environment, bacteria are exposed to phages, which can infect them. Bacteriophages are the most abundant living entities and exceed the number of bacteria by about 10 times. For these reasons, they are very important in the regulation of the microbial balance and pose their most

serious threat. Bacteria have developed defense systems against these viruses and conversely, viruses have devised escape mechanisms that allow the infection. Therefore, this continuous phage-host interaction is a strong selective pressure that triggers a rapid co-evolution of both entities. There are a remarkable variety of phages as a result of the diversity in bacteria and many different viral defense mechanisms. Recently, important progress has been made in the knowledge of these phage resistance mechanisms. However, our understanding of these processes is not yet complete due to our ignorance of many aspects of phage biology. A better understanding of these antiviral mechanisms will have an important economical and medical impact.

ACKNOWLEDGMENTS

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CHAPTER 2

THE EVOLUTION OF SELF DURING THE TRANSITION TO MULTICELLULARITY

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Abstract: The notion of "self" is intrinsically linked to the concepts of identity and individuality. During evolutionary transitions in individuality—such as, for instance, during the origin of the first cell, the origin of the eukaryotic cell and the origin of multicellular individuals—new kinds of individuals emerged from the interaction of previously independent entities. The question discussed here is: How can new types of individuals with qualities that cannot be reduced to the properties of their parts be created at a higher level? This question is addressed in the context of the transition to multicellularity and using the volvocine green algae—a group of closely related unicellular and multicellular species with various degrees of physiological and reproductive unity—as a model system. In this chapter, we review our framework to addressing the evolution of individuality during the transition to multicellularity, focusing on the reorganization of general life-traits and cellular processes and the cooption of environmentally-induced responses.

INTRODUCTION

In philosophy, "self" is broadly defined as the essential qualities that make a person distinct from all others; the particular characteristics of the self determine its identity. The notion of "self" is, thus, intrinsically linked to the concepts of identity and individuality. Individuals are entities that are distinct in space and time. In biology, individuals have been defined based on several additional criteria including genetic uniqueness, genetic homogeneity, or physiological autonomy.[1] Going back to the root of the word individual (i.e., "not divisible"), individuals can also be thought of as the smallest units that cannot be divided into parts that maintain the essential properties of

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the whole. Lastly, from an evolutionary perspective, individuals are units of selection that possess the properties of heritable variation in fitness.[2]

During evolutionary transitions in individuality—such as, for instance, during the origin of the first cell, the origin of the eukaryotic cell and the origin of multicellular individuals—new kinds of individuals emerged from the interaction of previously independent entities. Such associations can involve similar entities (such as during the transition to multicellularity) or rather distinct entities (such as during the evolution of the eukaryotic cell) and be based on a wide range of ecological interactions (from commensalism and mutualism to exploitation and parasitism; for discussion see ref. 3). The initial interactions can be facilitated by either aggregation (e.g., the formation of multicellular fruiting bodies in slime molds and myxobacteria) or the failure of offspring individuals to separate (which is the case during the development of most multicellular organisms). The long-term stability of these associations and the subsequent integration of previously independent units into higher-level individuals are dependent on the frequency of cooperative interactions and the mediation of the inherent conflicts among lower levels.[3] At a mechanistic level, during transitions in individuality, a new genotype-phenotype map has to be created to reflect the emergence of a new kind of individual (and a new "self"/identity) at the higher level. The way in which the lower-level genotype-phenotype maps are reorganized at the higher level can influence the potential for evolution of the newly emerged multilevel system.[4]

The question discussed here is: How can a new kind of individual with qualities that cannot be reduced to the properties of its parts be created at a higher level and how does this process affect the lower levels (i.e., the previously independent individuals) in terms of their own individualities and identities? We address this question in the context of the transition to multicellularity and using the volvocine green algae—a group of closely related unicellular and multicellular species with various degrees of physiological and reproductive unity—as a model system. For the purpose of this discussion, we define an individual as the smallest unit that is physiologically and reproductively autonomous. This definition restricts the term multicellular individual to organisms with two types of cells: reproductive (germ) cells and nonreproductive (somatic) sterile cells. In contrast to multicellular forms in which all cells have reproductive abilities—and thus each cell (part) can reproduce the group (the whole), in multicellular organisms with a germ-soma separation, not all cells are able to recreate the whole; the evolution of nonreproductive cells renders the group indivisible and thus a true individual.

We have approached the questions posed above from many perspectives: multilevel selection (in terms of cooperation, conflict and conflict mediation),[3] fitness trade-offs and fitness reorganization,[2] life history trade-offs,[5] reorganization of general life-traits and cellular processes[4] and the cooption of environmentally-induced responses.[6] Below, we review our framework to addressing the evolution of individuality during the transition to multicellularity, focusing on the two latter perspectives. Specifically, we have argued that the emergence of individuality at a higher level (and the emergence of a new genotype-phenotype map) requires (i) the dissociation of certain processes, traits and functions at the lower level and their reorganization at the higher level, (ii) the cooption of lower-level processes and pathways for new functions at the higher level and (iii) changes in gene expression patterns, from a temporal into a spatial context.[4,6,7] We have also suggested that some of the differences among extant multicellular lineages (including differences in their evolutionary potential) can be explained by the way in which the reorganization of these processes and traits (and the emergence of the new

genotype-phenotype map) has been achieved during the transition to multicellularity and the evolution of individuality at the higher level.[4] Volvocine algae exemplify well these suggestions. In this group, the transition to multicellularity embraced unique paths, partly due to the constraints inherited from their unicellular ancestors.

THE VOLVOCINE ALGAE AS A CASE STUDY

"Few groups of organisms hold such a fascination for evolutionary biologists as the Volvocales. It is almost as if these algae were designed to exemplify the process of evolution".[8]

Diversity

Volvocine algae are photosynthetic biflagellated green algae in the order Volvocales, comprising closely related unicellular (*Chlamydomonas*-like) and multicellular forms that show a progressive increase in cell number, volume of extracellular matrix per cell, division of labor and ratios between somatic and reproductive cells[9] (Fig. 1). Interestingly, somatic cell specialization and higher-level individuality evolved multiple times in this group and the different levels of complexity are thought to represent alternative stable states (among which evolutionary transitions have occurred several times during the evolutionary history of the group), rather than a monophyletic progression in organizational and developmental complexity.[9,10] The observed morphological and developmental diversity among volvocine algae appears to result from the interaction of conflicting structural and functional constraints and strong selective pressures.

CONSTRAINTS

All volvocine algae share the so-called "flagellation constraint",[11] which has a different structural basis than the one invoked in the origin of metazoans.[12] Specifically, in volvocine algae, because of their coherent rigid cell wall the position of flagella is fixed and thus, the basal bodies cannot move laterally and take the position expected for centrioles during cell division while still remaining attached to the flagella (as they do in "naked", wall-less green flagellates). Therefore, cell division and motility can take place simultaneously only for as long as flagella can beat without having the basal bodies attached (i.e., only up to five cell divisions).

The presence of a coherent cell wall is coupled with the second conserved feature among volvocine algae—namely, their unique way of cell division. In this green algal group, cells do not double in size and then undergo binary fission. Rather, each cell grows about 2^n-fold in volume, followed by a rapid, synchronous series of n divisions under the mother cell wall; this type of cell division is referred as to multiple fission or palintomy (i.e., the process during which a giant parental cell undergoes a rapid sequence of repeated divisions, without intervening growth, to produce numerous small cells). Because clusters, rather than individual cells, are produced in this way, this type of division was suggested to have been an important precondition facilitating the

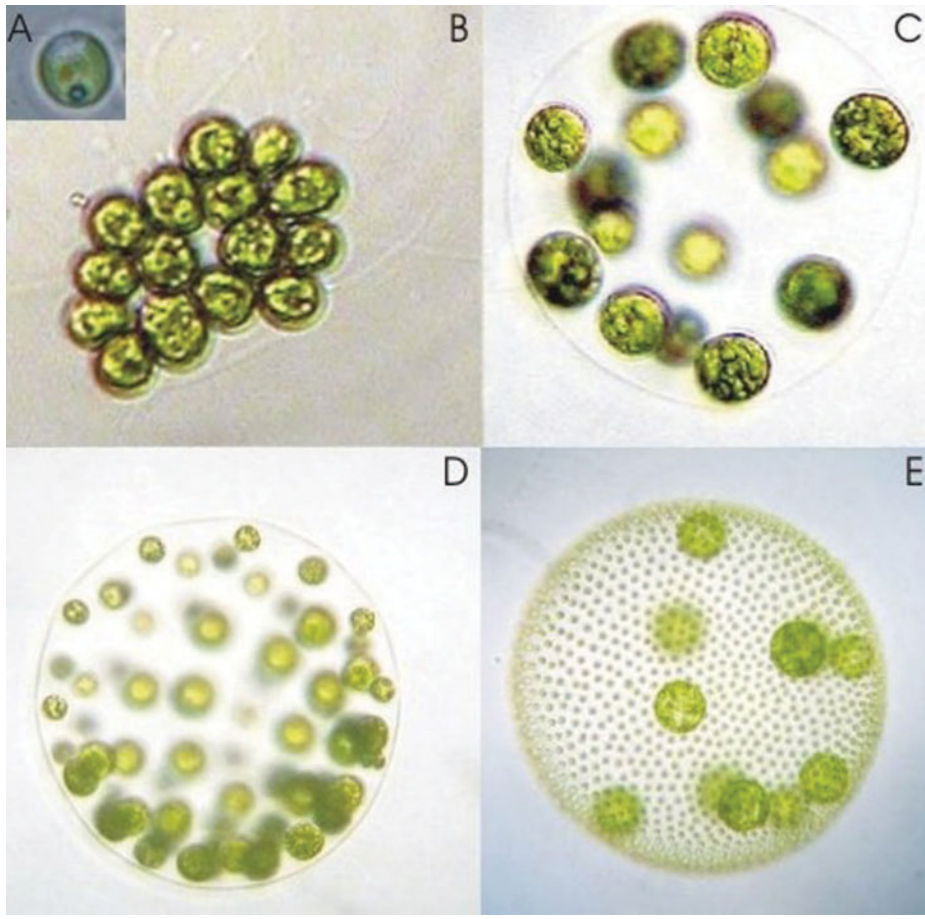

Figure 1. A subset of volvocine green algae that show a progressive increase in cell number, volume of extracellular matrix per cell, division of labor between somatic and reproductive cells and proportion of vegetative cells. A) *Chlamydomonas reinhardtii*; B) *Gonium pectorale*; C) *Eudorina elegans*; D) *Pleodorina californica*; E) *Volvox carteri*. Where two cell types are present, the smaller cells are the vegetative (somatic) cells, whereas the larger cells are the reproductive (gonidia) cells.

formation of multicellular colonies in this group.[13] In the unicellular species, such as *Chlamydomonas*, the daughter cells (2^2-2^4 cells) separate from each other after division. However, in many species, the cluster of 2^n daughter cells does not disintegrate and coenobial forms (i.e., a type of multicellular organization in which the number of cells is determined by the number of divisions that went into its initial formation, without any further cell additions)[13] are produced. For instance, in *Gonium*, the resulting cells (2^2-2^5) stay together and form a convex discoidal colony. In *Eudorina* and *Pleodorina* the cells (2^4-2^6, 2^6-2^7, respectively) are separated by a considerable amount of extracellular matrix and form spherical colonies. Finally, in *Volvox*, a high number of cells (2^{15}-2^{16}) form colonies up to 3 mm in size (Fig. 1).

SELECTIVE PRESSURES

The two selective pressures that are thought to have contributed to the increase in complexity in all volvocalean lineages are the advantages of a large size (potentially to escape predators, achieve faster motility, homeostasis, or better exploit eutrophic conditions) and the need for flagellar motility (e.g., to optimally position themselves in the water-column and to achieve better mixing of the surrounding environment).[8,14,15] Interestingly, given the background offered by the volvocalean type of organization presented above, namely the flagellar constraint and the multiple fission type of cell division, it is difficult to achieve the two selective advantages simultaneously. As the colonies increase in size and number of cells, also does the number of cell divisions (up to 15-16 in some *Volvox* species); consequently, the motility of the colony during the reproductive phase is negatively impacted for longer periods of time than are acceptable in terms of the need to access the euphotic zone. In larger species, this negative impact of the flagellation constraint is overcome by division of labor: some cells are involved mostly in motility, while the rest of the cells become specialized for reproduction. The proportion of cells that remain motile throughout most or all of the life cycle is directly correlated with the number of cells in a colony: from up to one-half in *Pleodorina* to >99% in *Volvox*.[9] In *Volvox*, the division of labor is complete: the motile (somatic) cells are sterile, terminally differentiated and undergo cellular senescence and death once the progeny is released from the parental colony;[16] only the reproductive cells (termed gonidia) form new colonies.[17]

THE GENETIC BASIS FOR CELL DIFFERENTIATION IN *VOLVOX CARTERI*

Volvox carteri is the most studied member of the multicellular volvocine algae[13] (Fig. 1). It consists of 2,000-4,000 permanently biflagellated somatic cells and up to 16 nonflagellated reproductive cells (Fig. 2). Terminal differentiation of somatic cells in *V. carteri* involves the expression of *regA*, a master regulatory gene that encodes a transcriptional repressor[18] thought to suppress nuclear genes coding for chloroplast proteins.[19] Consequently, the cell growth (dependent on photosynthesis) and division (dependent on cell growth) of somatic cells are suppressed. *regA* contain a SAND domain, which is found in a number of nuclear proteins, many of which function in chromatin-dependent or DNA-specific transcriptional control.[7] Proteins containing a SAND domain have been reported in both animal and land plants; one such protein, ULTRAPETALA1, acts as a key negative regulator of cell accumulation in *Arabidopsis* shoot and floral meristems.[20]

Mutations in *regA* result in the somatic cells regaining reproductive abilities—which in turn results in them losing their flagellar capabilities.[21,22] As motility is very important for these algae, the survival and reproduction of *V. carteri* individuals in which such mutant somatic cells occur is negatively affected.[14] Interestingly, although *regA* belongs to a gene family that comprises 14 members in *V. carteri,*[23] *regA* is currently known as the only locus that can mutate to yield Reg mutants.[18]

The expression of *regA* is strictly determined by the size of cells at the end of embryogenesis; cells below a threshold size will develop into somatic cells.[24] Which cells will not express *regA* and differentiate into germ cells is determined early in development

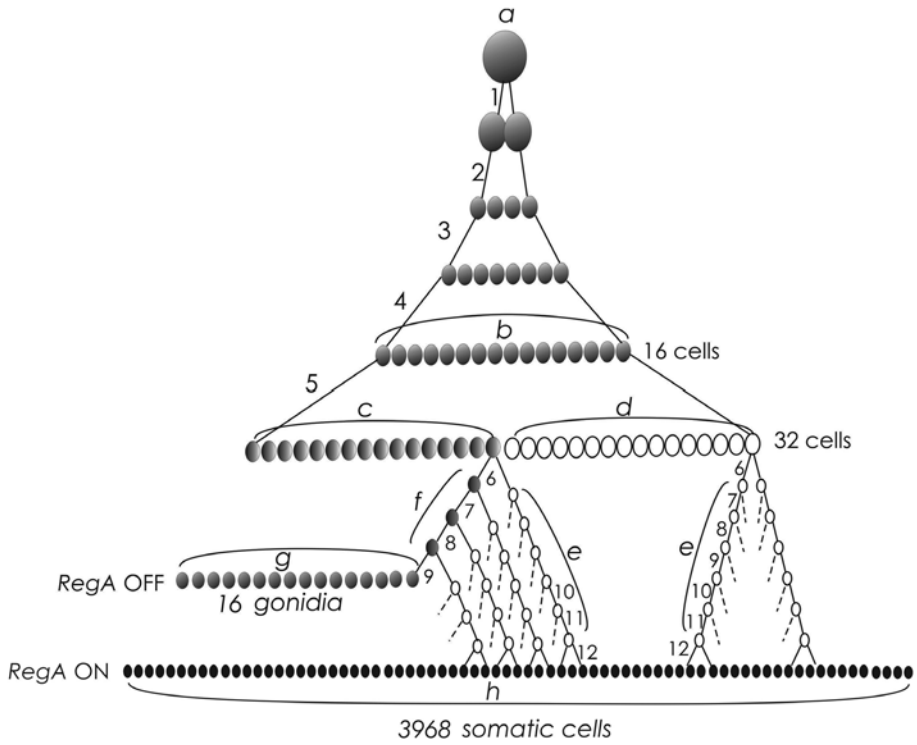

Figure 2. Schematic representation of asexual development and germ-soma separation in *Volvox carteri*. Gray ellipses denote totipotent (the "mother" gonidium—*a* and the 16 totipotent blastomeres—*b*), pluripotent cells (*c* and *f*) and the next-generation gonidia (*g*); white ellipses indicate unipotent (i.e., the somatic blastomeres and the somatic initials: *d* and *e*, respectively); black ellipses denote terminally differentiated somatic cells (*h*). Numbers mark the succession of cell divisions in the embryo. Cells are not represented at scale (*a* is ca. 2^9-fold larger than *g* and there is a ½-reduction in cell size with every symmetric cell division); all divisions take place under the mother cell wall, in a rather rapid fashion without intervening growth (i.e., palintomy and multiple fission) (adapted from ref. 4).

through a series of asymmetric cell divisions (Fig. 2). The asymmetric divisions ensure that some cells (i.e., the germ line precursors) remain above the threshold cell size associated with the expression of *regA*.[25] *RegA* is induced in very young somatic cells immediately after the end of embryogenesis but is never expressed in gonidia.[18] The mechanism underlying the differential expression of *regA* (i.e., ON in the somatic cells and OFF in the gonidia) is not known; it has been postulated that specific transcription factors bind to the *cis*-regulatory elements identified in three of the introns (i.e., two enhancers and one silencer) and act in concert to either silence or induce *regA* expression.[26]

UNICELLULARITY VERSUS MULTICELLULARITY

Many general life-properties and traits (such as immortality, totipotency, growth and reproduction) as well as cellular processes (such as cell division) are expressed differently

in unicellular versus multicellular individuals (see below). In the next section we discuss how these basic life properties and cellular processes have been reorganized during the transition to multicellularity and the emergence of individuality at the higher level, and apply these concepts to the evolution of multicellularity in volvocine algae.

General Life-Properties and Traits

Vegetative and Reproductive Functions

Any biological entity features two main sets of functions, vegetative (i.e., nutrition and growth) and reproductive, which correspond to the two basic components of fitness, survival and reproduction. These basic biological functions are coupled at the level of the individual, as a physiological and reproductive unit. However, the two sets of functions are realized differently between a unicellular and a multicellular individual. In unicellular forms, the same cell is responsible for both vegetative and reproductive activities (i.e., they are coupled at the cell level). Nevertheless, at the level of the individual, these functions do not take place simultaneously as they are dissociated in time: the vegetative phase precedes the reproductive phase. In undifferentiated multicellular forms, all cells perform both vegetative and reproductive functions and—as in their unicellular ancestors, these functions are separated in time, both at the cell level and multicellular entity level. On the other hand, in multicellular individuals with germ-soma separation, the two sets of functions are uncoupled at the cell level; some cells perform only vegetative functions, whereas other cells are specialized for reproductive functions. Consequently, the two sets of functions can take place simultaneously (i.e., they need not be separated in time anymore).

Growth is an important property of life. Interestingly, growth has different implications in unicellular versus multicellular individuals. In the former, growth is coupled with reproduction; growth to a specific cell size will generally trigger the reproduction of the individual and vice versa, reproduction requires achieving a preset cell size. In multicellular individuals, on the other hand, growth and reproduction of the individual can be uncoupled; reproduction is not necessarily dependent on growth and growth does not necessarily trigger reproduction.

Immortality and Totipotency

Immortality and totipotency are two basic life-traits. Here, immortality is used as the capacity of a cell to divide indefinitely and totipotency is defined as the ability of a cell to create a new individual. In contrast to totipotency, the term pluripotent denotes the ability of a cell lineage to produce cells that can differentiate into all cell types (but not into a new functional individual); lastly, multipotency refers to the potential of one cell to differentiate into more than one (but not any) cell type.

In unicellular forms, cells have both the potential to divide indefinitely (i.e., they are potentially immortal) and to create new individuals, either asexually or sexually (i.e., they are totipotent). In unicellular individuals, immortality and totipotency are thus coupled at the cell level. In differentiated multicellular individuals, on the other hand, only one or a few cell lineages manifest both immortality and totipotency; most other cell lineages have only certain degrees and combinations of potential for cell division and differentiation. For instance, in groups without an early segregated germ line (such

as plants and some simple metazoans like *Hydra*), the somatic cell lineages are incapable of continuous division or redifferentiation and thus they have to be replenished from one or a few pluripotent lineages that remain mitotically active throughout ontogeny and can also differentiate into germ cells (e.g., the interstitial I-cells in *Hydra*).[27] In lineages with a germ line that is terminally differentiated early in the development (such as in many animals), various degrees of mitotic capacity (approaching immortality in some stem cell lineages) and/or potential for differentiation are maintained in the many multipotent somatic stem cells (i.e., secondary somatic differentiation).[28]

Cellular Processes and Life-Traits

Cell division is a basic process in all cellular life-forms. The mechanisms controlling cell division are, however, different between unicellular and multicellular individuals. In unicellular individuals, cell division is strictly dependent on cell growth (cells divide when a specific set size is achieved). In many multicellular forms, however, this is not always the case: factors other than cell size (such as intercellular or systemic signals) can trigger or inhibit cell division. In addition, in unicellular forms cells have an unlimited division potential (cell division is strictly coupled with immortality), whereas in multicellular individuals, cells have limited and variable potential in most cell lineages (i.e., they are mortal) and their division potential is under the control of the higher-level individual.

Cellular Processes and Higher-Level Functions

Interestingly, cell division and cell growth have different roles and consequences at the level of the individual in unicellular compared to multicellular forms. In unicellular forms, every cell division results in the reproduction of the individual (cell division is strictly coupled with reproduction). In multicellular forms, on the other, hand, cell division is uncoupled from the reproduction of the individual in most cells (i.e., cell divisions do not necessarily result in the reproduction of the higher level). Also, whereas in unicellular forms, cell growth is the main contributor to the growth of the individual (with the exception of extracellular deposits in some lineages), in multicellular forms, the growth of the individual is mostly achieved through increasing the number rather than the size of cells (with some exceptions in lineages where there is significant increase in volume of extracellular matrix, internal space or even cell size).

TRANSITION TO MULTICELLULARITY: THE EMERGENCE OF A NEW SELF

We have argued that the unicellular-multicellular transition and the emergence of individuality at a higher level requires: (i) reorganizing basic life-traits (such as immortality and totipotency) between and within lower levels, (ii) decoupling processes from one another at the lower level (e.g., cell division from cell growth), (iii) decoupling certain cellular processes from functions and traits (e.g., cell division from reproduction and immortality), (iv) coopting them for new functions at the higher level (e.g., the cooption of cell division for multicellular growth) and (iv) changing the temporal expression of vegetative and reproductive functions into a spatial context.[4] Below, we discuss these concepts and apply them to our study case, the volvocine algae.

Reorganizing Immortality and Totipotency

During the transition to multicellularity and the emergence of individuality at the higher level, immortality and totipotency became restricted to one or a few specific cell lineages, namely those involved in the reproduction of the higher level. However, many cell lineages maintained various degrees and combinations of mitotic and differentiation potential. This required the reorganization (i.e., the differential expression) of these traits both among cell lineages and within a cell lineage. As discussed earlier, this reorganization has been achieved differently among the extant multicellular groups.

In *V. carteri*, immortality and totipotency are restricted to gonidia, the 16 cells following the first 4 embryonic cell divisions (*a* and *b* in Fig. 2)[24,29] and the zygote (after a sexual cycle; not shown). At the 32-cell stage, 16 cells (i.e., the germline blastomeres—*c* in Fig. 2) are pluripotent (i.e., they give rise to both germline precursors—*f* and somatic initials—*e*), while the other 16 cells (i.e., the somatic blastomeres—*d* in Fig. 2) are unipotent and produce solely somatic initials. The germline blastomeres divide asymmetrically for three or four times (each time renewing themselves and producing a somatic initial) and arrest mitosis two or three cell division cycles before the somatic blastomeres do. These 16 cells (*g* in Fig. 2) will differentiate into the germ cells of the next generation. After a total of 11-12 cell divisions, the somatic initials stop dividing and differentiate into somatic cells (*h* in Fig. 2), which have no mitotic or differentiation potential (they are terminally differentiated).

It is interesting that in *Volvox*, although immortality and totipotency have become fully restricted to the germ line (and reproduction and individuality at the higher level emerged), somatic lineages have no mitotic or redifferentiation potential. In other words, the two traits have been reorganized between germ and soma, but not within somatic cell lineages. The two sets of traits are still very linked in *V. carteri*; they are either both fully expressed (in gonidia) or both suppressed (in somatic cells). Noteworthy, the early-sequestration of the germ line was achieved without the evolution of secondary somatic differentiation processes; no multipotent somatic stem cells are present in the adult. This is rather surprising, because it has been suggested that the evolution of an early-defined germ line was possible because, due to the evolution of the multipotent stem cells and secondary somatic differentiation, the ancestral pluripotent germinative lineage was released from the task of producing the somatic tissues and was able to terminally differentiate into germ cells early in development.[28]

Decoupling Cell Division from Cell Growth

In multicellular individuals, to ensure the functionality of the soma, factors other than cell size must be used to determine which cells divide, when and how often. This requirement necessitates decoupling cell division from cell growth; consequently, a better and more finely tuned control on the replicative potential of the lower level can be achieved. However, this has not been accomplished in *V. carteri*; cell division is still strictly dependent on cell growth; reproductive cells have to increase $2^{10\text{-}12}$ fold in volume before dividing 10-12 times to produce the final number of cells in the multicellular individual.

Decoupling Cell Division From Cell Reproduction

Furthermore, to ensure the reproduction of a cell-group (and the heritability of the group traits), cell division has to be uncoupled from cell reproduction (i.e., the reproduction of the previously independent unicellular individual) and be coopted for the reproduction of the higher level (the group). The ability to reproduce the group can be achieved either by all or only some members of the group.

The case in which all cells have higher-level reproductive capabilities is best exemplified by a reproductive mode called autocolony, in which when the group enters the reproductive phase, each cell within the group produces a new group similar to the one to which it belongs; cell division no longer produces unicellular individuals but multicellular groups. This mode of reproduction characterizes the volvocine algae without a germ-soma separation, such as *Gonium* and *Eudorina* (Fig. 1).

In *Eudorina*, all cells (16 or 32) go through a vegetative (growth) and reproductive phase. However, cell division does not anymore result in a number of free unicellular individuals (such as in *Chlamydomonas*), but rather an embryo; cell division has been thus decoupled from cell reproduction and has been coupled with the reproduction of the group in all members of the group. Nevertheless, cell division is still strictly dependent on cell growth: each cell will start dividing only after a $2^{4\text{-}5}$-fold increase in size was attained, and once cell divisions are initiated they will continue synchronously until all the new embryos are formed. Although the stability, heritability and the reproduction of the higher level are ensured in this way, its individuality is not; because every member can be separated from the group and create a new group, such a group is not the smallest physiological and reproductive autonomous unit, thus is not a true individual in the sense used here (i.e., it is divisible).

The case in which only some cells have higher-level reproductive capabilities characterizes lineages with a separation between germ and soma. To achieve this, the coupling between cell division and reproduction is broken in most cells, namely the somatic cells; they reproduce neither themselves (as former free-living unicellular individuals) nor the higher-level unit; cell division is thus decoupled from the reproduction of both the lower and higher levels. In this way, somatic cells loose their individuality as well as the right to participate in the next generation; but in doing so they contribute not only to the emergence of individuality at the higher level but also to the emergence of a new level of organization, the multicellular soma. Soma is thus the expected consequence of uncoupling cell division from reproduction in order to achieve individuality at the higher level. *V. carteri* follows this pathway; however, the way in which germ-soma separation was achieved is rather unique among multicellular forms.

Coopting Cell Division for Growth at the Higher Level

By decoupling cell division from reproduction, this very important process became available for new functions. We suggested that this event was paralleled by the co-option of cell division for a new function at the higher level, namely the growth of the multicellular individual. Later, the use of cell division for more than cell multiplication, (i.e., which "gives rise to more entities of the same kind")[30] may have provided multicellular lineages with an additional advantage, namely cell differentiation; indeed, in many multicellular lineages asymmetric cell divisions are involved in cell differentiation.

Interestingly, in *V. carteri,* although the coupling between cell division and reproduction has been broken in the somatic cells, cell division was not coopted for the post-embryonic growth of the higher-level individual; rather, cell division was simply repressed in somatic cells. Specifically, the somatic cells lack the ability to divide post-embryonically; all the cell divisions responsible for the final number of cells in the adult take place during embryonic development (the further growth of the young spheroid is accomplished only through small increases in cell size and through a massive deposition of extracellular matrix). The implications of this outcome are multiple and profound. A direct implication is that soma in *V. carteri* differs from the soma of most multicellular organisms. Because somatic cells do not divide, further growth and/or regeneration of the individual are not possible during ontogeny; in addition, because the somatic cells undergo senescence and death at the age of 5 days,[16,17] the life span of the higher-level individual is limited to the life span of the lower-level somatic cell. Due to this unique type of soma, *V. carteri* is missing more than the ability to grow, regenerate, or live longer. Without a mitotically active multipotent stem cell lineage or secondary somatic differentiation there is less potential for cell differentiation and further increases in complexity.[4]

Changing Expression Patterns from a Temporal to Spatial Context

As discussed above, during the transition to multicellularity and the emergence of individuality at a higher level, some cells loose both their own individuality as well as the right to participate in the next generation. But why would cells give up their own reproduction (i.e., reproductive altruism)? The evolution of specialized somatic and reproductive cells can be understood in terms of the need to break survival-reproduction trade-offs, such that the survival and reproduction of a multicellular group can be maximized independently and simultaneously, and the benefits of a large size can be realized.[2] For instance, in undifferentiated multicellular flagellated algae, the reproductive phase is paralleled by the loss of motility—which can negatively affect the survival of the individual, especially in multicellualr groups whose reproduction will require a large number of cell divisions. On the other hand, in differentiated multicellular forms—such as *Volvox*, the spatial dissociation of reproductive and vegetative functions between gonidia and somatic cells allows the two sets of functions to take place simultaneously.

At a mechanistic level, we suggested that the evolution of germ-soma separation involved a change in the expression of vegetative and reproductive functions from a temporal (as in unicellular individuals) to a spatial context.[4] We have further argued that the evolution of soma in multicellular lineages involved the cooption of life-history trade-off genes whose expression in their unicellular ancestors was conditioned on environmental cues (as an adaptive strategy to enhance survival at an immediate cost to reproduction), through shifting their expression from an environmentally-induced context into a developmental context[4,7] (Fig. 3A).

Indeed, in volvocine algae—as in other photosynthetic organisms, nutrient-poor or stressful environments trigger a series of metabolic alterations—collectively known as acclimation, which favor survival when the potential for cell growth and division is reduced.[31] One of the consequences of this complex series of responses is a temporary inhibition of cell division (and thus reproduction), to ensure long-term survival. Acclimation involves both specific responses (e.g., scavenging for a specific nutrient) and general responses. The general responses include: a decline in the rate of photosynthetic activities, the accumulation of starch (diverting energy and fixed carbon from cell growth), a general

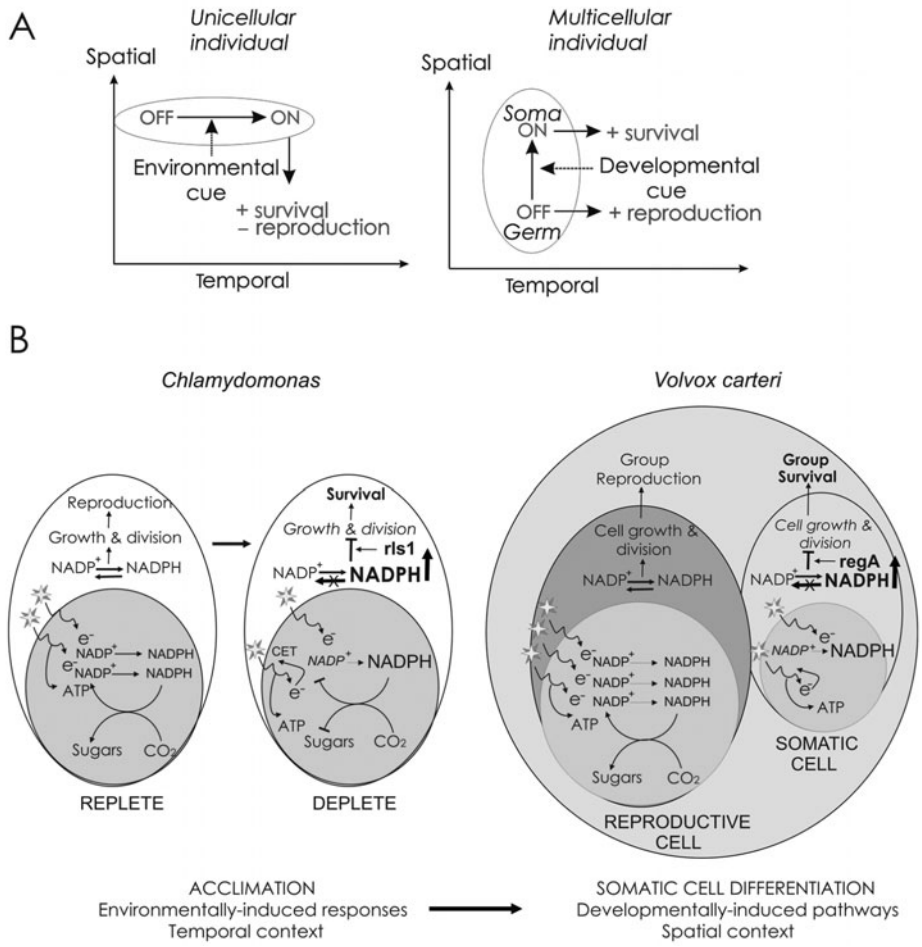

Figure 3. The evolution of germ-soma separation during the transition to multicellularity. A) General schematic representation of the change in expression pattern of a life-history trade-off gene from a temporal context (environmentally-induced)—in a unicellular individual, into a spatial context (developmentally-induced) in a multicellular individual. Adapted from Nedelcu AM et al. The evolutionary origin of an altruistic gene. Mol Biol Evol 2006; 23(8):1460-4; with permission of Oxford University Press. B) A model for the cooption of acclimation responses into somatic cell differentiation in *Volvox carteri*; see text for discussion. Although many components are involved, for simplicity, changes in redox status are symbolized by the over-reduction of the NADP pool due to either decreased NADPH consumption—in nutrient-deprived *Chlamydomonas*, or excess of excitation energy (owing to a higher surface/volume ratio)—in *Volvox* somatic cells. The switch to cyclic electron transport (CET), which can maintain ATP synthesis (and thus vital processes) in acclimated *Chlamydomonas* cells[35] and possibly in *Volvox* somatic cells, is also indicated (adapted from ref. 6).

metabolic slowdown and cessation of cell division.[31,32] Photosynthetic organisms use light energy to generate chemical energy (ATP) and reductants (NADPH) that are subsequently used to fix carbon dioxide (which will regenerate ADP and $NADP^+$). This coupling renders photosynthesis and its efficiency highly dependent on environmental conditions; changes in various abiotic factors—including light, temperature, water and nutrient

availability have an immediate impact on photosynthetic activities and subsequently on other metabolic processes.[33]

The down-regulation of photosynthesis is critical for sustaining cell viability under conditions of nutrient deprivation.[32,34] The lack of nutrients in the environment blocks cell growth and limits the consumption of NADPH and ATP generated via photosynthesis. Consequently, the photosynthetic electron transport becomes reduced and the redox potential of the cell increases.[31,32] Furthermore, because NADPH is not rapidly recycled (due to the slowdown of anabolic processes and the decreased demand for reductant in nutrient-poor environments), excited chlorophyll molecules and high potential electrons will accumulate and could interact with oxygen to create reactive oxygen species (ROS). ROS refer to a series of partially reduced and highly reactive forms of oxygen, including the superoxide anion (O_2^-), the hydroxyl radical ($OH\cdot$) and the hydrogen peroxide (H_2O_2). Although ROS are byproducts of normal metabolism and can act as secondary messengers in various signal transduction pathways (e.g., see refs. 35-37 for a review), increased intracellular levels of ROS (i.e., oxidative stress) can alter cellular functions and damage many biological structures, most importantly, DNA.[38]

Consequently, the regulation of the photosynthetic electron transport is an important hallmark of the general response to nutrient deprivation in *Chlamydomonas*. A series of processes including reduced photosynthetic electron transport and the redirection of energy absorbed from photosystem II to photosystem I can decrease NADPH production, favor ATP production through cyclic electron transport and allow a more effective dissipation of the excess absorbed excitation energy. Altogether these changes decrease the potential toxic effect of excess light energy (and thus serve to increase survival) and help coordinate cellular metabolism and cell division with the growth potential of the cell.[31,39]

We have identified in *C. reinhardtii* [7] the closest homolog of *V. carteri regA*—the gene responsible for the permanent suppression of division and reproduction in somatic cells (discussed earlier). Recently, we have also shown that this gene—currently known as *rls1*,[23] is induced under nutrient limitation (including phosphorus-, sulfur-deprivation and during stationary phase) as well as light deprivation.[6] Furthermore, we showed that the induction of *rls1* coincides with the down-regulation of a nuclear-encoded light-harvesting protein[7] and with the decline in the reproduction potential of the population under limiting conditions.[6] The fact that *rls1* is expressed under multiple environmental stresses and its induction corresponds with a decline in reproduction suggests that *rls1* is part of the general acclimation response and might function as a regulator of acclimation in *C. reinhardtii*. To support this suggestion is the finding that an inhibitor of the photosynthetic electron flow that triggers general acclimation-like responses,[32] also induces the expression of *rls1*.[6]

How can general acclimation responses in unicellular organisms be coopted for cell differentiation in multicellular groups? As we discussed above, in photosynthetic organisms, the flux of electrons through the electron-transport system (ETS) has to be balanced with the rate of ATP and NADPH consumption; imbalances between these processes can result in the generation of toxic ROS[32]. When a nutrient (e.g., sulfur, phosphorus) becomes limiting in the environment, ATP and NADPH consumption declines; this results in an excess of excitation energy and a subsequent change in the redox state of the photosynthetic apparatus, which will trigger a suite of short- and long-term acclimation responses[32,33] (Fig. 3B). Other environmental factors (e.g., cold, water stress) are also known to result in changes in the cellular redox status and trigger similar acclimation responses.[35] Thus, in principle, any factor that can elicit a similar redox change could prompt acclimation-like responses and ultimately induce cessation

of cell division. In a group context, if such a change is restricted to a subset of cells and if the suppression of reproduction in this subset of cells is beneficial to the group, sterile somatic cells can evolve and be fixed.

In *V. carteri*, the expression of *regA* is restricted (by an unknown mechanism) to cells whose size at the end of embryonic divisions falls below 8 μm.[24] As cell surface area and volume change at different rates, we proposed that in these small cells the ratio between membrane-bound proteins (including ETS and ETS-associated components) and soluble factors (including $NADP^+$ and ADP) becomes skewed—relative to the ratio in larger cells, towards the former.[6] Consequently, these small cells could experience an imbalance between the flux of electrons and the availability of final acceptors, which would result in a change in the intracellular redox status and the induction of acclimation-like responses, culminating with the suppression of division (Fig. 3B). To support this scenario is the fact that cytodifferentiation is light-dependent in *V. carteri*.[40]

Hence, by simulating the general acclimation signal (i.e., a change in the redox status of the cell) in a spatial rather than temporal context, an environmentally-induced trade-off gene can be differentially expressed between cell types, allowing for the two components of fitness to be maximized independently and simultaneously, and for individuality at the higher level to emerge. This hypothesis also predicts that somatic cell differentiation is more likely to evolve in lineages with enhanced acclimation mechanisms—or more generally, in lineages that can trade-off reproduction for survival in stressful environments. Because environments that vary in time (such as those volvocine algae live in)[13] will select for enhanced and efficient acclimation responses (note that temporally varying environments have been shown to select for phenotypic plasticity—i.e., generalists, in *C. reinhardtii*),[41] such environments are likely to be more conducive to the evolution of somatic cell differentiation.

A New Genotype-Phenotype Map

It is not known how the genotype-phenotype maps are formed nor how they are able to change in evolution.[42] During the unicellular-multicellular transition, a new genotype-phenotype map has to be created to reflect the emergence of individuality at the higher level. We argued that the way in which certain complex sets of traits and the genotype-phenotype maps associated with them are reorganized during the transition affects the flexibility and robustness of the new genotype-phenotype map at the higher level and can interfere with the potential for further evolution of the lineage.[4]

In this context, it is rather intriguing that in *V. carteri*, immortality can be regained and individuality can be destroyed by single mutations. As mentioned earlier, mutations in *regA* result in somatic cells regaining reproductive abilities. Although they start out as small flagellated cells, they later enlarge, loose flagella and redifferentiate into gonidia;[43] in other words, somatic cells regain both immortality and totipotency. In other multicellular lineages, such as humans, multiple mutations (each of which requires a minimum of 20-30 cell divisions) are required for immortality (i.e., cancer cells) to be regained.[44] The fact that single mutations have such large effects on individuality traits suggests that in *V. carteri*, the genotype-phenotype map at the higher level has been realized through a rather small number of genetic changes. Any attempt to increase the evolvability of these lineages has to first affect the current genotype-phenotype map to allow increased variability of the traits associated with immortality and totipotency (so as to decouple them in the somatic cells) without affecting the individuality of the system (e.g., by evolving

mechanisms to control these traits independently, thereby allowing cell replication and/or differentiation in the soma). In other words, the genotype-phenotype map has to at first become more robust (so that small genetic changes will not lead to the recreation of the maps associated with the previously independent lower levels, as it is currently the case) but flexible (so as to allow improvement through mutation and selection).

To gain such properties a number of small-effect mutations, in a very precise order (such that the viability of the individual under selection is not affected) is required. However, the way in which cell division, cell growth, immortality and potency have been reorganized in *Volvox*, as well as the way the genotype-phenotype map has been created at the higher level, makes the evolution of such traits more difficult. For example, the fact that i) the decoupling of cell division from reproduction in somatic cells was not achieved by inventing new ways to control cell division, but rather by blocking it altogether and ii) the suppression of cell division was not achieved through evolving some new mechanisms but rather through inhibiting the growth of the cell, strongly limits the evolution of traits that are dependent on these processes. These important complex sets of processes have not been decoupled from one another through their dissociation at the lower level and their cooption for new functions at the higher level, but rather through the suppression of some of the processes at the lower level; in this way, processes such as cell growth, cell division and differentiation are not represented in the higher-level map and thus cannot contribute to phenotypic variability.

Improvement is expected to come from mutations that, for instance, allow the somatic cells to regain controlled mitotic activity and some degree of differentiation potential during ontogeny. To achieve this, the multiple fission type of division should be replaced by a binary type, such that cell divisions during adulthood do not result in the duplication of the entire organism (as they do in the *V. carteri* mutants in which somatic cells regain mitotic capabilities); in addition, a binary type of cell division would allow a more finely tuned increase in body size, via small increments. In this way, more phenotypic variability can be achieved and become available for selection. It should be mentioned that the multiple fission type of division is a derived trait, which is thought to have evolved through the modification of the cell cycle via very conserved type of proteins involved in the key pathway that controls both cell division and differentiation in animal cells, namely, the retinoblastoma (RB) family of tumor suppressors.[45] Mutations of this gene in *Chlamydomonas reinhardtii* result in the initiation of the cell cycle at a below-normal size, followed by an increased number of cell divisions.[46] Such an alteration of the cell cycle might have been involved in the evolution of the multiple fission type of cell division, which is considered a precondition for the origin of multicellularity in *Volvox*.[13] If this is the case, it would argue for another example of achieving an important trait at the higher level (i.e., multicellularity) through a small number of genetic changes and thus for the potential instability/inflexibleness of the higher-level genotype-phenotype map emerged in this way.

CONCLUSION

During evolutionary transitions in individuality, a new identity (a new "self") emerges at the higher level from the re-organization of the properties displayed by the interacting entities. For instance, the transition from unicellular to multicellular individuals requires the re-organization at the higher level of certain basic life properties, such as growth, reproduction, immortality and totipotency, as well as of the cellular processes associated

with them (e.g., cell division and cell growth). The way in which this re-organization is achieved can affect the flexibility and robustness of the genotype-phenotype map that emerges at the higher level and can interfere with the potential for further evolution of the lineage. During the evolution of multicellularity, some cells gave up not only their own individuality but also their ability to reproduce. This form of extreme reproductive altruism is instrumental to the emergence of individuality at the higher level, as the presence of cells that lack the ability to reproduce the group (i.e., to recreate the whole) renders the multicellular group indivisible and thus an individual. The evolution of soma involved the co-option of life-history genes whose expression in their unicellular ancestors was conditioned on environmental cues (as an adaptive strategy to enhance survival at an immediate cost to reproduction), through shifting their expression from a temporal (environmentally-induced) into a spatial (developmental) context.[4,7] Interestingly, in eusocial insects, caste evolution is also thought to have involved the remodeling of pathways associated with basic life-history traits such as nutrition and reproduction present in their solitary ancestors,[47,48] which argues that the two distinct evolutionary transitions in individuality can be understood in a common framework.

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CHAPTER 3

GLYCONECTIN GLYCANS AS THE SELF-ASSEMBLING NANO-MOLECULAR-VELCROSYSTEM MEDIATING SELF-NONSELF RECOGNITION AND ADHESION IMPLICATED IN EVOLUTION OF MULTICELLULARITY

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Abstract: The goal of this chapter is to make a specific contribution about glyconectin glycan as the self-assembling nano-molecular-velcro system mediating initial steps of self-nonself recognition and cell adhesion in Porifera, the first descendants of the most simple primordial multicellular organisms. Two original findings will be described: (i) Velcro like concept based on highly polyvalent and specific intermolecular glycan to glycan associations with extremely low affinity of the single binding site and (ii) novel structures of the large and newly emerging family of glyconectin like glycan molecules. The emphasis will be put on the interdisciplinary approach for studying structure to function relationship at the different size scale levels by combining the knowledge and technologies (instrumentation and methods) of physics, chemistry, biology and mathematics. Applying such strategy which is crossing the boundaries of different science disciplines enabled us to develop a new Atomic Force Microscopy (AFM) based nano-bio-technology and perform the first quantitative measurements of intermolecular binding forces at the single molecular level under physiological conditions. We propose that nano-velcro systems of the glyconectin glycans, which are the constituents on the cell surface that are the most exposed to

†This work is dedicated to the memory of our mothers Milica and Elisabeta

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the environment, were responsible for the molecular self-nonself recognition and adhesion processes that underpinned the emergence of multicellular life forms.

INTRODUCTION

The classical field of cell adhesion and recognition is covering broad research on many different organisms, organs, tissues, cells and molecule types, as well as on the vast of physiological processes involving cellular interactions.[1] Unfortunately, as an unnecessary consequence, often monodisciplinary approaches were dominant. Technically limited methodologies, selective descriptions and classifications were done in many different ways and commonly were depending on the subjective interest, knowledge, available technology and influence of the particular group.[5-8] For the associated discipline of self-nonself recognition and adhesion similar research and reporting pattern is often evident. In this chapter we will present summary of our interdisciplinary approach with results about glyconectin glycan self-nonself recognition and adhesion system. Furthermore, we will compare glyconectins novel mechanisms and structures with other known types of cell recognition and adhesion molecules.

Our presentation will be based on practical but formally logical and simple Questions and Answers (Q and A) form: Why, What, Where, How and When.

Q AND A

Following the rules of didactic reasoning Q and A type description of self-nonself recognition and adhesion phenomenon will be presented here. The emphasis will be on a new and unique class of glyconectin glycans operating via novel mechanism of self-assembling nano-molecular-velcro and use of interdisciplinary approach which combines classical biochemical-molecular biology measurements with the new nano-bio-technology.

We will provide: (i) quantitative structure to function related Q and A comparisons between glyconectin glycans and other types of known molecules implicated in self-nonself recognition and adhesion processes relevant to evolution of multicellularity (ii) answers and discussion about the glyconectin glycans as the unique self-assembling nano-molecular-velcro and (iii) descriptions of interdisciplinary nano-sciences approaches combining physics, chemistry, biology and mathematics,

Following is the summary of the general Questions and contents to be answered and discussed in this chapter:

1. Why does self recognition and adhesion exist in complex multicellular organisms? Evolution of multicellularity, anatomical integrity with self-nonself distinguishing mechanisms, broader definition of self-nonself.
2. What is the nature of the molecules operating in self-nonself discrimination? Classification, Chemical nature, Sequences, 3D structure/molecular conformations, novel glyconectin structures, variability of structures.
3. Where are self-nonself cell recognition and adhesion molecules localized? Spatial distribution at molecular, cellular, tissue, organ and whole organism level (Cell, Extracellular Matrices, Tissues).

4. How do the cell recognition and adhesion molecules function in self-nonself discrimination? Description of different types of molecular mechanisms and principles of physiological functions during embryonal development (tissue and organ differentiation and formation), immunity (innate and acquired) associated with viral, bacterial and other parasite interaction with a host and pathological states such as tumor growth, metastasis, novel velcro like glycan to glycan self-assembly mechanisms of glyconectin mediated self recognition and adhesion.
5. When is cell recognition and adhesion active for self-nonself discrimination? Life cycles, fetus tolerance, fertilization, host-parasites, host-symbionts and auto immune disorders.

Q: WHY DOES SELF RECOGNITION AND ADHESION EXIST IN COMPLEX MULTICELLULAR ORGANISM?

A: Self recognition and adhesion in complex multicellular organisms enable functional and morphological identity throughout their life cycles as well as their reproduction.

During biological evolution two essential steps were necessary. The first was the emergence of cells via molecular self assemblies and the second subsequent one was the development of multicellular assemblies. Multicellular life is the most sophisticated form of changing patterns of matter and energy known so far. Their evolution required cellular self-nonself discrimination and adhesion. Cell adhesion defines the form, physical state and consequently the functioning of all multicellular living systems. Intermolecular binding forces between cell adhesion molecules are intrinsic properties of such cohesive structures. Differences in the degree of binding strengths between diverse types of molecules at the given environmental thermodynamic conditions determine selectivity of their associations providing molecular mechanism for cellular recognition and self-nonself discrimination.[2-5] Therefore, distinguishing self from nonself must operate via alogeneic and/or xenogeneic differences in types and/or spatial and temporal expression of cell recognition and adhesion molecules.

We proposed to use the intermolecular binding forces between cell recognition and adhesion molecules as the main quantitative criteria for assessing and defining their functional contribution to processes essential for distinguishing self from nonself during evolution, maintenance of the body architecture, reproduction and diversity of the complex biological life forms.[2-5]

Information technology engineers are implementing principles of biological self-nonself discrimination in order to ensuring the security of computer systems includes such activities as detecting unauthorized use of computer facilities, guaranteeing the integrity of data and preventing the spread of computer viruses.[6] These protection problems are instances of the more general problem of distinguishing self (legitimate users, corrupted data, etc.) from other (unauthorized users, viruses, etc.). A change-detection algorithm based on the way that natural immune systems distinguish self from other were developed.[6]

Self is commonly consider in biology as syngeneic specific (genetically identical) and nonself as either xenogeneic specific (derived from an organism of a different species—genetically not identical or allogeneic specific (derived from separate individuals of the same species—genetically not identical).

Definition of self and nonself in biology may also be considered in a more broad way where size/scale aspect play very important role. To elaborate this notion we shall first define a self unit and their integral components which differ or are identical to other units on the basis of their structural properties. Depending on the chosen size of the units and thus their components following are the size levels relevant to biological life: (i) molecule, (ii) organelle (iii) cell, (iv) tissue, (v) organ, (vi) organism, (vii) population of organisms or even (viii) ecosystems). As mentioned above biologists usually define self-nonself discrimination at the organism level due to the unique genome structure of each individual. From philosophical point of view which includes biochemical and physical aspects, any of the above unit level can be chosen for self-nonself discrimination without selecting the genome as the basis but rather just a pure structural properties of the unit and their components. Defining self and distinguishing from nonself requires the active process of interaction between two units which has to be established involving exchange of information of the components content and quality. Finally decision process has to follow as recognizing self from nonself and consequent reactions or interactions. In conclusion, living biological systems self non recognition is decision process based on positive iteration and active rejection reaction (immunity of higher animals) alternatively may be no interaction in terms of binding, no affinity between molecules and no cell adhesion.

Classical but pioneering experiment in the field of cell recognition and adhesion have used different experimental model system which usually did not involve careful vocabulary about the levels of self recognition which may occur within organism as well as between individual organisms. The example of the first case is liver and heart cell sorting from the same organisms. At this level of organizations cells of two organs are independent self entities or non directly interaction sums of cells which are united in higher order of organism self via immune system. Therefore, as the complexity of units arise the new systems guiding the self must co-evolve to enable self recognition of the larger or higher level unit.

Experimental and theoretical scientific knowledge, the use of logic and the common sense arguments results in the hypothesis that morphogenetic processes during embryonal development in each syngeneic individual life form, at least at the certain extent, follow the molecular and cellular self-assembly processes which have occurred during the evolution of unicellular and multicellular life forms involving important process self-nonself recognition at the molecular, cellular, tissue, organ and whole organism level.

New and emerging scientific disciplines may go through similar phases as the living reproducing organism by having generic developmental stage involving proliferation, growth and differentiation phase into sub-disciplines, followed by degeneration and redundantly carcinogenic proliferation with metastatic progressions useful only for itself. In the light of this book subject this may be biologically as well as sociologically interpreted as the new self creation within itself by rejecting the higher level of self.

Q: WHAT IS THE NATURE OF THE MOLECULES OPERATING IN SELF-NONSELF DISCRIMINATION?

A: Molecules operating in self-nonself discrimination are biological polymer molecules: (i) proteins, (ii) glycans, (iii) lipids and their naturally occurring covalently linked combinations known as (iv) glycoproteins and (v) glycolipids including few (vi) organic monomeric molecules.

The self-nonself recognition molecules are part of the larger family of cell recognition and adhesion molecules. They can be classified according to many different criteria such as chemical composition, sequence, 3D structures, spatial and temporal expression and function. The most common, simple and broad type of classification is based on the chemical nature of the cell adhesion molecules. There are five large groups of biological polymer molecules: (i) proteins, (ii) glycans (carbohydrates, polysaccharides), (iii) lipids, (iv) glycoproteins and (v) glycolypids. Last two groups are mostly common and naturally occurring combination of the first three groups.

Within each category of cell adhesion and recognition molecules further sub-classification can be done according to the sequence (primary structure) and size of the molecule. Sub-classification described here is for animal and bacterial cells only. Here we are listing all classes of cell adhesion and recognition molecules.

i. Sub-classification of proteins, which are linear amino acids polymers directly coded by genes, are done acceding to their primary structure motifs-gene families. Note that most of this molecules are glycosilated and thus belong to glycoproteins. However, here we are using the classification according to the protein sequence part. There are four classes of adhesion and recognition gene families coding specific protein sequences: (a) immunoglobulins,[7,8] (b), cadherins,[9] (c) integrins[10] and (d) lectins.[11] Each of the four classes has several sub-classes differing again in variation of primary sequences motifs.
ii. Glycans are linear and branched polymers of monosaccharides. They are not directly coded by genes. Sub-classification can be done as in the case of proteins according to their primary sequence 3D structure and size into: (a) N-lynked small glycans branched and non branched, (b) O-linked small branched and non branched glycans, (c) glycosaminoglycans large non branched glycans and (d) glyconectinoglycans large branched and nonbranched glycans.
iii. Lipids category of adhesion and recognition molecules are saturated or unsaturated hydrocarbon chains with terminal polar groups and organic cyclic hydrophobic rings. Sub-classification lipids in general is based on the chemical nature of the polar groups, size and chemical nature of the chain and its saturation degree into: (a) fatty acids, (b) glycerolipids, (c) glycerophospholipids, (d) sphingolipids, (e) sterol lipids, (f) prenol lipids, (g) glycolipids and (h) polyketides.
iv. Glycoproteins are glycoconjugates; covalently linked glycans to proteins or more commonly known post translational glycosylation modified proteins. Five subcategories based on glycosylation type, nature of glycan linkage to protein and glycan size and glycan structure (not protein sequence motifs) can be distinguished: (a) N-linked glycoproteins of molecular mass lower then 1×10^6 D, (b) O-linked glycoproteins of molecular mass lower then 1×10^6 D, (c) hybrids of (a) and (b) sub-category, (d) mucins large O-linked glycoconjugates of molecular mass larger then 1×10^6 D, (e) proteoglycans glycoconjugates O- and N- linked of molecular mass larger then 1×10^6 D, (f) peptidoglycans glycoconjugates crosslinked peptides and glycans found in bacteria of molecular mass larger then 1×10^6 D and (g) glyconectin glycoconjugates O- and N- linked of molecular mass larger then 1×10^6 D.
v. Glycolipids are lipids with covalently attached glycans. Major known sub-categories are: (i) glyceroglycolipids (galactolipids, sulfolipids), (ii) glycosphingolipids (cerebrosides, gangliosides, globosides, sulfatides, glycophosphosphingolipids), (iii) glycosylphosphatidylinositols.

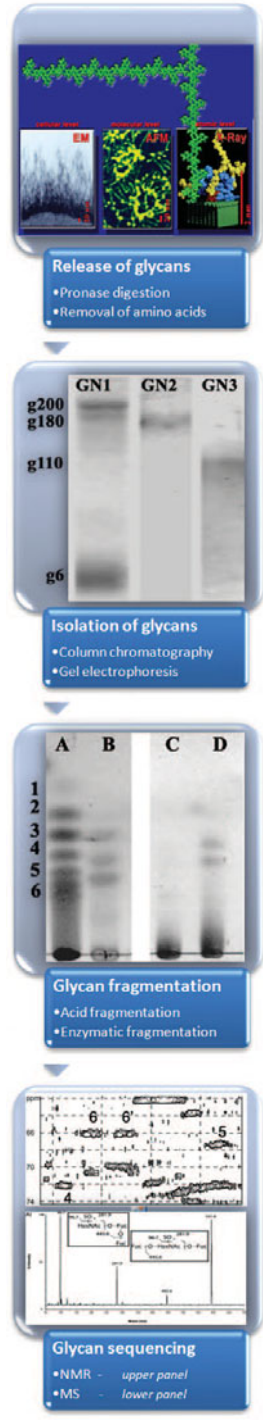

Figure 1. Please see figure legend on following page.

Figure 1, viewed on previous page. The fist panel shows Eelectron Microscopy (EM), Atomic Force Microscopy (AFM) and X-ray images of glycans dimensions at cellular, molecular and atomic level. EM; the Electron Microscope image of cells stained for acidic polysaccharides. These glycans are the most peripheral molecules (over 200 nm) from the cell surface with very high density and abundance. AFM; Atomic Force microscope image of glyconectin 1 sub-class with g200 glycan arms of 180 nm. X-ray; model of protein on plasma membrane in blue with small glycans in yellow and large glycan in green which is an order of magnitude longer then presented if the real length of g200 glycan would be taken in account. The second panel sows the second step of structural analyses by polyacrylamide gel electrophoresis of purified glyconectin glycan fraction is presented. Electrophoresis of glyconectin glycans was performed on a polyacrylamide gradient gel (7.5-15%). Gels were stained with 0.3% alcian blue in 3% acetic acid in aqueous 25% isopropanol. Lane a, 20 μg of glyconectin 1 glycans; lane b, 20 μg of glyconectin 2 glycans; lane c, 20 μg of glyconectin 3 glycans.[2-5] The third panel shows the third step of structural analyses of glycans by fingerprinting with trifluoroacetic acid hydrolyses. TLC analysis of hydrolyzed fractions of glyconectin 1 and glyconectin 2 stained by sulfuric orcinol. Lane 1, standard Glc degrees of polymerization (DP); lane 2, 0.1 M trifluoroacetic acid hydrolysis of glyconectin 1; lane 3, 0.1 M trifluoroacetic acid hydrolysis of glyconectin 2; lane 4, 1 M hydrolysis of glyconectin 2. The forth step in structural analyses using Nuclear magmetic resonance (NMR) and Mass Spectrometry (MS) sequencing are shown in the forth panel. Complex sequencing procedure in combined NMR and MS complementary approach requires sophisticated instrumentation and high skills.

Extensive multidisciplinary approach was employed to isolate and structurally characterize glyconectin glycan and protein structures. On one hand this involves classical analytical and preparative biochemical and molecular biology techniques: (i) sedimentation and density ultracentrifugations, (ii) chromatography (molecular sieving, ion exchange, affinity, reverse phase HPLC, gas chromatography), (iii) electrophoresis (SDS and glycan sieving), (iv) mass spectrometry (electrospray ionization, matrix-assisted laser desorption/ionization, secondary ion mass spectrometry), (v) and nuclear magnetic resonance spectroscopy and (vi) gene cloning with polymerase chain reaction (Fig. 1). The results have shown that glyconectins are new emerging class of glycoconjugates structurally different in their protein and glycan part from glycoproteins, proteoglycans and mucines molecules (Fig. 1).

Glyconectins are found in different animal phyla ranging from the most simple Porifera via Echinodermata to Mammals including humans. Although this early sub-classification of glyconectins is based on structural and functional analysis of the few above mentioned species from three phyla it is clear that some of glyconectin structures and shapes can either be xenogeneic specific (derived from an organism of a different species—genetically not identical), allogeneic specific (derived from separate individuals of the same species—genetically not identical) and syngeneic specific (genetically identical). Xenogeneic specific glyconectin subclasses found in Porifera have common structural properties such as large size of over 10×10^6 D (largest natural occurring glycoconjugate biopolymers beside mucins and proteoglycans), repetitive block-polymeric protein and glycan sequences, large linear and branched N- and O- linked glycan structures of 100×10^3 D, presence of fucose and/or arabinose within glycan chain together with sulfated and/or pyruvilated galactose and uronic acids (Table 1). Glyconectin glycan repetitive structures are novel sequences resistant to any so far known glycosidases and are not cross reactive with other anti glycan antibodies. Monoclonal antibodies raised against glyconectin glycans do not cross react with glycosaminoglycans, the most similar charged polysaccharides polymers.[2-5]

Some of the xenogeneic or species specific sub-classes of glyconectin glycans can be also defined as carciono-embryonal antigens mediating cell recognition and adhesion during embryonal development and tumor growth and metastasis within individual organism

Table 1. Glycan structures determined by nuclear magnetic resonance spectroscopy and mass spectrometry after mild hydrolyses of isolated glyconectin sub-classes of polysaccharides

Glyconectin Sub-Class 1	Glyconectin Sub-Class 2	Glyconectin Sub-Class 3
GlcNAc-Fuc- GlcNAc-(SO_3)Gal-Fuc Gal-(SO_3)Gal-GlcNAc- Py(4,6)Gal-GlcNAc-Fuc (SO_3)GlcNAc-[Fuc]Fuc	Py(4,6)Gal-$(Hex)_{0-1}$-Fuc Py(4,6)Gal-$(Hex)_{0-3}$-GlcNAc Py(4,6)Gal-$(Hex)_{1-5}$ Py(4,6)Gal-$(Hex)_{0-}$ Py(4,6)Gal-[Hex]Hex-Hex Py(4,6)Gal-Hex- $(Hex)_4$-[Py(4,6)Gal]Hex PyGal-Hex-[Hex]HexNAc	HexNAc-(SO_3)Ara/Fuc-Fuc Hex-HexNAc-(SO_3)Ara/Fuc-

without involving self-nonself recognition. because their expression in variety of species is controlled during embryogenesis and they are re-expressed in several types of tumors.[2-5]

Variability of the recognition structures is the essential for enabling their function of recognizing self from nonself. Immunoglobulin family of molecules achieves variability through recombination of the variable region part of the gene during differentiation of lymphocytes. Variability of glycan structures as the secondary gene products is achieved via differential expression of glycosylation and glycosidases enzyme battery as well as via regulation of their kinetics. When theoretical calculation of the potentially existing variability of protein chemical structures with glycan structures is made astonishing differences can be noted that glycans are potentially far more variable structures. Two examples can be illustrative for this statement: (i) for dipeptide (two different L amino acid each used once) 2 linear isomers are possible where as for two different D hexoses each used once 128 linear disaccharides structures are theoretically possible, (ii) hexapeptides will give 7×10^2 structures where as hexasaccharides has 3×10^9 isomers.

Q: WHERE ARE SELF-NONSELF CELL RECOGNITION AND ADHESION MOLECULES LOCALIZED?

A: Self-nonself cell recognition and adhesion molecules have to be localized on the external surface of plasma membranes and on extracellular matrices due to their function to keep the selectively cell assemblies together in a defined anatomical form and to be the first environmental chemical and physical sensor for self-nonself. Selectivity of their interactions is based on the intermolecular binding forces which provides thermodynamically sufficient affinity of interactions enabling cellular self from nonself discrimination.

The molecules guiding cellular self-nonself discrimination and adhesion should be present at the outermost cell surface. These sensors are the first physical and chemical encounters of changing environment. By gaining such sensors with recognition and adhesion properties the primordial multicellular organisms could preserve functional and morphological identity throughout their life cycles.

The physical state and form of all multicellular living systems and consequently their functioning are defined by cell adhesion. Intermolecular binding forces are intrinsic properties of such cohesive structures. The differences in binding strengths between

receptor to ligand molecules at the given environmental thermodynamic conditions determine selectivity of their associations. These differences in intermolecular binding strength are providing molecular mechanism for self-nonself discrimination. Therefore, cell adhesion molecules should play the role in cellular self-nonself recognition processes. Since this has been demonstrated in most cases and since cell recognition molecules without adhesion properties associated with direct cell to cell interactions are unknown we will use in this chapter term cell recognition and adhesion molecules. In spite of the fact that cell recognition and adhesion represent the basis for distinguishing self from nonself not all of cell recognition and adhesion molecules are relevant for this process because: (i) several of them may be allogeneic or xenogeneic identical and/or (ii) may also be used by viruses, bacteria and other parasites as adhesive docking during the infective entry into multicellular organisms. In the first case cell recognition and adhesion is essential for sorting of the differentiating cells during morphological process in developing embryos which are similar in different organisms.

In multicellular organisms spatial expression of a particular type of syngeneic, allogeneic and/or xenogeneic specific cell recognition and adhesion molecules in different tissues is genetically controlled. The resulting selectivity of expression pattern can together with intermolecular binding forces also provide molecular basis for recognition and adhesion function. Localization was usually determined in three ways: (a) direct biochemical isolation from the source tissues and cells, (b) indirect immunoassay by staining of cells and tissues with antibodies raised against particular cell adhesion and recognition molecules and/or by immunoprecipitation and (c) indirect RNA detection for a particular gene coding cell recognition and adhesion protein. This way does not provide evidence that the RNA is indeed translated into protein. However, this method enabled development of useful RNA tissue atlas for proteins.

Glyconectins are the largest cell surface and extracellular matrix macromolecules with length size of 1 micrometer and thus they are the most externally exposed molecules of the cell surface. Compared to glycoproteins, glyconectins are more then 200 times extended from the lipid bi-layer of cell membrane and are the first structures of cell surfaces encountering the environment (Fig. 1). Consequently the initial self-nonself recognition via cell recognition and adhesion processes is physically unavoidable to occur via this type of molecules.

Q: HOW DO THE CELL RECOGNITION AND ADHESION MOLECULES FUNCTION IN SELF-NONSELF DISCRIMINATION?

A: Cell recognition and adhesion molecules functioning in self from nonself discrimination are providing sufficient intermolecular binding strength between complementary receptor pairs and by these means also keep self in a defined anatomical form. The molecular mechanism may be based on either (i) classical and well known mono- to oligovalent moderate and/or high affinity binding between individual ligand and receptor protein to protein or protein to glycan molecules or (ii) glyconectin like highly polyvalent very low affinity of individual site homotypic or heterotypic intermolecular glycan to glycan binding. Therefore, subclass of glyconectin glycans with xenogeneic specificity are the newly discovered self-assembling nano-molecular-velcrosystem mediating self-nonself recognition and adhesion implicated in evolution of multicellularity.

During the evolution of primordial multicellular organism with a limited degree of cell differentiation primary function of most peripheral self surface molecules was self-nonself recognition and adhesion. The emergence of more complex multicellular organisms was based on the appearance of higher degrees of complexity and the multistep nature of self-recognition and adhesion systems. These can be related to (1) allogeneic self-nonself discrimination in the divergence of species,[11] and (2) syngeneic organ and tissue specificity during morphogenesis with cell recognition and adhesion molecules which may be similar or identical between different species and genetically different individuals (xenogeneic and allogeneic non specific and not involved in self-nonself discrimination).[2-5]

The major cell adhesion and recognition glycoprotein classes of immunoglobulin, cadherin and integrin families function via protein to protein intermolecular binding. These may be calcium ion dependent or independent. These interactions may be either heterotypic involving specific receptor and ligand bindings or homotypic. Lectins and some immunoglobulins function via heterotypic protein to glycan binding. In both cases the molecular mechanism is based on monovalent higher affinity interactions.

In mammals immunoglobulins play a crucial role in self-nonself discrimination by specific binding to nonself protein and glycan antigen structures. Lectins may play an important role during fertilization (which is allogeneic nonspecific xenogeneic specific), symbionts and parasite interactions.

Cadherins and integrins and many lectins are mediating cell recognition and adhesion associated with cell differentiation during development and in pathological cases such as tumor growth and metastasis which may not be xenogeneic and allogeneic specific and thus not relevant for self-nonself discrimination in the classical definition. It is important to note that these molecules may carry glycan modification, irrelevant for their primary cell recognition and adhesion function, which however may be target antigen for allogeneic and xenogeneic self-nonself discrimination via immunoglobulins.

Contrary to the above classes of cell recognition and adhesion molecules glyconectins operate via glycan to glycan interactions. These binding is calcium ion dependent and may either be homotypic or heterotypic. The molecular mechanism is based on highly polyvalent and specific intermolecular associations with extremely low affinity of the single glycan binding site. Therefore, species specific (xenogeneic specific) sub class of primordial Porifera glyconectin glycans can be defined as a self-assembling nano-molecular-velcrosystems mediating self recognition by adhesion and nonself discrimination by no adhesion.[2-5]

The small structural xenogeneic and allogeneic variability in some subclasses of glyconectin glycans together with the velcro type intermolecular association mechanism provides and ideal and reversible cell recognition and adhesion mechanism relevant for cell recognition and adhesion during embryonal development and in tumor growth and metastasis as well as in self-nonself discrimination at the organism level.

Multidisciplinary approach was employed to functionally characterize glyconectins glycan and protein structures (Figs. 1-3). On one hand these involve semi quantitative classical in vitro inhibition and promotion of cell aggregation assays by using isolated recognition and adhesion molecules and specific aggregation blocking monoclonal and polyclonal antibodies against these molecules. On the other hand a new type of assay using of color coded beads covalently coupled to different xenogenecally specific glyconectin glycans structures from three Porifera species was introduced. Adhesion assays with various combinations of these isolated glycans in a bead-bead and bead-cell assays under variable shear forces were used to more quantitatively test for self-nonself recognition

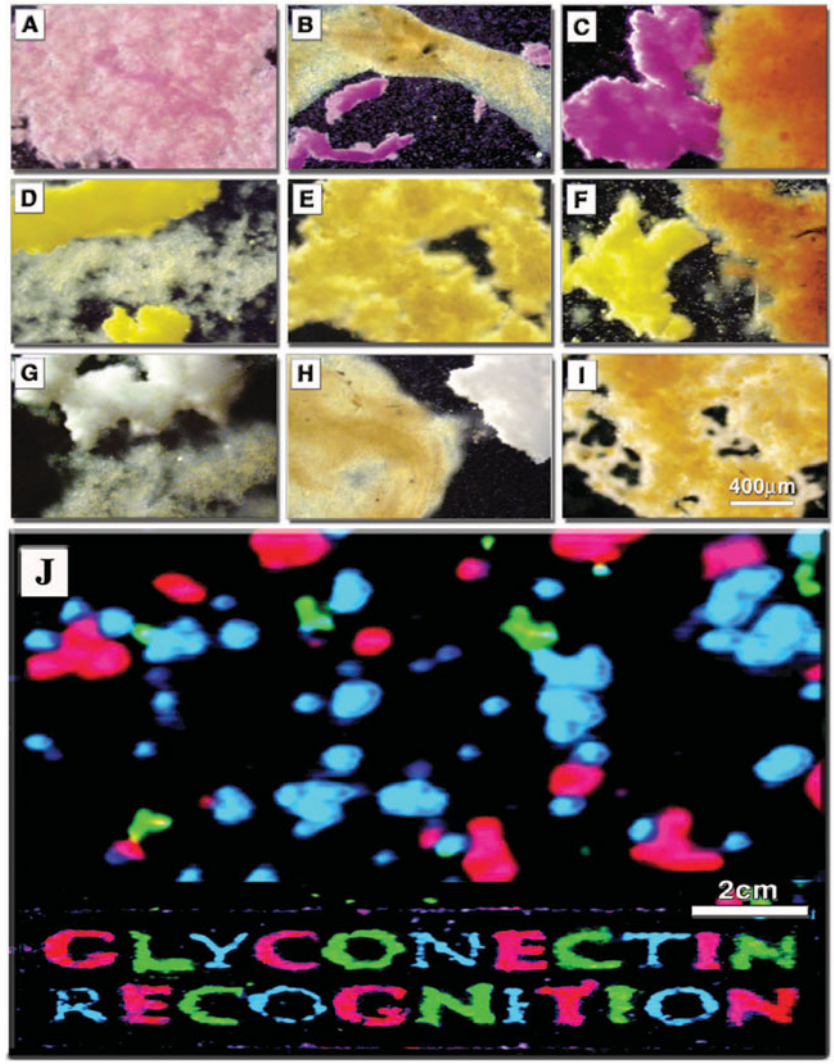

Figure 2. Species-specific glyconectin to glyconectin interactions mediate bead-cell recognition and adhesion. Xenogeneic glyconectin self-recognition in a mixture of glutaraldehyde-fixed cells and glyconectin-coated beads in seawater buffered with Tris in the presence of 10 mM Ca^{2+}. M. prolifera cells bearing glyconectin 1 were incubated with: glyconectin 1 (pink beads) (A), glyconectin 2 (yellow beads) (D) and glyconectin 3 (white beads) (G). H. panicea cells bearing glyconectin 2 were incubated with: glyconectin 1 (B), glyconectin 2 (E) and glyconectin 3 (H) color-coded beads. C. celata cells bearing glyconectin 3 were incubated with: glyconectin 1 (C), glyconectin 2 (F) and glyconectin 3 (I) color-coded beads (glutaraldehyde fixation changes cell colors, i.e., M. prolifera, orange to yellowish white; H. panicea, white to yellowish brown; and C. celata, brown to brownish orange. We did not observe differences in adhesion properties between fixed and live metabolically attenuated cells in a rotary assay. (J) Simultaneous species-specific glyconectin to glyconectin recognition in suspension and blotting assay. Letters were drawn using 4 µl of 1.5 mg/ml glyconectins on a Hybond-C extra nitrocellulose membrane (Amersham Biosciences) and probed in seawater buffered with Tris with pink, green and blue fluorescent beads coated with glyconectin 1, 2 and 3, respectively. A, seawater buffered with Tris without 10 mM Ca^{2+}. B, seawater buffered with Tris with 10 mM Ca^{2+} All photographs were taken after 30 min of mixing.

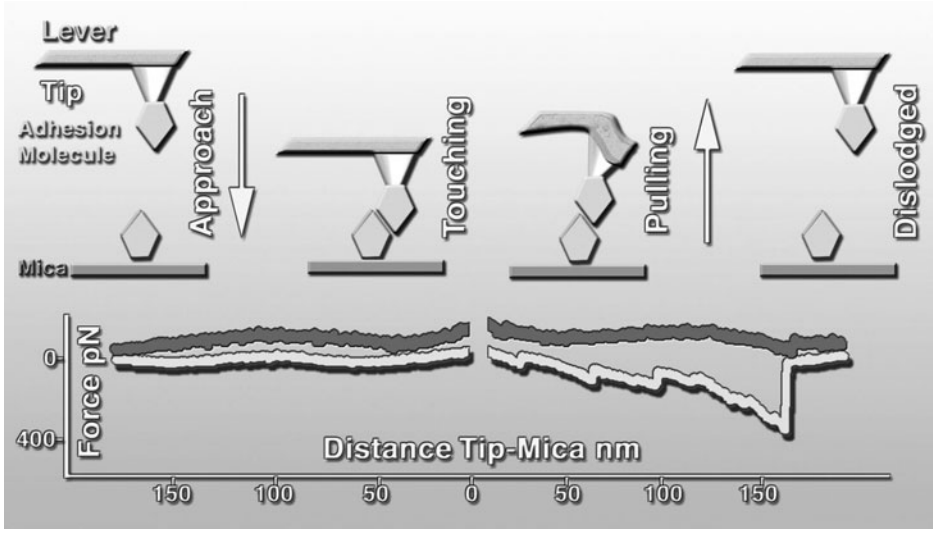

Figure 3. Schematic presentation of AFM measurements of intermolecular binding strength between glyconectin 1 glycans.

functions (Figs. 2,3) Multiple color codes of beads and cells enabled us to perform triplet and quadruplet aggregation assay with different glyconectin subclasses and show their role in Porifera xenogeneic self-nonself discrimination.[2-5]

Finally we have introduced for the first time a new nanotechnology based Atomic Force Microscopy (AFM)[2,12] measurements of intermolecular binding strength between a single pair of complementary and non complementary adhesion glyconectin molecules in physiological solutions (Fig. 3). This direct approach provided the first quantitative evidence for glyconectin glycan to glycan self-nonself selective cohesive function.

Interactions between individual adhesion molecules (immunoglobulin, selectin, cadherin, integrins and extracellular matrix adhesions), were usually investigated by kinetic binding studies, calorimetric methods, X-ray diffraction, nuclear magnetic resonance and other spectroscopic analyses. These methods do not provide direct measurement of the intermolecular binding forces which are fundamental for ligan-receptor association related to cell adhesion and recognition. To measure glyconectin to glyconectin interaction forces, we covalently attached glyconectins via their protein part to an AFM sensor tip and a flat mica surface. The attachment process involved only glyconectin proteins thus leaving functional carbohydrate adhesion sites unmodified. The cantilever tip having attached glyconectin molecules was moved with AFM piezo motor toward the glyconectin modified surface and a series of approach-and-retract cycles were collected in physiological liquid medium (Fig. 3). Glyconectin glycan to glycan binding was characterized by measuring both the force necessary to separate the glyconectin-functionalized AFM sensor tip from the glyconectin modified mica surface and the percentage of interaction events under different ionic conditions. These two indicators of glyconectin activity varied reversibly with the Ca^{2+} concentration, in agreement with glyconectin promoted cell adhesion and Glyconectin-coated bead aggregation. At a physiological Ca^{2+} concentration of 10 mM (physiological for marine organisms as sea water sponges), the average force between

glyconectin glycans was 125 pN, ranging up to 400 pN, with high probability of binding (60 ± 10%). At a Ca^{2+} concentration of 2 mM, cell adhesion and glyconectin-promoted bead aggregation were sharply reduced and the force (40 ± 15 pN) and probability of binding (12 ± 5%) were also reduced. The interaction between glyconectin glycans is Ca^{2+}-selective, as reported with a cell aggregation assay. Indeed, 10 mM Mg^{2+} could not replace Ca^{2+} in AFM experiments or in adhesion of glyconectin-coated beads.[2-5]

AFM measurements of intermolecular binding between several homotypic pairs of Porifera, xenogeneic specific subclass 1, 2 and 3, showed that intermolecular binding forces per pair of molecules are all the range of 400 pN. Heterotypic combination of subclasses 1, 2 and 3 revealed intermolecular binding strength of 20 pN. Therefore, glycan to glycan interaction is responsible for self-nonself recognition and adhesion. In conclusion, measurement of binding forces intrinsic to adhesion molecules is necessary to assess their contribution to the maintenance of the anatomical integrity of multicellular organisms. Our atomic force microscopy results of the binding strength between cell adhesion glyconectin glycans under physiological conditions showed that homotypic adhesive force of 400 piconewtons per molecular pair could hold the weight of 1600 cells assuring the integrity of the multicellular sponge organism. Interaction forces between heterotypic molecules were 20 times lower and are thus not sufficient to sustain existence of heterotypic aggregates under physiological hydrodynamic conditions of natural sea environment. Furthermore, this data also explain why small and loose unspecific aggregation was sometimes observed during the initial stage of heterotypic mixing under mild agitation.[2-5]

In conclusion xenogeneic subclass of glyconectin glycans, as the most peripheral cell surface molecules of sponges (today's simplest living Metazoa), are proposed to be the primary cell adhesive molecules possessing self-nonself discrimination essential for the evolution of the multicellularity.

Our novel AFM-based nanobiotechnology opened a molecular mechanic approach for studying structure- to function-related properties of any type of individual biological macromolecules related to self-nonself discrimination.

It is very important to note that the rupture force of a single covalent C–C bond is about 10 nN, whereas the strongest noncovalent binding forces measured with adhesion glyconectins are about 25 times weaker (400 pN).[2-5] These findings explain why the adhesion structures remain intact throughout AFM experiments. It is essential to covalently crosslink ligands and receptors to base supports and to the cantilevers via self-assembly layer of lipids and NOT to use (quick and dirty) noncovalent adsorptions of ligands. The use of covalent crosslinking and lipid shielding procedure ensures optimal measurements of intermolecular binding strength by minimizing the influence of unwanted and unrelated forces resulting from de-adsorption/adsorption of ligands and receptors and long-distance electrostatic forces between the mica and the cantilever and/or their interactions with the ligand and receptor. Unfortunately, several studies are ignoring this chemical-physical facts and are using adsorption methods without shielding where adsorption forces are wrongly declared as covalent attachment on gold.[13,14] Do NOT use such procedure because they result in meaningless results, which poisons the literature and are very confusing and discouraging for biologists who are seeking to use these superb techniques of measurements of intermolecular binding strength and evaluation for their functional contribution. Ideally, measurements between single pair of ligand to a receptor should be performed by AFM. Considering the size of the AFM tip and its curvature angle in comparison with the size of the crosslinked molecule, caution must be taken when determining the number of molecules that are crosslinked to the tip. Usually

few nanometer diameter of smaller protein molecule will allow crosslinking under the standard described conditions of at least ten molecules per 10 nm^2 surface of available curvature of the tip. Therefore, under these conditions it will be difficult to interpret how many ligand–receptor molecules will be contributing to the measured force.[13-15] Use of serial dilution experiments is recommended to solve this problem.

Q: WHEN IS CELL RECOGNITION AND ADHESION ACTIVE FOR SELF-NONSELF DISCRIMINATION?

A: Self-nonself discrimination in multicelluar organisms must be operational through the life cycle via cellular recognition and adhesion. During the embryonal life of most mammals the rejection of the nonself fetus "mysteriously" does not occur.

The function of the adaptive immune system in the case of humans is the recognition of self and rejection of nonself. Fetus has different genome from a mother. Therefore, it is the new developing self different from mother self. However, fetus is tolerated and not rejected. Several non proven theories are suggested that non immunological barrier of uterus exist and that immunosuppression is activated with assumptions of using also endogenous retroviruses.[16,17]

In some pathological cases in mammals altered self recognition occurs. This leads to loss of tolerance of self and finally results in auto immune disease and self rejection.

Unicellular and multicellular symbionts, parasites and other pathogens may avoid rejection by the host either by (i) using of a molecular mimicry principle which involves expression of similar host self antigens, thus enabling recognition as the host self and/or (ii) hiding of host nonself antigens and thus avoiding nonself rejection.

For propagation and diversity of species via sexual reproduction the essential fertilization step involved mixing of two genomes must be xenogenic specific and not allogeneic specific.

CONCLUSION

Self-nonself recognition and adhesion is fundamental prerequisite for evolution of multicellularity. The initial step of this process is based on carbohydrate to carbohydrate self-assembling nano-molecular-velcro system.We are proposing that large variability of carbohydrates as the most exposed and dominant components of plasma membranes are involved in many cellular interactions essential for self-nonself recognition. Unfortunately, chemical complexity of carbohydrates and need for interdisciplinary nano-technology approach resulted in the past decades to silent ignorance of carbohydrate role in biological self-nonself recognition processes.

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CHAPTER 4

NEGLECTED BIOLOGICAL FEATURES IN CNIDARIANS SELF-NONSELF RECOGNITION

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Abstract: Cnidarian taxa, currently of the most morphologically simplest extant metazoans, exhibit many salient properties of innate immunity that are shared by most Animalia. One hallmark constituent of immunity exhibit by most cnidarians is histocompatibility, marked by wide spectrum of allogeneic and xenogeneic effector arms, progressing into tissue fusions or inflammatory rejections. Scientific propensity on cnidarians immunity, while discussing historecognition as the ground for immunity in these organisms, concentrates on host-parasitic and disease oriented studies, or focuses on genome approaches that search for gene homologies with the vertebrates. Above tendency for mixing up between historecognition and host-parasitic/disease, highlights a serious obstacle for the progress in our understanding of cnidarian immunobiology. Here I critically overview four 'forgotten' cnidarian immune features, namely, specificity, immunological memory, allogeneic maturation and natural chimerism, presenting insights into perspectives that are prerequisite for any discussion on cnidarian evolution. It is evident that cnidarian historecognition embraces elements that the traditional field of vertebrate immunology has never encountered (i.e., variety of cytotoxic outcomes, different types of effector mechanisms, chimerism, etc.). Also, cnidarian immune features dictating that different individuals within the same species seem to respond differently to the same immunological challenge, is far from that recorded in the vertebrates' adaptive immunity. While above features may be connected to host-parasitic and disease phenomena and effector arms, they clearly attest to their unique critical roles in shaping cnidarians historecognition, calling for improved distinction between historecognition and host-response/disease disciplines. The research on cnidarians immunity still suffers from the lack of accepted synthesis of what historecognition is or does. Mounting of an immune response against conspecifics or xenogeneic organisms should therefore be clearly demarcated from other paths of immunity, till cnidarian innate immunity as a whole is expounded.

Self and Nonself, edited by Carlos López-Larrea.

INTRODUCTION

Ancestors of the vertebrates' adaptive and innate immunities have recently been downgraded to simple multicellular marine invertebrates, thus reviving the questions as to the evolution of self-nonself recognition phenomenon. Although many salient properties of innate immunity systems are shared by all multicellular animals, the evolutionary origin remains poorly understood and some of the shared properties have been less explored.[1] Since cnidarians are currently of the most morphologically simplest extant metazoans, they are also of the most suitable for studying the evolutionary origins of self-nonself recognition.[1-3] This is further manifested by their biological features. Most cnidarians (anthozoans and hydrozoans) are sessile creatures, with negligible competence to move away from points of settlement, sometimes living in densely populated communities. This leads to copious allogeneic and xenogeneic encounters with other permanently attached-to-hard-surfaces organisms, which settle in close proximity. When allogeneic and xenogeneic contacts start, they either develop tissue fusions (forming chimeric entities) or aggressively develop inflammatory rejections (the most common outcomes recoded in nature), the latter resembling events occurring following allogeneic interactions in mammalian pregnancy and vertebrate transplantation.[2-5] These agonistic reactions that demarcate physical barriers between genetically distinct organisms are hallmark to (a) competitive exclusion of other organisms that compete for limited available free substrate and (b) needs for keeping genome's integrity, maintaining each organism's individuality and preventing somatic and germ cell parasitism.[1,5,6]

Despite of the central dogma of immunologists stating that all invertebrates, which do not possess specialized lymphocytes (some of them, like cnidarians, even lack circulatory systems), rely entirely on innate immunity and the obvious cnidarians morphological simplicity, cnidarians do express complex epithelial cell-based effector mechanisms for allorecognition and xenorecognition as well as behavioural responses and specialized immune apparati. The effector mechanisms range from contact avoidance involving chemical sensing, allelopathy, barrier formation, tissue and skeletal overgrowths, development of sweeper tentacles, employment of mesenterial filaments, growth of hyperplastic stolons, creation of pseudofusions, morphological resorption of chimeric individuals, bleaching, retarded growth rates, transitory fusions, nematocyst shooting, developing of delayed responses, necrosis formation, tissue growth without calcification, attraction of motile phagocytic cells, retreat growths, allogeneic reversals and more (details and reviews in refs. 2,3-5,7-31).

Much of the largely phenomenological outcomes that are associated with cnidarians immunity[1-31] offer little in terms of the cellular and molecular constituents that clarify morphological observations. As a result, while there are unequivocal results attesting to the existence of adaptive-like immune features in cnidarians (specificity, memory and maturation;[2,5,6,22,26,27] i.e., innate immunity with adaptive features[32]), much of the recent research on the Cnidaria has been neglecting these biological features in lieu of molecular comparisons with mammalian immune genes and by concentrating on host defence aspects, overlooking allorecognition. This emanates from recent genome sequence projects performed on marine invertebrates, including Cnidaria,[33-35] that, while providing much improved sensitivity for the detection of gene homologs with the vertebrates (a usual deliverable considers the catalogue assemblage of immune related genes from a genome sequence), they do not delve into analyzing of unique immunological features. This chapter aims to provide a critical overview of four 'forgotten' cnidarian immune

features (specificity, immunological memory, allogeneic maturation and chimerism), presenting insights into perspectives that are prerequisite for any discussion on cnidarian evolutionary immunity.

SPECIFICITY

One of the hallmark characteristics of self-nonself recognition systems in the vertebrates is their high specificity, conferred by highly diverse loci and receptors that infer the recognition factors, regulatory factors and effector proteins.[36] Whereas very little is known about the actual underlying mechanism or the cells involved in this process in the Cnidaria, there are numerous examples for the wide existence of highly specific self- nonself recognition in this phylum. A general paradigm is that since immune responses are costly, potentially destructive to the host, generating an unlimited pool of recognition molecules that encompass many cell types, it will be a waist to have highly specific immunity in most marine invertebrates that are small creatures compared to the vertebrates (with limited numbers of cells) and are short living organisms.[32,37] This was envisioned by the tenet manifesting that invertebrate immune systems are pathogen manipulated through immune evasion,[3] not allorecognition inspired.

The ability of cnidarians to distinguish accurately between self and nonself had been clearly documented in anthozoans and hydrozoans.[2,5,6,38] Such distinction could be exerted by recognizing either the presence or the absence of nonself attributes or by detecting the presence or absence of self-molecules.[5,6,39] Several studies[8,10,25] have already demonstrated in allogeneic and xenogeneic assays that specific coral genotypes may elicit simultaneously or separately an array of different effector mechanisms, revealing the capacity for 'nonself recognition pattern over 'self recognition'.[6] When evaluating allorecognition systems in cnidarians (mainly on corals) it was evident that the effector arms are characterized by very fastidious and specific recognition events, are mounted by extreme polymorphism (i.e., there was no single fusion obtained on hundreds of allorecognition assays performed between adult colonies of *Stylophora pistillata*, as opposed to young colonies; unpublished), are capable of detecting fine differences even among kin organisms and express arrays of genotype specific effector mechanisms as well as a variety of cellular-morphological characteristics.

One of the best assays for elucidating specific innate immunity is through analyzing of allorecognition transitivity among interacting coral conspecifics. The simplest transitivity panel involves the interactions between three candidates (labeled as A, B, C) and a specific effector mechanism that is relevant as an interactor for all possible combinations emerged between the three interacting partners and carries the property of fusion/rejection or an hierarchy. Transitivity will be determined in cases when A = B (fusion) and B = C then A = C; or when A = B, but A ≠ C (rejection) then B ≠ C. Nontransitive relationships will be established when A = B and A = C but B ≠ C. The same rationale is implied for the hierarchy patterns in the expression of effector mechanisms. Linear hierarchy will be established when A > B (A dominates B) and B > C, then A > C, or A < C for circular hierarchy.[6,39,40] Allorecognition assays, specifically tailored for the tested species have revealed the nontransitive nature of corals' effector mechanisms,[10,11,22,26,40] indicating nonself recognition.[40] In these nontransitive hierarchies, the most dominant colony in a panel of allorecognition assays, could be found the subordinate or equal in aggression to the inferior colony. Following that, networks of hierarchies were established between

interacting conspecifics and interacting colonies could be clustered into several distinct allorecognition groups according to their allorecognition responses.[6] In a study on the coral *Stylophora pistillata* from Eilat, the Red Sea,[22] three allorecognition groups (marked I to III) of nine interacting genotypes, were detected by pairwise combinations assays. Within each group, colonies overgrew each other in a linear hierarchy pattern. Between groups, they either rejected or overgrew each other; each of the four Group I colonies was engaged in nonfusion/overgrowth interactions with Group III and unilaterally rejected each of the three colonies of Group II. Interactions between Groups II and III were only rejections and directionality was dependent on allogeneic partner types. This complicated pattern of incompatibility in *S. pistillata* cannot be explained by the concept of simple "self recognition", since incompatibility here seems to be coded as a series of discrete alternatives resulting from complex genetic elements of the partners involved. Results revealed also that all allogeneic effector arms and their directionality in *S. pistillata* were highly reproducible[9,22] (Fig. 1). Therefore, types of allogeneic responses can be considered as internal, specific outcome of recognition and are clearly not a causative outcome of external biological (i.e., predation, competition) cue or responses to environmental parameters. The same is implies in another study on nontransitive type of xenogeneic interactions developed between four species of marine sessile invertebrates, two of which were scleractinians[10] and in other studies on reef corals[16,41] (Fig. 1). Xenogeneic interactions, while nontransitive in nature,[10,16,41] are also subjected to physiological/behavioral responses in addition to histocompatibility borne outcomes. Results revealed

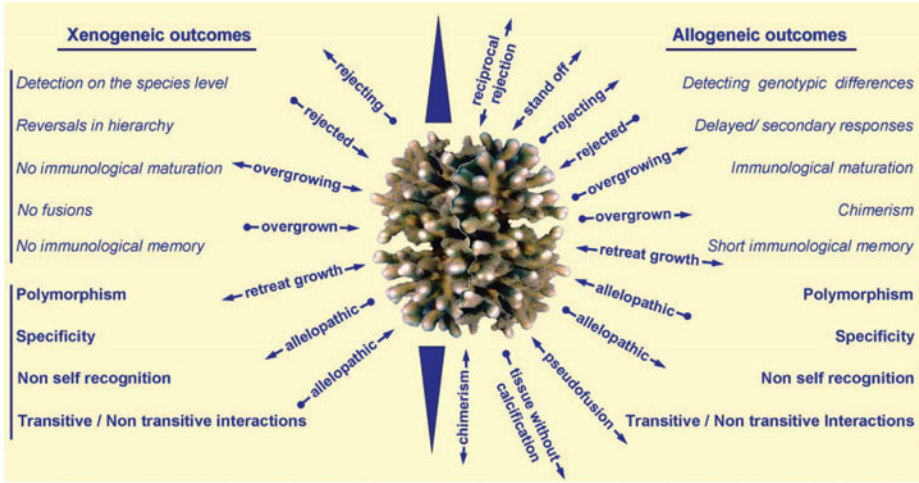

Figure 1. A schematic illustration that reveals the high diversity and the exact specificity for historecognition in the Cnidaria. A colony of the branching coral *Stylophora pistillata* is naturally encountered in various allogeneic (n = 11; centre, right panel of arrows) and xenogeneic (n = 9; left panel) types of interactions. Thus, a single coral genotype is not limited to a single mode of interaction in response to allogeneic/xenogeneic encounters. The coral's wide catalogue of effector mechanisms is capable therefore of dealing, specifically, with unlimited array of 'nonself' attributes, mounting against each nonself entity a specific response. Arrowheads point to the allogeneic/xenogeneic hierarchy. On both sides (up, italics) five major nonshared coral properties to allogeneic and xenogeneic responses are listed (immunological memory, chimerism, maturation, types of secondary responses and the ability to detect differences on the colony vs. the species levels). Bottom list (in bold) reveals four major properties shared between xenogeneic/allogeneic responses.

that expressions are also modified by environmental regulators, such as water flow and temperature,[42,43] the size of the neighboring xenogeneic partner[25] and quantity and the length of mesenterial filaments.[13,44]

A detailed historecognition test on the hydrocoral *Millepora dichotoma*[26] has further elucidated the existence of specific secondary effector mechanisms. While a reproducible unilateral tissue and skeleton overgrowths were recorded within the first 10 weeks in a set of 42 allogeneic interactions, four additional types of secondary responses were later observed among most incompatible combinations: reversals in overgrowth directionality, tissue necroses, stand-offs and abnormal growth patterns, all characterized by high variability in type and intensity of response. Based on the outcomes of the primary overgrowths, a complex nontransitive hierarchy was constructed for this set of colonies.

Innate immunity is still considered as devoid of specific and highly polymorphic effector arms, one of the most prominent features of adaptive immunity. However, recognition of 'self' vs 'nonself' in the cnidarians, while still representing two different enigmatic ways that immune systems may elicit (by detecting the presence or absence of attributes that define self or by detecting the presence or absence of nonself attributes[39]), could challenge traditional views on innate immunity. Mounting results attest to high degree of polymorphism in allogeneic and xenogeneic responses (albeit in some cases they are less polymorphic[45]), wide repertoire of responses, precise directionality of effector arm mechanisms, specific reactions in types, magnitudes and hierarchies, also characterized by hypervariable allodeterminant.[46] In fact, each allogeneic partner may be 'treated' differently and specifically by its confrere (Fig. 1), selectivity and reproducibility, with surprising delayed allogeneic responses such as cytotoxicity, overgrowth, reversal and secondary responses, which differ from a primary elicited outcome,[26] altogether revealing that these organisms, lacking circulatory systems and excess immune cells, define precisely their allogeneic and xenogeneic world. This complicated and complex nature of cnidarians histocompatibility, cannot be explained thoroughly by the tenet of 'self recognition', since it recognizes many nonselves, differently and only a single self. Moreover, incompatibility seems to be coded by a series of discrete alternative effector arms,[6] resulting from complex allogeneic genetic elements.[46] The self recognition for allorecognition implies that all different types of nonselves will be grouped into one uniform 'nonself' entity, without individualizing each of these nonself attributes.[6] This is not the case with the Cnidarian immunity. It should, however, be specified that all above documentations that 'rely' on recognition elements (not yet disclosed in cnidarians) are purely phenomenological, lacking our understanding on the molecular and cellular levels.

IMMUNOLOGICAL MEMORY

The exhibition of alloimmune memory, which is derived from selective amplifications of surviving antigen-specific lymphocyte clones, is another fundamental and hallmark characteristic of the vertebrate immune systems. Immunological memory enables an organism to benefit from past experience with foreign antigens, by responding to a repeated identical immunological challenge, quicker and more efficiently. In many mammals, an enhanced secondary immune response to a previously encountered antigen may last a lifetime.[47] Since immunological memory had been primarily defined using mammalian models, it may lead to potential errors in our interpretation of immunological phenomena in other groups of organisms, especially in invertebrates.[48] As a result, innate immunity is

still generally considered as devoid of memory component, because the mechanisms and the effector arms (i.e., memory B cells, CD4 memory T cells, cytotoxic CD8 memory T cell) establishing this memory in adaptive immunity of the vertebrates are clearly missing in innate immunity systems.[47] All memory components in the vertebrates are associated with research on adaptive immunity and concentrated in tuned reactions that enable the host to adapt to pathogens during its lifetime (xenogeneic interactions), not allorecognition. Clearly, the rationale of memory is tightly linked to the inherent nature of specificity (as discussed above). Without specificity, immunological memory could not be established.

The issue of immunological memory in invertebrates in general[5,32,47,48] and in cnidarians, in particular,[32,49] is a controversial issue as various studies clearly showed that allorecognition in these organisms is not completely devoid of induced effector arms.[5,32,47-50] It is reasonable, however, to assume that immune memory is advantageous only if there is a chance of being exposed to a previously encountered challenge; a highly expected scenario for marine sessile organisms, like the cnidarians. A coral colony may spend decades in the same specific spot, interacting with same allogeneic and xenogeneic specific attributes. This should select for a memory component in historecognition (as memory will be advantageous where repeated encounters are commonly presented for long terms. This is not unlikely scenario[1,2,5-7,9-11,16,19,20,22,23,25,26,28-31,41-45]), following the capacity of specific recognition of previous immune challenges.

Years ago,[49] a suggestion had been raised for three minimal functional criteria necessary for defining such immunological competence: (1) antagonistic or cytotoxic reaction after sensitization; (2) selective or specific reactivity; and (3) inducible memory on secondary contacts. Following this definition, several studies during the 70′ and 80′ (reviewed in ref. 50) have demonstrated short memory component in corals' and gorgonians' allorecognition, as well as on a biochemical level in a sea anemone.[51] More recently, a study on the gorgonian coral *Swiftia exerta* histocompatibility[50] has revealed an allograft specific rejection pattern, exemplified by rapid and progressive bleaching, necrosis and loss of tissue in the immediate contact area. First contacts required 7-9 days to produce 1 mm of necrosis; second contacts at a new tissue area, following a resting period, resulted with speedy (3-4 days) reactions. Intervals of up to eight weeks still produced a significantly accelerated secondary response, as opposed to third party allografts, all demonstrating recognition specificity. In contrast, a study performed on the Red Sea hydrocoral *Millepora dichotoma*[27] could not demonstrate a memory component, as second-set and third-party grafts were indistinguishable from the first sets in all allogeneic combination tested.

Studying immunological memory in other invertebrates,[47,48] may reveal aspects not yet recorded in the Cnidaria. For example,[52] exposure of the copepod *Macrocyclops albidus* to its tapeworm parasite, *Schistocephalus solidus*, reduced the chances of re-infection of the same host by siblings of the infecting tapeworm but not by unrelated genotypic parasites. Similarly, different *Daphnia* clones were found to be differently protected against diverse strains of the same pathogenic bacteria.[53] Thus, this instrument of learning and memory could allow better protection.[48]

IMMUNOLOGICAL MATURATION

Possible ontogeny in immune responses of the cnidarians could shed an additional light on the specificity and the complex nature of allorecognition in this phylum. Several studies have documented allogeneic maturation in scleractinians,[29,31,54-56] in soft corals[57]

and in colonial hydrozoans[58,59] without identifying or characterizing the biological mechanisms that perpetuate immunological maturation. This is of special interest because allorecognition is thought to reduce costly tissue fusion with individuals other than self.[1] Two studies[29,57] demonstrated that a window in ontogeny allows fusion between newly settled polyps (before the allorecognition system matures), but a recent study[56] has surprisingly revealed erratic histoincompatible responses in the same coral species studied before[29] (see below).

Generally speaking (not considering recent[56] surprising results), allogeneic maturation comes about at earlier stages of development. Best example is the coral *Stylophora pistillata*, where adult colonies always distinguish accurately between "self" and "nonself" attributes and respond selectively to specific allogeneic challenges, never by tissue fusions.[2,5,6] By establishing allogeneic contacts within groups of siblings or nonrelated offspring, three types of allogeneic responses, depending on the age of the interacting partners, had been documented.[29] The first was tissue fusion and the formation of a stable chimera, observed in partners less than two-month old. The second developed in contacts of 2-4-month old partners, started with tissue fusion and followed with separation between chimera partners when the oldest partner in the chimera reached the age of four months. The third type revealed regular histoincompatibility responses, as documented in allogeneic interactions of adult colonies,[2,5,9,22] recorded in all allogeneic encounters with partners over the age of four months. Maturation of allorecognition in this species was therefore achieved through three time-dependent stages, four months following metamorphosis. Combinations of siblings or genetically unrelated partners did not affect the results. A follow-up study[57] on soft corals further confirmed the existence of allogeneic maturation in the Cnidaria by documenting that tissue fusions could occur only between allogeneic young colonies. The lack of an active historecognition system in the early stages of ontogeny in the scleractinian and soft corals studied is probably universally connoted in other recent studies,[31,54] including colonial hydrozoans.[58-60]

As aforementioned, a new study[56] has revealed erratic responses in coral maturation. Whereas about half of kin allogeneic interactions of the coral *Stylophora pistillata* led to tissue fusions and chimera formations, none of the 83 nonsibling pair combinations was histocompatible and rejections between young siblings at the age of less than two months were documented, in contrast to previous results.[29] More surprising were the results documenting fusions between siblings at ages older than four months (in contrast to former study[29]), even between more than one-year-old partners. It was suggested[56] that the results reflected reduced genetic heterogeneity and continuous partial inbreeding (caused by chronic anthropogenic impacts on shallow water coral populations), where planulae originating from the same mother colony or from different mother colonies that are genetic related, share increasing parts of their genomes. Offspring born to related parents may also reveal an increasing genomic homozygosity; altogether imposing erratic immunity. It is interesting to note that defined genetic lines of the hydroid *Hydractinia symbiolongicarpus* have revealed that the hydroid's allorecognition resides in a single chromosomal region contains at least two loci,[61] further illuminating the important role of heterozygosity in self/nonself expression. Ontogeny of historecognition was also found in two species of *Hydractinia*, where immature offspring fuse to parental strains and remained fused as opposed to reproductively mature offspring that initially fused with parental strains, only to separate 3-21 days post fusion.[58] Late larval stage and early post metamorphosis are the stages at which individuals became allogeneic competent.[59]

A similar phenomenon of erratic histoincompatible responses was described for allogeneic interactions in *Pocillopora damicornis*, another genus of the same family. While earlier studies[55] had documented fusions between all combinations of young spat (sibling, nonsiblings and different colour morphs), a subsequent study[31] documented a novel rejection type between young sibling colonies, a result that had not been previously recorded. This rejection was termed as 'incompatible fusion', marked by seemingly regular fusion between partners that develop into rejection and/or separation between individuals. A hypothesis was put forward[31] that ontogenetic changes in *Pocillopora damicornis* histoincompatibility were variable, occurring in earlier or later stages of development. In contrast to *Stylophora*,[29,56] allogeneic interactions between siblings or nonsibling partners of *P. damicornis*, *Seriatopora caliendrum* and *Seriatopora hystrix*[31,62] had not been affected by the partners' age.

The ontogeny of allorecognition responses in the Cnidaria is not only interesting as a fundamental topic of invertebrate immune systems, but is also of ecological and evolutionary significance regarding the existence of widely distributed natural chimerism and the evolution of 'self' versus 'nonself' recognition.[5,47]

CHIMERISM

A wide spectrum of colonial organisms (i.e., sponges, cnidarians, bryozoans, tunicates, algae) often settle and grow beside conspecific individuals, an ecological state followed by direct tissue contacts between allogeneic individuals. Often, self/nonself recognition mechanisms allow the development of tissue fusions between allogeneic disparate conspecifics and the development of natural chimeras[2,57,63-68] (a chimera = an organism containing tissues or cells of at least two genetically distinct conspecifics). Many studies have documented the existence of naturally occurring chimeras in these phyla and it has been proposed that several types of ecological and biological benefits are associated with chimeric colonies.[63-68] As found in a variety of marine invertebrates, including the cnidarians,[63-65] chimerism is a tool that precisely discriminates between "self" and "nonself", even between closely related conspecifics. Chimerism and the events expressed morphologically by the effector arms differ fundamentally from one invertebrate group to another, best studied in botryllid ascidians.[63,64,66-75]

Studies on hard and soft corals[31,45,55-57,62,65,76,77] showed high proportions of fusions between young partners that are engaged in allogeneic interactions, an outcome not documented in adult alloreacting partners. This is of special interest because allorecognition is thought to reduce costly tissue fusion with individuals other than self.[1] Fusion between juveniles of scleractinian corals and the formation of chimeric entities were first detailed in *Pocillopora damicornis*[55] and then in other pocilloporid corals like *Stylophora pistillata*,[45] *Seriatopora caliendrum* and *S. hystrix*.[62] Fusion between conspecifics is not restricted to corals and is commonly found in hydrozoans.[26,78,79]

Several studies[64,67,72,73,75,80-82] tried to define evolutionary significance in natural chimeras by evaluating the fitness costs and benefits of chimerism as compared to the state of genetically homogeneous entities, or by analyzing chimeras of two partners vs. multi-partners chimeras. These include the expression of heterosis, increased genetic repertoire, reduced onset of reproduction, increased competitive capabilities, enhanced growth and survivorship rates, synergistic complementation and assurance of mate location. Not a single benefit has been documented in studied cnidarian chimerism

(although detailed studies on tunicates chimeras have revealed some interesting benefits; i.e., ref. 72). On the other hand, scientific interest has been focused on the possible event of germ cell parasitism, particularly relevant for organisms like cnidarians in which germ cell sequestration remains undetermined until late in ontogeny or when it is never fully attained along the life span of the organism (above references). No single study on cnidarians chimerism has revealed yet germ/somatic cell parasitism. On the other hand, several other costs were found. Gild et al,[79] that followed long-term repeated fusion and separation cycles in *Hydractinia* colonies, revealed a slow-down of colonial growth rates following fusion and recovery in growth rates following separation. The result for rapid transfer of stained material between partners in the *Hydractinia* transitory chimeras, raised doubts whether separation guarantees protection against cell parasitism and whether there are benefits associated with transitory fusion. In soft coral chimeras,[57] chimerical partners were detached, or chimerism resulted in the death of one or more partners, or in morphological resorption of the partners. Long-term observations on soft corals chimeras (450 d) further documented slower growth and growth-retarding disorders such as disruption of the structural pattern of polyp budding and polyp configuration. In hard corals, mortality of some of the *Maeandra* partners within the chimera occurred 17 days after fusion[83] and another earlier study[84] did find that the growth of aggregated colonies were slower compared with that of isolated individuals. One of the allogeneic responses in the hydrocoral *Hydractinia*[79] effector arms is transitory fusion, where colonies undergo tissue fusion, followed by tissue death at contact areas and colony separation. Long-term observations on repeated fusion and separation cycles in clones featuring transitory fusion showed a slow-down of growth rates following fusion and recovery in growth rates following separation. Experiments with mixed sex chimeras[58,85] have revealed that in transitory chimeras both colonies expressed only their own sex whereas in permanent chimeras, deviations from the expected sex ratio was recorded. As in *Hydractinia*, multi-partner entities made of *Stylophora pistillata* aggregated spat increased in size, as compared to individual spat, especially in the first two months.[65] However, at the single genotype level, the multi-partner entities were the smallest, revealing highest-cost-per-genotype also when compared, for example, to bi-partner chimeras.[65]

Observations (unpubl.) documented frequent spat aggregations of the branching coral *Stylophora pistillata* in the wild. However, although widespread in marine invertebrates, the extent of chimerism in wild populations of reef corals is unknown. A recent study[76] has estimated about 5% chimerism in natural populations of *A. millepora* from Australia, suggesting that chimerism is more widespread in corals than previously thought.

CONCLUSION

This chapter presented an overview of four 'forgotten' cnidarian immune features, namely, specificity, immunological memory, allogeneic maturation and chimerism, providing insights into perspectives that are prerequisite for any discussion on comparative and evolutionary immunity. It is evident that historecognition systems of the cnidarians are highly polymorphic at the morphological level.[1,2,5] Cnidarian inducible historecognition responses (both allorecognition and xenorecognition) are further characterized by a wide catalogue of effector mechanisms, by the employment, in many cases, of specialized structures that are constructed de novo or undergo changes upon contact and by using (not always, i.e., refs. 20,25) distinctive lines of nematocyst populations.[85] This is

performed by effector arms that demonstrate all the variety of features found in the complex structure of the vertebrate immune systems.[2] The literature clearly attests that the cnidarians possess highly diverse historecognition systems and effector mechanisms capable of dealing, in a specific manner, with unlimited numbers of "nonself" attributes and challenges.[5-7,10-15,19-23,25-31,39-51,55-62,65] It is therefore unfortunate that in contrast to discipline of main stream immunology, where availability of methods for studying genome-wide expression profiles has led to impressive achievements, the research on cnidarians immunity suffers from the lack of accepted synthesis of what historecognition is or does.[2,3,86-88]

Part of the ambiguity in historecognition synthesis emerged from the outcomes that cnidarian historecognition embraces elements that the traditional field of vertebrate immunology has not encountered[1,5,6] (such as the variety of cytotoxic outcomes, different types of effector mechanisms, chimerism, etc.; Fig. 1). The hallmark cnidarian immune feature (also found in other marine invertebrates[5,63,67,75,93]) that different individuals within the same species seem to respond differently to different individuals[5,48] (Fig. 1), is also far from that recorded in the vertebrates' immune response repertoire. Same imply to terms like 'memory', 'maturation' and 'specificity' that are broadly used without clear definition or carry very limited mammalian-oriented definitions.[47,52]

Above difficulties lead to another scientific obstacle. While it is believed that cnidarian innate immunity may offer key insights into the complexities of higher metazoans' immune systems,[3-6,89] recent studies on cnidarians immunity, while discussing historecognition as the ground for immunity in these organisms, concentrate on diverse host-parasitic and disease oriented issues, including melanin-producing signalling pathways and inflammatory responses of cnidarians,[3,87,88,90-92] also linking between climate change, anthropogenic stress and coral diseases. Other studies focus on molecular biology aspects of innate immune repertoire in Cnidaria, including genome approaches and search for gene homologies with the vertebrates,[3,33-35,86,93-97] underscoring our limited understanding of the mechanisms for self-nonself recognition in the cnidarians (the sequence data leave much to be deciphered with respect to relevant biological features[93]). This scientific propensity for mixing up between historecognition and host-parasitic/disease routes of immunity has been a serious obstacle for progress in our understanding of cnidarian immunology. Historecognition issues such as intraspecific competition, allotypic diversity, effector arms, specificity, immunological memory, allogeneic maturation and chimerism may be connected or not to host-parasitic/disease responses in the cnidarians.

Immunity of cnidarian taxa, the most ancient groups of metazoans, is poorly known. However, despite the ubiquity of historecognition in hydrozoans and anthozoans, its molecular basis has not been identified in comparison with currently known histocompatibility genes in vertebrates and colonial tunicates, except for the unique case of *Hydractinia*.[46,61] This *Hydractinia* historecognition system is made of hypervariable molecule bearing three extracellular domains with greatest sequence similarity to the immunoglobulin superfamily. Therefore, while the molecular study on Cnidaria historecognition needs an assemblage catalogue of immune genes from the genomes, first it requires an allorecognition assay that may help in the classification scheme of transcripts emerged. Histocompatibility genes should encode for recognition factors, regulatory factors and effector proteins,[33] with great diversity and routes. However, only historecognition function-based approach may elucidate the cascade of events participating in self-nonself recognition in the Cnidaria. By blurring the borders between above two disciplines, all 'ecological immunology' approaches (i.e., refs. 98, 99) associated with host-parasitic and disease oriented studies in the cnidarians[3,87,88,90-92,98,100] cannot be helpful in revealing the

secrets of cnidarian historecognition. The art of mounting an immune response against conspecifics or xenogeneic antigens should be clearly defined and demarcated from other paths of immunity, till cnidarian innate immunity as a whole is elucidated.

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CHAPTER 5

INTRACELLULAR INFLAMMATORY SENSORS FOR FOREIGN INVADERS AND SUBSTANCES OF SELF-ORIGIN

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Abstract: In order to survive, all organisms must recognize and eliminate foreign invaders such as infectious pathogens, chemicals, ultraviolet rays, metabolites and damaged or transformed self-tissues, as well as allogenic organs in cases of transplantation. Recent research in innate immunity has elucidated that there are versatile inflammatory sensors on spatiotemporal 'sentry duty' that recognize substances derived from both 'nonself' and 'self', e.g., Toll-like receptors, retinoic acid-inducible gene-I-like receptors, nucleotide oligomerization domain-like receptors and c-type lectin receptors. Having acquired high-level functions through the development of multiple molecules, higher organisms have established both extracellular and intracellular sensors that can discriminate danger-associated molecular patterns from promiscuous, but biologically similar, molecular patterns. In addition, 'loss-of-function' or 'gain-of-function' mutations in these inflammatory sensors have been linked (at least in part) with the etiology and severity of autoimmune diseases, autoinflammatory diseases and immunocompromised diseases in humans. Further studies focusing on the role of these inflammatory sensors in the development of immune disorders would highlight new avenues for the development of novel diagnostic and therapeutic applications with regard to these diseases.

INTRODUCTION

The mammalian innate immune system possesses a variety of 'arms' that sense pathogens and dangerous substances derived from self-components and metabolites. Upon microbial infection, soluble factors such as antibodies and complement recognize

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various components of pathogens, while cell-surface receptors such as c-type lectin receptors (CLRs), scavenger receptors and immunoglobulin Fc receptors either directly or indirectly capture pathogens to facilitate intracellular bactericidal processes. Also, multi-cellular organisms higher than plants possess cell-surface signaling receptors that sense pathogen components and allosterically activate intracellular signaling through common adaptor molecules shared by such receptors. Recent research studies have found that several different types of intracellular sensors recognize both pathogen-derived (nonself) and native (self) substances as danger-associated molecular patterns and trigger divergent signaling pathways leading to the activation of innate immune responses. Although different types of intracellular sensors recognize various forms of nucleic acids, i.e., single-stranded (ss) DNA, double-stranded (ds) DNA, ssRNA and dsRNA, they discriminate between the distinct motifs, modifications and structures of nucleic acids irrespective of their origin. Thus, extensive investigations have been undertaken into whether these intracellular sensors also play a role in the pathogenesis and severity of autoimmune diseases, autoinflammatory diseases and immunocompromised diseases.

LIGANDS FOR INFLAMMATORY SENSORS

Lipid Metabolites

There are many types of lipid metabolites produced by bacteria, since each bacterium has genus- or strain-specific enzymes that synthesize or degrade various types of fatty acids. These enzymes are essential for membrane lipid homeostasis, which allows bacterial cells to adapt to different environments.[1] From an immunological point of view, several lipid metabolites have been investigated as potential stimuli of innate immunity, e.g., lipopolysaccharide (LPS), mycolic acid, lipoteichoic acid, lipoarabinomannan, lipoprotein/lipopeptide and glycosylphosphatidylinositol (GPI)-anchored lipids.[2] These pathogen-specific components are recognized by Toll-like receptor heterodimers (TLR2/TLR1 or TLR2/TLR6) present on the host cell surface (Table 1). According to previous studies, TLR2 appears to be the primary signaling receptor for lipoprotein and lipopeptides. A dimerizing partner, TLR1, predisposes TLR2 to sensing of triacylated lipoproteins and both TLR1 and TLR6 to sensing diacylated lipopeptides.[3,4] The role of TLR1 in the physical interaction between TLR2 and lipopeptide was confirmed by crystal structure analysis of the extracellular domain of TLR2, together with TLR1 and a synthetic triacylated lipopeptide.[5] Although having agonistic potential, other TLR2 ligands do not share structural similarity with triacylated lipoproteins, suggesting that different molecular mechanisms underlie the ligand-specific interaction with TLR2 that depend on the type of ligand, especially for crude lipid metabolites containing strongly hydrophobic moieties.[6]

LPS, a glycolipid present in the outer membrane of Gram-negative bacteria, is an amphiphilic molecule comprising two components: a hydrophobic lipid A and a hydrophilic polysaccharide. Lipid A is a phosphorylated diglucosamine disaccharide decorated with multiple fatty acids and plays an important role in anchoring LPS to the bacterial cell membrane. Lipid A is recognized by TLR4 together with coreceptors, MD2, CD14 and LPS-binding protein (LBP) (Table 1). LBP and CD14 facilitate the transmission of LPS to TLR4, while MD2 facilitates the dimerization of TLR4, culminating in the activation of cell signaling pathways.[7,8]

Table 1. TLRs

Name	Ligands	Signal Activation	Adaptor(s)
TLR1	Triacylated lipoprotein (with TLR2)	NF-κB, MAPK	MyD88
TLR2	Di- or triacylated lipoprotein, lipoteichoic acids, GPI-anchored lipids, peptidoglycan	NF-κB, MAPK	MyD88, Mal
TLR3	dsRNA, poly(I:C)	NF-κB, MAPK, IRF3, IRF7	TRIF
TLR4	LPS, lauric acid, fatty acids-metabolites	NF-κB, MAPK, IRF3, IRF7	MyD88, Mal, TRIF, TRM
TLR5	Flagellin	NF-κB, MAPK	MyD88
TLR6	Diacylated lipopeptide (with TLR2)	NF-κB, MAPK	MyD88
TLR7	ssRNA, imidazoquinolin compounds	NF-κB, MAPK, IRF3, IRF7	MyD88
TLR8	ssRNA, imidazoquinolin compounds	NF-κB, MAPK, IRF3, IRF7	MyD88
TLR9	CpG motif-contiaining ssDNA	NF-κB, MAPK, IRF3, IRF7	MyD88
TLR11	Profilin-like protein	NF-κB, MAPK	MyD88

*Intracellular TLRs are in bold.

It is also known that TLR2 and TLR4 recognize nonbacterial lipid metabolites (Table 1). Lee et al revealed that saturated fatty acids (SFAs), such as lauric acid, activate NF-κB and expression of cyclooxygenase-2 via TLR4.[9] TLR4 recognition of lauric acid is co-operative with MD2 and possibly CD14, indicating that the sensing of lauric acid by TLR4 is processed by a similar mechanism to that of bacterial LPS.[10] More direct evidence shows that SFAs released from adipocytes activate cocultured macrophages in a TLR4-dependent manner.[11] In addition, lauric acid activates TLR2-mediated inflammatory signaling along with TLR1 and TLR6, suggesting that TLR2 and TLR4 have the potential to recognize lipid metabolites originating from both the host and bacteria.[12,13] In contrast, SFAs are an important risk factor for metabolic syndromes such as obesity, insulin resistance and atherosclerosis. Previous studies reveal that chronic inflammation is associated with the progression of some types of metabolic syndrome, suggesting that continuous activation of TLR2 and TLR4 by abundant SFAs and their metabolites leads to the onset of metabolic syndrome.[14] Some studies show that, in contrast to SFAs, poly-unsaturated fatty acids (PUFAs) such as docosahexanoic acid (DHA) antagonize the signaling mediated by TLRs 2, 3, 4, 5 and 9.[10,12] So, although the molecular mechanisms underlying the association of SFAs/PUFAs with TLR-mediated inflammatory responses are still unclear, further in-depth studies may explain how metabolic syndrome is triggered by inflammatory responses mediated by TLRs.

Peptidoglycan

Peptidoglycan (PG) is a component of bacterial cell walls. It comprises sugars and amino acids that form 3-dimentional mesh-like layer structures, thereby providing structural strength and stability. Since PG is not present on host cells, it is one of the key molecular

patterns that triggers host innate immunity.[15] It has been demonstrated that PG is recognized by multiple sensors such as TLR2, CD14, nucleotide oligomerization domain (NOD)-like receptors (NLRs) such as NOD1 (also known as NLRC1 and CARD4) and NOD2 (also known as NLRC2 and CARD15), PG recognition proteins (PGLYRPs) and c-type lectin receptors (CLRs) (Table 2).[16-20] The exact mechanisms of PG recognition used by each sensor have been studied. Inamura et al found that chemically synthesized PG is not recognized by TLR2 expressed by HEK293 cells.[21] It has also been shown that pretreatment of crude bacterial cell wall extracts with NaOH or H_2O_2 abolishes their ability to activate TLR2. This suggests that thioethers present in lipoproteins or lipopeptides, known to be a key element of macrophage activating lipopeptide 2 (MALP-2), are crucial for TLR2 activation.[22] Dziarski et al prepared extensively purified insoluble and soluble PGs and demonstrated that polymeric PG, but not muramidase-treated monomeric PG, is an essential element for cellular activation via TLR2 and CD14.[23] While chemically synthesized PG does not activate TLR2, it does activate inflammatory responses mediated by NOD1 and NOD2.[21] It has been shown that NOD1 recognizes PG containing *meso*-diaminopimelic acid (*meso*-DAP), present in the cell wall of Gram-negative bacteria, while NOD2 senses muramyl dipeptide (MDP) that is a common PG motif in most bacteria.[18-20] However, though these studies are well accepted, an important mechanism remains to be clarified: How are those partial components of PG processed and delivered for recognition by the intracellular cytosolic sensors, NOD1 and NOD2? It has been shown that extracellular ATP treatment activates both the P2X7 receptor and pannexin-1 to open an endogenous membrane pore, promoting the translocation of MDP to the cytosol.[24] It is not clear, however, whether this happens in vivo during natural bacterial infections. Although one human transporter protein, hPepT1, has been shown to support MDP internalization, other carrier or transporter proteins for PG or its components have not yet been identified.[25]

Several reports show that PG is involved in the pathogenesis of autoimmune diseases such as psoriasis and arthritis.[26,27] Psoriatic skin lesions characterized by hyper-proliferation and altered differentiation of keratinocytes by association with leukocytes infiltrating the dermis and epidermis, which are caused and maintained by populations of $CD8^+$ and $CD4^+$ T cells, respectively.[28,29] Dermal $CD4^+$ T cells in psoriasis patients are continuously activated by streptococcal cell wall components such as PG, which is supposed to be

Table 2. NLRs

Name	Ligands	Signal Activation	Adaptor(s)
NOD1	*meso*-diaminopimelic acid (*meso*-DAP)-containing PG	NF-κB, MAPK	RIP2
NOD2	MDP	NF-κB, MAPK, caspase-1	RIP2, CARD9
NLRC4	Flagellin	Caspase-1	ASC
NLRP1	MDP, anthrax lethal toxin	Caspase-1	ASC
NLRP3	LPS, PG, MDP, flagellin, aerolysin, bacterial RNA, viral DNA, poly(I:C), MSU, CPPD, hemozoin, imidazoquinolin compounds, aluminum salt, urushiol, TNCB, asbestos, silica, UVB	Caspase-1	ASC

a crucial mechanism for establishing psoriatic skin lesions.[26,27] Interestingly, epidermal $CD8^+$ T cells in psoriasis recognize the autoepitopes presented by keratinocytes, which are homologous to peptides derived from streptococcal M protein.[30,31] Taken together, it appears that the presence of streptococci leads to the exacerbation of psoriatic skin lesions through molecular mimicry between autoantigens and bacterial cell wall components and that such autoinflammatory responses are enhanced and maintained by innate immune responses activated by PG.

Arthritis is frequently seen in patients with microbial infections such as rheumatic fever, urinary tract infections, gonorrhea, inflammatory bowel syndrome and Lyme disease. Although the pathogenesis of arthritis associated with such infectious diseases is poorly understood, the common aspect is the presence of bacterial components deposited in the local joint lesions, despite the absence of live bacteria. Among many bacterial products, the administration PG is sufficient to induce aseptic poly-arthritis in mice and the severity of the arthritis correlates with the amount of PG deposited in the joints. Saha et al precisely analyzed PG-induced acute arthritis in several lines of knockout mice and found that PGLYRP-2 and NOD2 are both required for the development of joint lesions. They clearly demonstrated the sequence of arthritis progression: PG activates NOD2 leading to the local expression of PGLYRP-2. PGLYRP-2 then enhances the level of chemokine expression, which facilitates neutrophil recruitment into the tissues, resulting in arthritis.[16]

Proteins

Flagellin is a well-characterized bacterial protein, constituting the polymerized flagellar filament that forms the flagellum, an organelle involved in bacterial motility. Monomeric flagellin has four domains termed D0, D1, D2 and D3.[32,33] While the D0 and D1 domains contribute to polymerization, the D2 and D3 domains are exposed on the surface of the flagellar filament and so, in general, antibodies to these regions develop after infection with flagellated bacteria.[34] Although flagellin is recognized by the acquired immune system as a protein antigen, it is also recognized by the extracellular sensor TLR5 and the intracellular sensors NLRC4 (also termed as IPAF, CARD12 and CLAN) and NLR family apoptosis inhibitory protein (NAIP, also known as NAIP5/BIRC1/NLRB1) (Tables 1 and 2).[35-37] TLR5 localizes to the cell surface and studies using polarized cells such as intestinal epithelia show that it is expressed exclusively on the basolateral surface, acting as a basolateral sensor of flagellated bacteria in the epithelia.[38,39] In addition, studies examining flagellin mutants show that TLR5 recognizes the conserved D1 domain of flagellin that resides inside the flagellar filament, suggesting that TLR5 gets access to the D1 domain of monomeric flagellin once the naïve flagellar filament is depolymerized.[40] Furthermore, a recent study using the human alveolar Type II epithelial cell line A549 shows that activation of TLR5 takes place in the intracellular compartment and is mediated by lipid raft formation.[41] Taken together, these results suggest that the flagellar filaments are transferred into the cell through the cellular endocytic machinery, where they are depolymerized to expose the D1 domain of flagellin, thus facilitating recognition by TLR5. In contrast, the cytosolic sensors NLRC4 and NAIP have been shown to transmit cellular signals for the processing of interleukin-1β (IL-1β) in response to flagellin stimulation.[36,37] Previous studies have shown that the virulence-associated Type III and Type IV secretion systems (T3SS and T4SS) of salmonella and listeria, respectively, facilitate flagellin transfer into the cytosol of the host cell. In fact, strains

of *S. Typhimurium* deficient for T3SS, or *L. pneumophila* deficient for T4SS, could not induce NLRC4-dependent inflammatory responses.[36,37,42,43]

Profilin-like protein is also reported to stimulate innate immunity. Profilin is an actin-binding protein involved in polymerization of actin filaments for cellular locomotion and shape changes. Profilin is found in most eukaryotic cell types, while profilin-like protein is found in the parasite, *Toxoplasma gondii*. Yarovinsky et al showed that a profilin-like protein in *Toxoplasma gondii*, TgPRF, induces production of IL-12 from mouse splenic dendritic cells (DCs) in a TLR11-dependent manner (Table 1).[44] Although a previous study showed that TLR11 also senses uropathogenic *E. coli* infections, the component of *E. coli* responsible for this has not yet been identified.[45]

While TLR5 and NLRC4 recognize bacterial flagellin, a recent study suggested that flagellin-mediated immune activation is involved in the pathogenesis of Crohn's disease (CD).[46] CD is an inflammatory bowel disease caused by immune-dysfunction specific to the intestinal mucosa, which is triggered by bacterial microflora in the intestine. Such dysfunction leads to loss of tolerance to the microflora antigens and disruption of mucosal barrier function, resulting in the development of enteritis and/or colitis.[47] Lunardi et al have shown that anti-flagellin antibodies, which they found by screening a random peptide library with CD patients' sera, also recognize TLR5 as an autoantigen.[46] Interestingly, a portion of such antibodies activate TLR5 per se, thereby inducing activation of monocytes and enhancement of intestinal permeability in CD. This suggests that the generation of such inflammatogenic autoantibodies contribute to the etiology and severity of CD through the activation of intestinal lamina propria DCs that express high levels of TLR5 and control Type 17- and Type 1-T helper cells and IgA-producing B cells.[46,48] In contrast, several groups have identified the *Nod2* gene as the susceptible gene for CD by showing that mutations in the leucine-rich repeat (LRR) domain cause 'gain-of-function' of NOD2 to induce inflammatory responses.[49,50] Although mutations in the *Nod2* gene have been identified in a minority of CD patients, these findings allow us to accept that the pathogenesis of CD is strongly associated with the disruption of homeostasis, which maintains the magnitude of the inflammatory responses against bacterial microflora in the intestine.

Nucleic Acids

Obligate intracellular pathogens replicate within the host cell by utilizing the host cellular machinery. During the early incubation period of viral infections, components of viral particles such as the capsid, envelope and other appendices disappear in the host cell and viruses only exists as their replicating genomes within the cell. Thus, it has been suggested that to detect viral infection and induce antiviral immune responses, intracellular sensors have been developed that recognize peculiar molecular patterns expressed by the genomes and replication byproducts of pathogens. These include GU- or AU-rich sequences, CpG motifs, 5′-triphosphospate RNA, dsRNA and right-handed helical structures of B-form DNA (B-DNA) (Fig. 1).[2] Such molecular patterns of nucleic acids are sensed by diverse family members of TLRs, RLRs and NLRs and, as yet, unidentified sensors, most of which have been shown to play essential roles in eliciting subsequent protective immune responses against pathogens (Tables 1, 2, 3 and Fig. 1). Further analysis of knockout mice and disease-associated gene mutations in humans suggests that these inflammatory sensors are, at least in part, either directly or indirectly involved in the pathogenesis, development and severity of several types of autoimmune diseases.

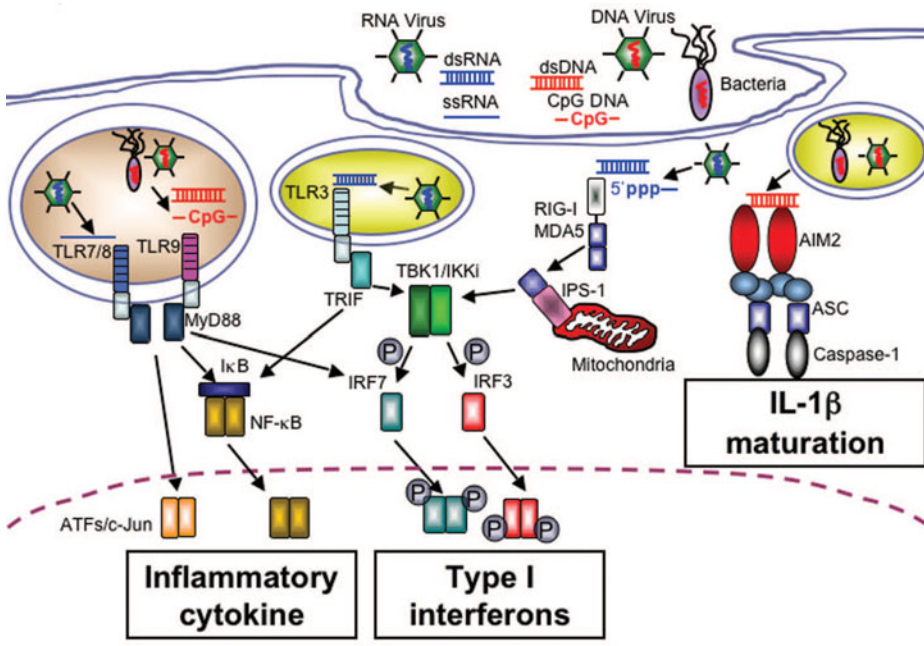

Figure 1. Intracellular sensors for nucleic acids.

INTRACELLULAR SENSORS

TLRs 3, 7, 8 and 9 for the Recognition of Nucleic Acids

It has been suggested that TLR3 preferentially recognizes dsRNA derived from the viral genome presented extracellularly, i.e., the viral genome released from damaged host cells and/or viral particles (Table 1 and Fig. 1).[51] TLR7 recognizes ssRNA viruses such as influenza virus and vesicular stomatitis virus (VSV) as well as synthetic GU-rich ssRNA and synthetic small molecule compounds such as imidazoquinoline, imiquimod and R-848 (Table 1 and Fig. 1).[2] Human TLR8 also recognizes AU-rich ssRNA and R-848. However, the cellular and tissue distribution of TLR8 expression is different from that of TLR7. Human TLR7 is highly expressed in plasmacytoid DCs (pDCs) that produce robust amounts of Type I interferons (IFNs), while no, or low, levels are expressed in myeloid cells. In sharp contrast, the level of TLR8 expression is high in monocytes and in monocyte-derived DCs (mDCs), whereas no, or low, levels are expressed in pDCs.[52] TLR9 recognizes ssDNA expressing unmethylated CpG motifs whose frequency is higher in the genomic DNA derived from pathogens compared with that from vertebrates, suggesting that the CpG motif is a key signature that discriminates the origins of DNA (Table 1 and Fig. 1).[53] Although the nucleic acid-sensors, TLRs 3, 7, 8 and 9 are highly homologous to each other, the subcellular localization pattern of TLR3 is different from that of TLRs 7, 8 and 9. While TLR3 is present in early endosomes even after ligand stimulation, TLRs 7, 8 and 9 reside in the ER under physiological conditions and recruit to late endosomes after ligand stimulation, which is a critical step for triggering

consequent cellular signaling.[54-56] In addition, it has been suggested that TLR3 plays diverse roles in a wide variety of viral infections caused by ssRNA-type viruses such as encephalomyocarditis virus (EMCV), influenza virus, respiratory syncytial virus, West Nile virus and by dsDNA-type viruses such as MCMV and HSV1. This indicates that TLR3 plays an, as yet, uncharacterized role in viral infection other than recognition of dsRNA ligands.[57-62] These TLR nucleic acid sensors mediate virus-induced Type I IFN production. TLR3 allosterically transmits signaling through its interaction with the Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), whereas TLRs 7, 8 and 9 do so through myeloid differentiation factor 88 (MyD88).[63] As is the case for TLR5, TLR9 is also expressed in the intestinal epithelia. Basolateral TLR9 transmits signals through a canonical NF-κB activation pathway by degrading IκB-α, whereas apical TLR9 induces the accumulation of ubiquitinated IκB in the cytoplasm, which prevents activation of NF-κB.[39] This suggests that basolateral TLR9 plays an important role in evoking inflammation in response to the submucosal infection by pathogens, while apical TLR9 acts as a suppressor of inflammation to maintain intestinal homeostasis by recognition of the intestinal microflora.[64]

In addition to the anti-pathogen properties of these TLRs, their in vivo roles in the pathogenesis of autoimmune diseases have been examined. Systemic lupus erythematosus (SLE) is a major autoimmune disease characterized by production of autoantibodies such as anti-DNA antibodies, high levels of Type I IFNs in sera and systemic vasculitis in multiple organs.[65] SLE autoantigens are composed of RNA, DNA and their associated proteins having the potential to activate TLR7 and/or 9, suggestive of the involvement of TLRs 7 and 9 in the etiology and development of SLE.[66] Although abundantly present in the sera, such autoantigens must be transferred to the late endosome to activate TLRs 7 and 9. Also, such nucleic acid-containing autoantigens are vulnerable to the degradation process mediated by extracellular nucleases before associating with TLRs 7 and 9.[54,55] Notably, 'loss-of-function' mutations in DNaseI are found in SLE patients, suggesting that undigested DNA fragments are involved in the pathogenesis of SLE.[67,68] Tian et al found that high mobility group box protein 1 (HMGB1) binds to DNA-immune complexes, facilitating delivery of the complex to the target cells expressing the receptor for advanced glycation end-products (RAGE), which plays a pivotal role in triggering TLR9 activation in response to DNA-immune complexes.[69] Moreover, FcγRIIA and the anti-microbial peptide LL37 produced by keratinocytes and neutrophils in the injured skin, have been shown to contribute to the delivery of autoantigens for efficient activation of TLRs 7 and 9.[70-72] Finally, it has been suggested that production of Type I IFNs induced by autoantigens promotes the differentiation and survival of auto-reactive B cells to augment autoantibody production, which is supposed to be the malignant feedback cycle taking place in SLE patients.[73]

The subcellular localization of TLR9 is also crucial for DNA sensing. The artificial redistribution of TLR9 to the plasma membrane confers cellular activation by self-DNA, suggesting that the TLR9 localization to the endosome, but not to the plasma membrane, is crucial for prohibition of self-DNA recognition and consequent innate immune activation. Previous studies identified three adaptor molecules, Unc93B1, PRAT4A and gp96 that play important roles in the maturation and trafficking of TLRs to their site of action.[55,74-76] Unc93B1 is a trans-membrane protein that controls trafficking of TLRs 3, 7 and 9 from the ER to the endosome. PRAT4A is present in the ER and acts as a regulator of the subcellular distribution of most TLRs, except TLR3. Gp96 is the member of the HSP90 family and resides in the ER where it controls the maturation of TLRs 2, 4, 5, 7 and 9.

Studies of mice deficient for either TLR7 or 9 crossed with a mouse model of lupus provide us with complex evidence of their role in autoimmune disease. Christensen et al showed that deficiency of TLR9 results in exacerbation of disease activity in a mouse model of lupus, despite the level of antibody production against DNA and chromatin being downregulated.[77] In contrast, TLR7 deficiency results in the attenuation of lupus symptoms.[78] Although both TLR7 and TLR9 are expressed in the same cell types, i.e., pDCs and B cells and induce Type I IFN production via similar signaling machinery, a couple of studies show that TLR9-mediated production of IFN-α is suppressed by TLR7 agonists, suggesting that activation of one is counteracted by the other.[79] Furthermore, a pathogenic role for TLR7 in autoimmune disease has been suggested by demonstrating that the Y-chromosome-linked autoimmune accelerator (Yaa) mutation in mice results in an increase in TLR7 expression levels concomitant with the frequent development of lupus-like symptoms.[80,81] In addition, a recent study shows that TLR7-mediated production of Type I IFNs is essential for the development of a mouse model of lupus induced by pristane, while disease progression is independent of cellular internalization of anti-DNA antibodies, which is a critical process for TLR9 activation.[82]

Retinoic Acid Inducible Gene-I (RIG-I)-Like Receptors (RLRs)

TLR nucleic acid sensors recognize a diverse set of virus-associated molecular patterns. However, Type I IFN production is induced by most viral infections independent of these TLRs. Yoneyama et al demonstrated for the first time that a cytoplasmic RNA helicase, RIG-I, senses infection by RNA viruses and induces innate antiviral immune responses mediated by Type I IFN production (Table 3 and Fig. 1).[83] They also identified two additional cytoplasmic helicases structurally similar to RIG-I; melanoma differentiation factor-5 (MDA5) and laboratory of genetics and physiology-2 (LGP2), both of which have been referred to as RLRs (Table 3 and Fig. 1). RLRs localize to the cytosolic compartment, while TLR nucleic acid sensors are trans-membrane proteins associated with the endosome. This suggests that RLRs preferentially sense replicating viral genomes and/or their byproducts in the cytoplasm, while TLRs sense nucleic acids of pathogens taken up into the endosome. All RLRs contain a DExD/H box RNA helicase domain.[83,84]

Table 3. RLRs and other nucleic acid sensors

Name	Ligands	Signal Activation	Adaptor(s)
RIG-1	5'-triphosphate RNA, poly(I:C)	NF-κB, MAPK, IRF3, IRF7	IPS-1
MDA5	dsRNA, poly(I:C)	NF-κB, MAPK, IRF3, IRF7	IPS-1
LGP2	5'-triphosphate RNA, dsRNA, poly(I:C)	Inhibit signaling mediated by RIG-1 and MDA5	?
DAI	dsDNA?	NF-κB, IRF3	TBK1
AIM2	dsDNA	Caspase-1	ASC
Histone H2B	dsDNA	NF-κB, IRF3	CIAO

RIG-I and MDA5, but not LGP2, have two caspase recruitment domains (CARDs). The CARD of these molecules plays an essential role in transmitting downstream signals through homophilic interaction with the CARD of a signaling adaptor molecule, IFN-β promoter stimulator-1 (IPS-1 and also named as MAVS, Cardif and VISA). This leads to the production of Type I IFNs upon viral infection.[83-88]

Knockout mice studies show that RIG-I senses ssRNA virus infections such as influenza virus, VSV and Japanese encephalitis virus (JEV). However, MDA5 plays an important role in inducing innate antiviral immune responses to other types of RNA virus such as encephalomyocarditis virus, Mengo virus and Theiler's virus.[89] Further analysis using synthetic RNA determined that the 5′-triphosphate end is one of the critical elements in the recognition process of inflammatogenic RNAs by RIG-I, but not MDA5.[90,91] Both RIG-I and MDA5 recognize the synthetic dsRNA analog, poly(I:C), which has no phosphates at its 5′-end. RIG-I, however, preferentially recognizes a shorter form of poly(I:C), while MDA5 recognizes a longer form.[92] Interestingly, RIG-I is involved in the recognition of transcribed RNA byproducts of poly(dA×dT)×poly(dT×dA) and Epstein-Barr virus genomic DNA generated by DNA-dependent RNA polymerase III endogenously present in human cells.[93,94] In contrast, another member, LGP2, does not have the CARD and knockdown of LGP2 results in increased production of IFN-β in response to viral infection in vitro, suggesting that LGP2 serves as a negative regulator of RIG-I- and MDA5-mediated immune responses.[95] Consistent with this, LGP2-deficient mice are resistant to VSV infection and higher anti-viral responses induced by poly(I:C). However, these mice are more susceptible to EMCV infection. Furthermore, a recent study demonstrated that V protein of paramyxovirus, a well-characterized suppressor of host innate immune responses, interacts with MDA5 as well as LGP2, but not with RIG-I and interferes with their ATP hydrolysis activity.[96] Taken together, these data suggest that LGP2 modulates innate antiviral responses depending on types of invading viruses.

Accumulating evidence obtained from both basic scientific and clinical research strongly suggests that excessive and chronic inflammatory responses, such as the hyper production of Type I IFNs, are associated with the development of autoimmune diseases. A recent study examining disease-associated single nucleotide polymorphisms (SNPs) in the human genome has revealed that 'loss-of-function' mutations in MDA5 correlate with resistance to Type I diabetes.[97] Although the precise mechanism has not been elucidated, it may suggest the involvement of viral-induced production of Type I IFNs in the pathogenesis of autoimmune isletitis. Cellular homeostasis is, in general, strictly controlled by negative-feedback machinery, as are inflammatory responses. Our studies have revealed that the Atg12-5 conjugate, an essential molecule for the canonical autophagic process, interferes with signaling mediated by RLRs by intercalating into the CARD-CARD interaction between RLR and IPS-1 under physiological conditions, suggesting a noncanonical role for the Atg12-5 conjugate as a suppressor of inflammatory responses.[98,99]

Nucleotide-Binding and Oligomerization Domain (NOD)-Like Receptors (NLRs)

NLRs are categorized as a family of cytosolic inflammatory sensors having structurally conserved domains. Twenty-three members in humans and 34 members in mice make up this family according to a genome-wide analysis of the putative genes and all have several common domains. NACHT (also known as NBD or NOD) was originally identified in NAIP, MHC class II transactivator (CIITA), incompatibility locus protein from *Podospora anserine* (HET-E) and telomerase-associated protein (TP1). Each LLR consists of 20-29

amino acids and the number of LLRs vary in each NLR.[100] The NACHT domain is required for ATP-dependent oligomerization (presumably 6–8-mer formation), which culminates in an ~700 kDa protein complex called an 'inflammasome',[101] while LRRs are supposed to be crucial for interaction with a variety of inter- and intra-molecular domains, as is the case with TLRs for their interaction with ligands.[100] In addition, NLRs have a signaling domain that interacts with downstream adaptor molecules to transmit signals. Three different types of the signaling domain divide NLR family members into subfamilies. An NLRB subfamily member, NAIP, contains the Baculoviral IAP repeat (BIR) domain as a signaling domain. NLRC subfamily members contain the CARD, while NLRP subfamily members contain the pyrin (also known as PAAD, PYD, or DAPIN) domain. Typically, CARD and pyrin domains have six α-helices and signal via homophilic interactions, e.g., CARD–CARD and pyrin–pyrin. Therefore, NLRs interact with downstream adaptor molecules containing CARD and/or pyrin domains: RIP2 contains CARD and interacts with NOD1 and NOD2; CARD9 contains CARD and interacts with NOD2; CARD8 (also known as DACAR/CARDINAL/TUCAN) contains CARD and interacts with NLRP2 and NLRP3 (also known as CIAS1/cryopyrin/Pypaf/NALP3);[102] apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC) contains both CARD and pyrin domain and interacts with NLRP1, NLRP2, NLRP3 and absent in melanoma 2 (AIM2) through the pyrin domain.[103] These adaptors subsequently interact with other CARD-containing molecules, such as caspase-1, leading to NF-κB activation and IL-1β processing during inflammatory responses.

The Inflammasome

The inflammasome is a cytosolic multiprotein complex that acts as an essential platform for activation of several inflammatory caspases, such as caspase-1, responsible for the maturation of the proinflammatory cytokines, IL-1β, IL-18 and IL-33. Inflammasomes are formed and activated once the cell recognizes inflammatory stimuli: (1) pathogen components such as LPS, PG, MDP, flagellin, aerolysin, anthrax lethal toxin, bacterial RNA and viral DNA, (2) inflammatory metabolites and chemicals such as crystals of monosodium urate (MSU), calcium pyrophosphate dihydrate (CPPD) and hemozoin, poly(I:C), R848, aluminum salts, urushiol, trinitrochlorobenzene (TNCB), asbestos and silica and (3) ultraviolet B (UVB).[2,104] These inflammatory substances need to be transferred into the cytosol to initiate the process of inflammasome formation, since addition of extracellular ATP (at millimolar concentrations), or transfection of substances such as nucleic acids, confers the ability of some of the above stimuli to trigger inflammasome responses. In fact, ATP triggers the opening of the nonselective purinergic cation channel P2X7 and the hemichannel pannexin-1 present in the membrane.[105] While potassium efflux and reactive oxygen species are also known to contribute to the activation of inflammasomes, metabolite crystals such as MSU and CPPD can activate the NLRP3 inflammasome in the absence of transducing agents. Activation of caspase-1 and ASC, a hallmark of inflammasome responses, also promotes cell death processes, called 'pyroptosis' and 'pyronecrosis', respectively, which are distinct from apoptosis and necrosis. Pyroptosis proceeds via activation of caspase-1 and is accompanied by DNA damage and plasma membrane breakdown, while pyronecrosis is not accompanied by DNA damage nor caspase activation, but is mediated by ASC and lysosomal enzymes.[106,107] Interestingly, cells seem to undergo either of these two different processes depending on the type and amount of invading bacteria such as shigella, listeria and salmonella. Therefore,

these processes are supposed to promote the inhibition of bacterial dissemination as a cell-autonomous anti-pathogen response.[108,109]

Genetic studies using mice deficient for different NLRs have characterized distinct inflammasomes formed in response to different types of inflammatory stimuli. The NLRC4 inflammasome is processed upon infection of the intracellular pathogens *Salmonella typhimurium*, *Legionella pneumophila*, *Pseudomonas aeruginosa* or *Shigella flexineri*.[36,37,108] NLRC4 senses flagellin present in the cytosol, transmitting signals that activate caspase-1 (Table 2). As described previously, TLR5 also senses flagellin and elicits cytokine production; however, it has been demonstrated that caspase-1 activation and IL-1β production is normally induced in response to flagellin or flagellated bacteria in TLR5-deficient macrophages, suggesting that the NLRC4 inflammasome is specialized to activate caspase-1-dependent IL-1β production. Both MDP and anthrax lethal toxin engage the NLRP1 inflammasome in the caspase-1 activation process, which is not mediated by adaptor proteins such as ASC, suggesting that NLRP1 directly interacts with caspase-1, as is the case for NLRC4 (Table 2).[101,110,111] The NLRP3 inflammasome promiscuously mediates IL-1β production induced by a diverse set of inflammatory stimuli such as LPS, MDP, nucleic acids, metabolite crystals, chemical irritants and UVB (Table 2).[112,113] In contrast to the other inflammasomes, the efficient activation of the NLRP3 inflammasome, by certain stimuli, which is mediated by P2X7 and pannexin-1 as described previously, requires additional treatment with millimolar concentrations of ATP.[24] IL-1β production is crucial for eliminating fungal infection, while exacerbating malarial infection. Although the direct interaction between NLRs and their cognate stimuli has not yet been determined, recognition of *Candida albicans* (*C. albicans*) by NLRP3 is independent of either P2X7 or potassium efflux. Genetic analyses elucidated that Dectin-1, a member of the CLR family, senses *C. albicans* and transmits signals through the tyrosine kinase, Syk and an adaptor, CARD9, thereby activating the NLRP3 inflammasome without direct association between *C. albicans* components and NLRP3.[114] Similarly, hemozoin, a byproduct of malarial infection, activates NLRP3 through Syk and Lyn.[115] Activation mechanisms of the NLRP3 inflammasome engaged by aluminum salts, asbestos and silica seem to be very complicated. The requirement of LPS for optimal aluminum salt-mediated activation of the NLRP3 inflammasome is controversial.[116-118] Recently, AIM2 was found to sense cytosolic dsDNA and form a distinct inflammasome with ASC (Table 3 and Fig. 1).[119-122] AIM2 is a member of hematopoietic interferon-inducible nuclear proteins with a 200-amino acid repeat (HIN-200) family that contains a pyrin domain at the NH_2 terminus. Among the molecules that have been characterized as inflammasome components, AIM2 is the only 'sensor' to directly interact with a cognate stimulus, i.e., dsDNA, through a HIN-200 domain present at the COOH terminus. Roberts et al also found that another member of the HIN-200 family, p202, binds to cytosolic dsDNA.[122] According to their results, p202 appears to be a negative regulator of the AIM2 inflammasome, because p202 lacks a pyrin domain and knockdown of p202 results in increased levels of caspase-1 activation in response to dsDNA stimulation. Interestingly, recent reports show that leukocytes from SLE patients express higher levels of AIM2 relative to control individuals, suggesting the involvement of AIM2 in SLE pathogenesis.[123] There also seem to be other bacterial components and corresponding NLR inflammasomes that trigger ASC- and caspase-1-mediated cellular activation processes. *Francisca tularensis* is a Gram-negative bacteria and replicates in the host cytosol after escape from the phagosome. Mariathasan et al showed that mice deficient

for either ASC or caspase-1, but not for NLRC4 and NLRP3, are more susceptible to *Francisca tularensis* infection.[124] Atg16L, a regulator of the autophagic process, was found to be a negative regulator of LPS-induced inflammatory responses mediated by inflammasomes, which is associated with the intestinal inflammatory responses in a mouse model of dextran sodium sulfate-induced colitis and is consistent with the observation of various 'loss-of-function' mutations in the *Atg16l* gene in patients with CD.[125-128]

Recent research on NLR family members has focused on SNPs associated with inflammatory diseases. A variety of 'loss-of-function' and 'gain-of-function' mutations within NLR family members and their adaptor molecules have been identified in patients with autoinflammatory diseases, also known as hereditary periodic fever syndrome. Most of these diseases manifest with systemic skin rashes, abdominal pain, arthritis and prolonged episodes of periodic fever with no apparent infection. SNPs associated with familial Mediterranean fever were found in the *Pyrin* gene, whose native function is a negative regulator of inflammasomes through the interaction with ASC and caspase-1. 'Loss-of-function' mutations in mevalonate kinase, which regulates the metabolic pathways involved in cholesterol synthesis, are frequently detected in patients with hyper IgD with periodic fever, also known as mevalonic aciduria. Although a precise inflammatogenic role of this kinase remains to be elucidated, some metabolites accumulated in the cells might have the potential to stimulate inflammatory sensors. 'Gain-of-function' mutations in NOD2 found in the NACHT domain are associated with autosomal dominant Blau syndrome, which manifests as systemic granulomatous lesions. A variety of mutations of the *Nlrp3* gene have been seen in patients with familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome and chronic infantile neurologic cutaneous and articular syndrome (CINCA), also known as neonatal onset multisystem inflammatory disease (NOMID).[129] In this series of autosomal dominant autoinflammatory diseases, the latter has additional symptoms such as deafness, uveitis and neurologic dysfunction. Mutations in the *Nlrp12* gene have also been found in several patients with hereditary periodic fever syndrome.[130] Most symptoms of patients with such autoinflammatory diseases are improved by treatment with anti-IL-1β e.g., Anakinra® (Amgen), Rilonacept® (Regeneron) and canakinumab (Novartis), suggesting that IL-1β is a pivotal pathogenic factor in these autoinflammatory diseases.

Cytosolic dsDNA Sensors That Trigger Production of Type I IFNs

While AIM2 senses dsDNA in the cytosol triggering production of IL-1β, TLR9 recognizes unmethylated CpG motifs in ssDNA in the endosomal compartment and induces the production of versatile proinflammatory cytokines, especially Type I IFNs in pDC. In human cells, RIG-I senses the transcribed RNA byproducts of DNA templates that are generated by RNA polymerase III (as is case for poly(dA·dT)·poly(dT·dA) and EBV genomic DNA), while we found that histone H2B acts as a sensor of cytosolic dsDNA, triggering cellular signaling through the newly identified adaptor, COOH-terminal importin 9-related adaptor, which organizes histone H2B and IPS-1 (CIAO) in human cells (Table 3).[131] However, accumulating evidence strongly suggests that other inflammatory sensors are essential for triggering Type I IFN production in response to cytosolic dsDNA, for example B-DNA. DNase II-deficient mice show the accumulation of undigested self-DNA in the cytoplasm of macrophages, triggering high levels of Type I IFN production, thereby developing lethal anemia in utero.[132] Mice deficient in both the *Dnase II* and *Ifnrα/β* genes are born healthy, but develop rheumatoid arthritis (RA)-like symptoms.[133] Furthermore, it has also been shown that deficiency, or

mutation, of other *Dnase* genes such as *Dnase I* and *III* (also known as the *Trex* gene) results in the development of either systemic, or local, autoimmune diseases similar to SLE, RA or inflammatory myocarditis.[67,68,134-136] Serum levels of IFN-α correlate with disease activity in SLE and mutations in the *Dnase I* gene are frequently found in SLE patients. Taken together, these observations suggest that the accumulation of aberrant self-DNA and exaggerated responses to this DNA, correlate with the etiology of autoimmune diseases (Fig. 2). Further studies have confirmed that cytosolic B-DNA induces higher levels of Type I IFN production in a wide variety of cell types compared with the Z-form of dsDNA through the cellular signaling axis directing TANK-binding kinase-1 (TBK1) and its substrate, interferon regulatory factor 3 (IRF3), independent of TLRs, RLRs and NLRs.[137] This phenomenon is biologically relevant to protective immune responses against infection by pathogens such as vaccinia virus and *Listeria monocytogene* and to the immunogenicity of DNA vaccines.[137-139] One putative candidate receptor for the recognition of cytosolic dsDNA and the subsequent triggering of Type I IFN production is Z-DNA binding protein 1 (ZBP1), also known as DNA-dependent activator of IFN-regulatory factors (DAI), which associates with both TBK1 and IRF3 (Table 3).[140] However, DAI-deficient mice responded normally to cytosolic dsDNA stimulation, suggesting that ZBP1 acts as a DNA sensor in a cell-type specific fashion.[139] Ishikawa et al demonstrated that stimulator of interferon genes (STING, also known as TMEM173, MPYS and MITA) plays a critical role for the production of Type I IFNs in response to cytosolic dsDNA.[141] STING is an ER-resident transmembrane protein and translocates from the ER, together with TBK1, to the Sec5-containing vesicles upon dsDNA stimulation, suggesting that STING is an essential trafficking guide for TBK1 to initiate IRF3-dependent Type I IFN production.

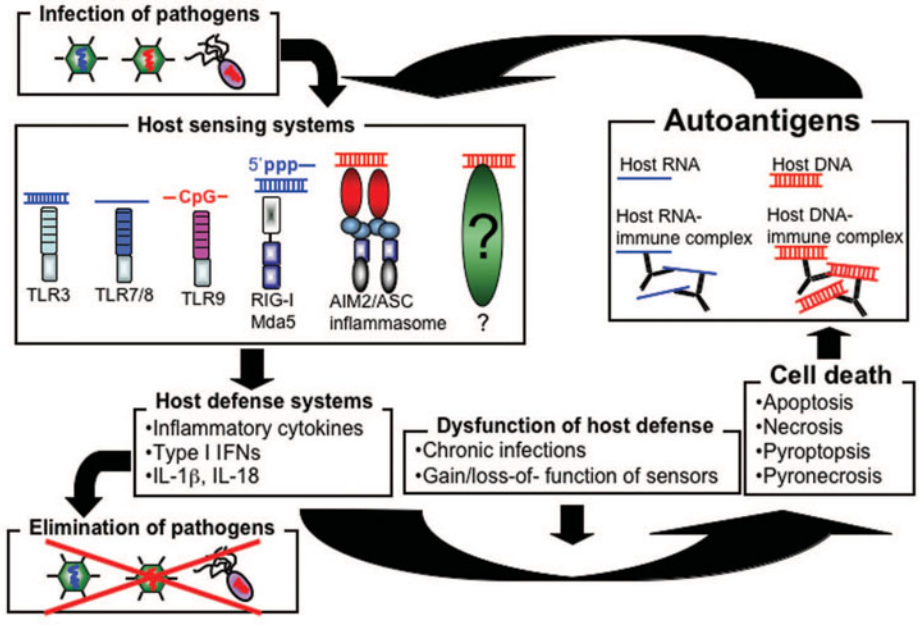

Figure 2. Vicious cycle in autoimmune disease caused by nucleic acids.

CONCLUSION

A number of inflammatory sensors and their interactive adaptor molecules have been identified and characterized. Technological progress in both the basic sciences and clinical research also provide us with a great opportunity to develop novel methodologies, especially for laborious investigations such as genome-wide SNP analysis. We have now started to obtain comprehensive knowledge about malfunctions of such sensors that result in compromised resistance to pathogens as well as vulnerability to autoinflammatory and autoimmune diseases. Further progress in both basic science and clinical research into inflammatory sensors will grant us knowledge and ideas to develop novel diagnostic and therapeutic applications that may benefit patients with inflammatory disorders.

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CHAPTER 6

NONSELF PERCEPTION IN PLANT INNATE IMMUNITY

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Abstract: The ability to distinguish 'self' from 'nonself' is the most fundamental aspect of any immune system. The evolutionary solution in plants to the problems of perceiving and responding to pathogens involves surveillance of nonself, damaged-self and altered-self as danger signals. This is reflected in basal resistance or nonhost resistance, which is the innate immune response that protects plants against the majority of pathogens. In the case of surveillance of nonself, plants utilize receptor-like proteins or -kinases (RLP/Ks) as pattern recognition receptors (PRRs), which can detect conserved pathogen/microbe-associated molecular pattern (P/MAMP) molecules. P/MAMP detection serves as an early warning system for the presence of a wide range of potential pathogens and the timely activation of plant defense mechanisms. However, adapted microbes express a suite of effector proteins that often interfere or act as suppressors of these defenses. In response, plants have evolved a second line of defense that includes intracellular nucleotide binding leucine-rich repeat (NB-LRR)-containing resistance proteins, which recognize isolate-specific pathogen effectors once the cell wall has been compromised. This host-immunity acts within the species level and is controlled by polymorphic host genes, where resistance protein-mediated activation of defense is based on an 'altered-self' recognition mechanism.

INTRODUCTION: THE AGE OLD QUESTION OF "WHAT IS SELF?"

The ability to distinguish self from nonself is the most fundamental aspect of an immune system. Although expressing an apparent passivity associated with their sedentary lifestyle and, being simultaneously exposed to evolving pathogens as well as environmental stresses, plants have evolved a unique metabolic plasticity that allows them to perceive pathogens and

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unleash effective defense strategies.[1] Since a plant's entire immune response is not based on an adaptive/acquired system as seen in mammals, it would appear to be an evolutionary ancient defense mechanism able to genetically distinguish 'self' from 'nonself' and result in downstream cascades to counter pathogen attack or eliminate the pathogen. The question therefore arises how such a system could perceive so many diverse pathogen-derived signals when the mechanism originated before the evolving variables in potential invaders and when plants are unable to acquire or adapt specifically, whilst simultaneously being exposed to environmental stresses? In this context it is important to define 'self' and 'nonself' during a defense response and to focus on how plants distinguish between self and nonself/damaged-self/altered-self using the perception and signal transduction mechanisms available.

Self

The philosophical debate regarding self appears to be mute when considering plant cells that are surrounded by a cell wall. In this biological context everything originating from within the wall is self and all molecules of foreign origin outside the cell wall are nonself. This definition applies to the self recognition (i.e., the identification of the same genetic homology) that is observed in the specific example of pollen recognition in the same species, from the same plant during self incompatibility (SI).

The established system of self/nonself recognition in SI systems utilizes receptor-ligand type interactions to perceive, recognize and reject incompatible pollen when the same S-haplotype is expressed by both pollen and pistil. Thus, SI prevents self-pollination. Although SI responses are generally comprised of a self/nonself recognition process, SI systems have evolved independently (as exemplified in the Solanaceae, Papaveraceae and Brassicaceae) and do not utilize one molecular mechanism exclusively. Rather, SI encompasses a collection of divergent cellular responses leading to pollen rejection.[2-4] In Brassicaceae, the recognition of self and self-incompatibility are components of a receptor-ligand based mechanism that utilizes an S receptor kinase (SRK) to perceive and reject self-pollen. SRK is an S-domain RLK, which in turn is part of the RLK family, some members of which represent PRRs involved in the detection of P/MAMPs (discussed below). S-domain RLKs also occur in species that do not exhibit self-incompatibility and exhibit up-regulated gene expression profiles in response to wounding and pathogen recognition, suggesting that they may fulfill a role in perception and/or defense. Although evolution may have driven expansion of certain RLK families to serve roles in particular physiological processes, this may not exclude these receptor types from functioning in different programs.[1] The evolutionary origins of plant SI, centered on the hypothesis that SI evolved from a defense pathway, was discussed by Nasrallah.[5] Parallels exist where plant SI and plant immunity have similar outcomes, such as the elimination of undesirable cells or organisms. Interestingly, the process of pollen rejection is closely associated with rapid and effective proteolytic events, including the ubiquitin-proteasome pathway and the vacuolar sorting pathways; processes that are also of great importance in plant defense. SI is not further discussed and the reader is referred to Zhang et al[4] for a recent review on the subject and to Sanabria et al[1] for the conceptual and mechanistic links between microbial recognition and self-incompatibility.

Nonself

The definition for self, as described above, is not entirely sufficient. However, with regards to immune responses, it appears to be more constructive to define what plants

perceive as nonself. In the same biological context, nonself should be redefined as a biological molecule/organism that the plant perceives to be (i) of different origin, e.g., pathogenic species recognized during a defense response (discussed in detail below) or (ii) of different state, e.g., altered or damaged cellular components recognized during routine 'house-keeping' maintenance and regulatory metabolism (discussed in detail below).

THE CONSTANT BATTLE BETWEEN SELF AND NONSELF: PRINCIPLES OF IMMUNITY

During co-evolution with pathogens, plants have evolved systems to distinguish self and nonself based on the detection of P/MAMPs. PAMPs may be described as invariant epitopes within molecules that are fundamental to the pathogens' fitness. They are widely distributed among different microbes, absent from the host and recognized by a wide array of potential hosts (Table 1). PAMP-triggered immunity (PTI), constitures the first line of inducible defense against infectious disease.[10,11] In response, many Gram-negative bacteria inject effector proteins, previously termed avirulence (Avr) proteins, into the host cells, through Type III secretion systems, which suppress the P/MAMP-mediated immune responses.[12-15] As a counter move, plants have co-evolved specific resistance (R) proteins to recognize the effector proteins.[10,13,14] This then leads to effector-triggered immunity (ETI) and/or the hypersensitive response (HR) representing a form of programmed cell death.[14,16] Moreover, host inhibition of bacterial virulence effectors can trigger immunity to infection.[17] And so the cycle continues, thus perpetuating the constant battle between pathogens and plants, described as an "arms race between pattern recognition receptors in plants and effectors in microbial pathogens."[18] (See Fig. 1.)

Innate Immunity

Plants possess an innate immune system consisting of PTI and ETI that detects and defends against potentially dangerous microbes.[14,16,18,19] It draws its origins from a phylogenetically ancient form of immunity that is common to all Metazoa and Viridiplantae,[20] which precedes SI.[1,21] The innate immune system in plants is unable to acquire or specifically adapt like the animal adaptive immune system.[22] Rather, it relies on a spectrum of predetermined receptors expressed in nonmobile cells. These receptors may be proteins with similar morphologies or proteins that are able to multi-task between different functions, in order to compensate for the inability to acquire antibodies.

Basal Resistance, Nonhost and Host Immunity

Nonhost immunity refers to an evolutionary ancient, multilayered resistance mechanism consisting of constitutive and inducible components.[23] Non-host immunity remains operative even in susceptible plants to limit pathogen growth and is associated with the release of molecules (ligands or elicitors) derived from the pathogen.[24] In addition, it is also associated with peptides or oligouronides originating from hydrolytic events during the interaction between plants and pathogens and acting as endogenous elicitors, analogous to the 'danger signals' of the vertebrate immune system, such as heat-shock proteins, nucleotides, reactive oxygen intermediates, extracellular-matrix breakdown products, neuromediators and cytokines.[25] Basal resistance is the innate immune response that protects plants against

Table 1. Summary of selected PAMPs* recognized by plants[6,7]

PAMP	Plant	Pathogen(s)	Active Epitope
β-glucans	Rice Legume	Fungi (*Pyricularia oryzae*), Oomycetes (*Phytophthora* spp.), Brown algae	Tetraglucosyl glucitol, branced hepta-β-glucoside, linear oligo-β-glucosides
Cerebrosides A, C	Rice	Fungi (*Magnaporthe* spp.)	Sphingoid base
Chitin/Chitosan	Arabidopsis, rice, tomato and wheat	All fungi	Chitin oligosaccharides (degree of polymerization > 3)
Cold shock protein	Solanaceae	Gram-negative bacteria, Gram-positive bacteria	RNP-1 motif (amino terminal fragment of cold shock protein)
Elongation factor (EF-Tu)	Brassicaceae	Gram-negative bacteria	elf18 (N-acetylated amino terminal fragment of EF-Tu
Ergosterol	Tomato	All fungi	
Flagellin	Most plants (except rice)	Gram-negative bacteria	flg22 (amino terminal fragment of flagellin)
Harpin (HrpZ)	Various plants	Gram-negative bacteria (Pseudomonads, *Erwinia*)	Undefined
Invertase	Tomato	Yeast	N-mannosylated peptide (fragment of the peptide)
Lipid-transfer proteins (elicitins)	Tobacco	Oomycetes (*Phytophthora* spp., *Pythium* spp.)	Undefined
LPS	Arabidopsis, pepper and tobacco	Gram-negative bacteria (Xanthomonads, Pseudomonads, *Burkholderia* spp.)	Lipid A/Inner core/Glucosamine backbone/ Combinations of motifs?

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Table 1. Continued

PAMP	Plant	Pathogen(s)	Active Epitope
Necrosis-inducing protein	Many dicot plants	Bacteria (*Bacillus* spp.), Fungi (*Fusarium* spp., *Verticillium* spp.) Oomycetes (*Phytophthora* spp., *Pythium* spp.)	Undefined
Peptidoglycan	Arabidopsis and tobacco	Gram-positive bacteria	Muramyl dipeptide
Rhamnolipids**	Grapevine	*Pseudomonas* species	Mono-/dirhamnolipids
Siderophores***	Tobacco	Undefined	*Pseudomonas fluorenscens*
Sulphated fuctans	Tobacco	Brown algae	Fucan oligosaccharide
Transglutaminase	Parsley and potato	Oomycetes (*Phytophthora* spp.)	Pep-13 motif (surface-exposed epitope of the transglutaminase)
Xylanase	Tobacco and tomato	Fungi (*Trichoderma* spp.)	TKLGE pentapeptide (suface-exposed epitope of xylanase)

*PAMPs may be described as invariant epitopes within molecules that are fundamental to the pathogens' fitness. They are widely distributed among different microbes, absent from the host and recognized by a wide array of potential hosts. **Varnier et al,[8] ***van Loon et al.[9]

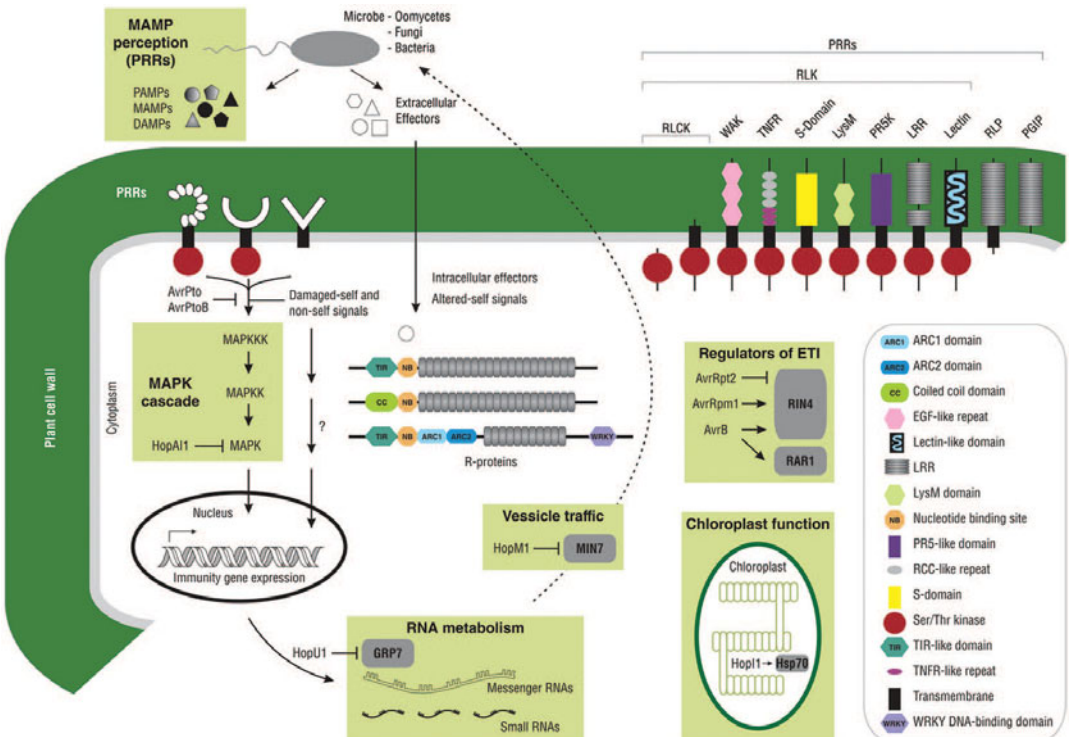

Figure 1. Perception systems for damaged-self, altered-self and nonself signals in plants. Pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and pathogen-derived effectors are perceived as nonself danger signals. Extracellular P/MAMPs originating from prototypical microbes and DAMPs generated by their enzymes, are recognized via pattern recognition receptors (PRRs). Pathogen effectors injected into the cell are detected, directly or indirectly, by intracellular resistance (R) proteins and associated proteins. The domain organization of typical extracellular and intracellular receptors in plants is shown.[21,141] Successful pathogens have acquired the ability to interfere or suppress generated signals and to circumvent plant defenses (e.g., AvrPto, AvrProB, AvrRpt2, HopAI1, HopU1, HopM1, HopI1).[146] Six possible targets for interference or suppression include: (1) MAMP perception through PRRs, (2) the MAPK cascade, (3) RNA metabolism, (4) vesicle trafficking, (5) regulation of PTI and (6) chloroplast function (adapted from Boller and He,[18] Boller and Felix,[19] as well as Tör et al[147]).

the majority of pathogens. In addition, another, more recently evolved form of immunity is operative in plants. Host-immunity acts within the species level and is controlled by polymorphic host genes, such as the *R* genes, the products of which interact, directly or indirectly, with secreted avr proteins or effectors of the pathogen.[20,26]

Current Models of Plant Immunity; PAMP- vs Effector-Triggered Immunity

Two branches to the plant immune system are now recognized: PTI and ETI, associated with different perception mechanisms in the host.[14,16]

PTI

PTI refers to the inducible responses activated upon recognition of conserved P/MAMPs such as lipopolysaccharides (LPS), peptidoglycan and flagellin of bacteria, or chitin and glucan of fungi, etc. It has been reported that P/MAMPs can interact, either directly or indirectly, with each other as well as the cell wall matrix of the host. This interaction would influence the speed, magnitude, versatility as well as the organization of the defense response.[27] Recent evidence indicates that some identified PRRs are members of the RLK family, e.g., the flagellin receptor (FLS2) and elongation factor Tu (EF-Tu)-receptor (EFR).[28,29] The work regarding flagellin and EF-Tu, indicates that there must be a requirement for numerous such signal perception and transduction systems in plants able to recognize all potential invaders.[29,30] Indeed, sequencing of the *Arabidopsis thaliana* genome has revealed the presence of >400 RLK sequences with various receptor configurations, of which those containing a leucine-rich repeat (LRR) in the extra-cellular domain constitute the largest group with 216 members.[30,32] The diversity and large number of plant RLKs suggest that they may be involved in the perception of a wide range of stimuli. Other PRRs are also found amongst nonRLK proteins such as *Glycine max* beta-glucan elicitor binding protein (GmGBP), *Lycopersicon esculentum* ethylene-inducing xylanase (LeEIX2) and chitin elicitor-binding protein (CeBIP) for perception of beta-glucans (soybean), xylanase (tomato) and chitin fragments (rice) respectively.[33-35]

ETI

The second branch of the plant immune system, ETI, in contrast acts mostly inside the cell, using polymorphic resistance proteins encoded by *R* genes, reviewed by Liu et al[36] as well as Tameling and Takken.[37] *R* gene-mediated resistance is a form of host-immunity activated upon recognition of an avirulence factor, a pathogen effector protein that elicits resistance (via direct recognition of the effector by the plant, or via their action on targeted host molecules, i.e., indirectly). Since *R* genes act in a race-specific manner, there are few that confer broad-spectrum resistance.

'Zigzag' Model and ETS

A 'zigzag' model to illustrate the quantitative output of the plant immune system as well as to illustrate the evolutionary relationship between PTI and ETI was recently proposed.[16] In Phase 1, P/MAMPs are recognized by PRRs, resulting in PTI that can stop further colonization. In Phase 2, successful pathogens deploy effectors that contribute

towards pathogen virulence. When effectors suppress or interfere with PTI, it results in effector-triggered susceptibility (ETS).[15,38] In Phase 3, an effector is specifically recognized by an R protein, which results in ETI. ETI is regarded as an accelerated and amplified PTI response, which results in disease resistance and may lead to an HR at the infection site. In Phase 4, natural selection drives pathogens to avoid ETI. This is achieved by either shedding or diversifying the recognized effector gene, or by acquiring effectors that suppress ETI. Thereafter, natural selection results in the evolution of new *R* specificities leading to ETI being triggered again.

Down-Stream Signaling

Similar to PTI, R-protein triggered immunity is also linked to reactive oxygen intermediate accumulation and activation of defense genes, but the two responses differ quantitatively and kinetically. The outcome of ETI can lead to programmed cell death of the host cell in the form of the HR in order to limit the spread of the infection. It results in local induced/acquired resistance (LAR), acting at the site of infection to contain the invader and systemic acquired resistance (SAR), which induces defenses in distal, non-infected parts of plants after activation of local resistance. Due to the fact that PTI and ETI have similar output responses, it is possible that the downstream signaling pathways converge.[39] It should be noted that SAR has also been demonstrated to be induced by recognition of PAMPs like LPS[40] and that certain PAMPs like flagellin and flg22 can cause an HR.[41] Both salicylic acid (SA) and jasmonic acid (JA) are required for P/MAMP-induced defense responses.[42] P/MAMP, as well as effector-triggered processes are linked to SA pathways, therefore, SA-mediated responses may be an important part of *R* gene-mediated defense.[43]

BIOCHEMISTRY OF PERCEPTION AND RECOGNITION: NONSELF DETECTION

Perception of Pathogens by Plants

The ability to monitor microbial presence at the cell surface is essential for plant defense mechanisms. Plant innate immunity is activated either upon perception of P/MAMPs by PRRs or upon recognition of pathogen race-specific effector molecules by R protein-mediated processes (Fig. 1). Recognition of potential pathogen-derived molecules or pathogen activity *in planta*, results in signal initiation and signal transduction, culminating in the activation or de-repression of defense-associated genes. The recognition specificities in the different kingdoms probably arose independently in order to recognize highly conserved molecules.[14,16]

The Plant Cell Wall as a Sensor of Integrity

The structure of the plant cell wall distinguishes it from all other eukaryotic cells. It represents the first barrier to an invading pathogen. If the invasion is halted, cellular damage is minimized and no other defensive actions are required. In this context the plant cell wall is not only a rigid or static structure used for mechanical support; it exists as a highly dynamic and responsive structure in a relationship with the plasma membrane and cytoskeleton, where the external and internal environments are joined and where

information from external stimuli is relayed.[44] The plant cell is able to perceive changes to the cell wall, be responsive and adapt with regards to growth and development, as well as stresses, e.g., wounding and pathogen attack, which was reviewed by Humphrey et al.[44]

Pathogen attack may lead to cell damage and influence the cell wall integrity. When the cell wall responds to stress or change, it may be due to the recognition of its 'damaged-self', through damage-associated molecular pattern molecules (DAMPs). The stress or change is perceived by a sensor or sentinels and the plant responds to the change in a defensive manner. An example of recognition of the plants' 'damaged-self' is when pathogen-secreted or endogenous plant polygalacturonases or pectate lyases cause enzymatic degradation of pectin in the plant cell wall, i.e., an altered/damaged state of self. Here, the polygalacturonase-inhibiting protein, an extracellular LRR R protein, interacts to generate oligogalacturonides, which are perceived by a sentinel in order to generate a signal, triggering defense related responses.[45-47] Other potential DAMPs include cellodextrins and cutin monomers, originating as degradation products from the plant cell wall cellulose and cutin layers. Plants can perceive modifications of the cuticle and activate a multi-factorial defense response.[48] However, sentinels that alert the plant to activate defense responses in response to DAMPs has only recently been explored.

In addition to signals generated due to cell wall degradation, conditions that lead to a decrease in cellulose content (e.g., due to loss of function mutations in cellulose synthase (*CESA)* or chemical inhibition of cellulose synthase/cell wall synthesis) are associated with a corresponding increase in defense-associated cell wall strengthening through lignin and callose synthesis.[49-52] This implies a feedback mechanism involving sensors of wall integrity. In addition, these conditions cause constitutive expression of genes associated with JA or ethylene signalling.[53] The synthesis of these hormones is usually associated with responses to pathogens, wounding and drought.[54-56]

The protein components in the cell wall probably play a determining role in perception and these include a variety of potential sensors. Arabinogalactan proteins (AGPs) are regarded as potential sensors of wall integrity.[44] AGPs are glycosylphosphatidylinositol (GPI)-anchored proteins (GAPs). GAPs may play a role in cell surface signaling, adhesion, matrix remodeling and pathogen response.[57] In addition, leucine-rich extensin (LRX) proteins that bind to the cellulose microfibrils, have also been identified as potential cell wall sensors. The wall-associated kinases (WAKs) are the best characterized of the potential cell wall receptors and are ideally situated for sensing and signaling from the cell wall.[58] WAK expression can be induced in response to pathogen attack and, being able to bind pectin fragments and oligogalacturonides,[59] may serve as potential sensors of damaged-self. Other RLKs found associated with the cell wall include lectin receptor kinases, a subset of which are found in plasma membrane-cell wall adhesions and proline-rich extensin-like receptor kinases (PERKs) involved in sensing of cell wall damage due to wounding by pathogens.[60] THESEUS1, a receptor kinase, is a new candidate for sensing cell wall integrity, but has not been proven to play a role in defense.[44] Furthermore, various plasma membrane proteins with extracellular domains, such as RLPs and RLKs important in relaying information from external stimuli (discussed below), interact with and within the wall matrix.

Sentinels of Nonself: Pattern Recognition Receptors

Plants have evolved a large range of potential immune receptors (refer to Tables 2-5) that recognize P/MAMPs as determinants of nonself, or mediate effector perception in the form of 'sentinels'. Sentinels may contain pattern recognition domains combined with

Table 2. Plant LRR-RLKs associated with plant-microbe interactions, innate immunity and defense

Gene	Plant	Class	Type	Function	Perception	Putative Ligands	Ref.
CURL3	*Solanum esculentum*	LRR	RD-kinase	Putatively systemin perception during wounding; possibly brassinosteroid perception *Orthologue of SR160	Nonself recognition: altered self	Systemin BL	107
DIPM1 to 4	*Malus x domestica*	LRR	Non-RD-kinase	Disease resistance and plant-pathogen interaction signalling	Nonself recognition: different origins	DspA/E	108
EFR	*Arabidopsis thaliana*	LRR	Non-RD-kinase	Disease resistance and plant-pathogen interaction signalling	Nonself recognition: different origins	EF-Tu (elf18)	29
ERECTA	*Arabidopsis thaliana*	LRR	RD-kinase	Resistance to *Ralstonia solanacearum*	Nonself recognition: different origins		109
FLS2	*Arabidopsis thaliana*	LRR	Non-RD-kinase	Flagellin perception	Nonself recognition: different origins	flg22	28
HAR1	*Lotus japonicas*	LRR	RD-kinase	Nodule development during nitrogen fixation symbiosis *Orthologue of NARK	Symbiotic relationship, recognition of different origins		110

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Table 2. Continued

Gene	Plant	Class	Type	Function	Perception	Putative Ligands	Ref.
LRPKm1	*Malus x domestica*	LRR	RD-kinase	Disease resistance and plant-pathogen interaction signalling. Highly induced expression during incompatible reactions and lower induction during compatible interactions with *Venturia inaequalis*	Nonself recognition: different origins		111
Nork	*Medicago sativa*	LRR	Non-RD-kinase	Bacterial symbiosis. Root nodule and mycorrhiza formation *Orthologue of SYMRK	Symbiotic relationship, recognition of different origins		112
PEPR1	*Arabidopsis thaliana*	LRR	RD-kinase	Activation of defence and possible positive feedback loop mechanism to amplify PAMP-induced responses.	Nonself recognition: altered self	AtPep1	113
SIRK	*Arabidopsis thaliana*	LRR	RD-kinase	Up-regulated during senescence and pathogen challenge	Nonself recognition: different origins		114
SR160	*Lycopersicon esculentum*	LRR	RD-kinase	Putatively systemin perception during wounding; possibly brassinosteroid perception *Orthologue of CURL3	Nonself recognition: altered self	Systemin BL	115
SYMRK	*Lotus japonicus*	LRR	RD-kinase	Bacterial symbiosis. Root nodule and mycorrhiza formation *Orthologue of Nork	Symbiotic relationship, recognition of different origins		116
Xa21 and Xa26	*Oryza sativa*	LRR	Non-RD-kinase	Specific disease resistance to *Xanthomonas oryzae* pv *oryzae*	Nonself recognition: different origins	AvrXa21 elicitor	117, 118

Table 3. Non-LRR RLKs and RLPs associated with plant-microbe interactions, innate immunity and defense

Gene	Plant	Class	Type	Function	Perception	Putative Ligands	Ref.
At-RLK3	*Arabidopsis thaliana*	DUF-26	RD-kinase	Expressed under oxidative stress and pathogen attack	Nonself recognition: altered self		119
CERK1	*Arabidopsis thaliana*	LysM	RD-kinase	Chitin elicitor signalling *Homologue of LysM RLK1	Nonself recognition: different origins	chitin	120
CHRK1	*Nicotiana tabacum*	Chitinase	RD-kinase	Induced by tobacco mosaic virus, *Phytophthora parastitica*	Nonself recognition: different origins		79
LecRK-a1	*Arabidopsis thaliana*	Lectin	RLP	Expressed during wounding	Nonself recognition: altered self		121
LRK10	*Triticum aestivum*	'Other'/S-domain	Non-RD-kinase	Specific resistance to wheat rust fungi	Nonself recognition: different origins		122, 123
LYK3	*Medicago truncatula*	LysM	RD-kinase	Involved in early events during nitrogen fixation symbiosis (symbiotic interactions) *Orthologue of NFR1	Symbiotic relationship, recognition of different origins	Nod factors	124
LYK4	*Medicago truncatula*	LysM	RD-kinase	Involved in early events during nitrogen fixation symbiosis (symbiotic interactions)	Symbiotic relationship, recognition of different origins	Nod factors	124
LysM RLK1	*Arabidopsis thaliana*	LysM	RD-kinase	Chitin elicitor signalling *Homologue of CERK1	Nonself recognition: different origins	chitin	125
NARK	*Glycine max*		RD-kinase	Nodule development during nitrogen fixation symbiosis *Orthologue of HAR1	Symbiotic relationship, recognition of different origins		126

continued on next page

Table 3. Continued

Gene	Plant	Class	Type	Function	Perception	Putative Ligands	Ref.
NFR1	*Lotus japonicas*	LysM	RD-kinase	Involved in early events during nitrogen fixation symbiosis (symbiotic interactions) *Orthologue of LYK3	Symbiotic relationship, recognition of different origins	Nod factors	127
NFR5	*Lotus japonicas*	LysM	RD-kinase	Involved in early events during nitrogen fixation symbiosis (symbiotic interactions) *Orthologue of SYM10	Symbiotic relationship, recognition of different origins	Nod factors	128
PBS1	*Arabidopsis thaliana*	RLCK	RD-kinase	Specific resistance to *Pseudomonas syringae* pv *phaseolicola*	Nonself recognition: different origins		129
PERK1	*Brassica napus*	RLK	RD-kinase	Sensing of cell wall damage due to wounding by pathogens	Nonself recognition: altered self		60
Pi-d2	*Oryza sativa*	LecRK/S-domain	Non-RD-kinase	*R* gene that confers resistance to blast disease	Nonself recognition: different origins		130
PnLPK	*Populus nigra var. Italic (Lombardy poplar)*	Lectin-like	RD-kinase	Expressed during wounding	Nonself recognition: altered self		131
PR5K	*Arabidopsis thaliana*	PR5	Non-RD-kinase	Disease/stress response	Nonself recognition: altered self		78
PvRK20-1	*Phaseolus vulgaris*	DUF-26	RD-kinase	Expressed during wounding and plant-microbe interactions	Nonself recognition: altered self		132

continued on next page

Table 3. Continued

Gene	Plant	Class	Type	Function	Perception	Putative Ligands	Ref.
RFO1	*Arabidopsis thaliana*	WAK/EGF	RD-kinase	Involved in resistance response to *Fusarium oxysporum*	Nonself recognition: different origins		133
RKC1	*Arabidopsis thaliana*	DUF-26	RD-kinase	Salicylic acid inducible	Nonself recognition: altered self		100
RKS1, 2	*Arabidopsis thaliana*	S-domain	RD-kinase	Salicylic acid inducible	Nonself recognition: altered self		100
RLK1	*Arabidopsis thaliana*	S-domain	RLP	Salicylic acid inducible	Nonself recognition: altered self		134
RLK4	*Arabidopsis thaliana*	DUF26	RD-kinase	Salicylic acid inducible	Nonself recognition: altered self		135
SFR1, 2	*Brassica oleracea*	S-domain	RD-kinase	Defense response signalling, wounding, pathogenic (*Xanthomonas campestris*, *Ralstonia solanacearum*) and nonpathogenic (*Escherichia coli*) bacterial infection	Nonself recognition: different origins and altered self		136
SYM10	*Pisum sativum*	LysM	RD-kinase	Involved in early events during nitrogen fixation symbiosis (symbiotic interactions) *Orthologue of NFR5	Symbiotic relationship, recognition of different origins	Nod factors	128
WAK1 to 4	*Arabidopsis thaliana*	WAK/EGF	RD-kinase	Cell expansion and disease response, *Pseudomonas syringae* (compatible), repressed by wounding	Nonself recognition: altered self		137, 138, 139, 140

Table 4. Some plant R proteins associated with plant-microbe interactions and innate immunity[141]

Class	Protein Structure	Gene	Plant	Function	Perception	Putative Ligands
1	TIR-NBS-LRR	***EILP***	*Nicotiana tabacum*	Non-host disease resistance, induced by hyphal wall component elicitor, *Pseudomonas syringae* pv. ***glycinae,*** *Pseudomonas syringae* pv. ***tabaci***	Nonself recognition: different origins and altered self	
		L	*Linum usitatissimum*	Resistance against *Melampsora lini*	Nonself recognition: altered self	AvrL567
		M		Resistance against *Melampsora lini*	Nonself recognition: altered self	AM
		P		Resistance against *Melampsora lini*	Nonself recognition: altered self	AvrP4, AvrP123
		N	*Nicotiana tabacum*	Resistance against Tobacco mosaic virus	Nonself recognition: altered self	TMV replicase
		RPP1	*Arabidopsis thaliana*	Resistance against *Peronospora parasitica*	Nonself recognition: altered self	ATR1
		RPP5		Resistance against *Peronospora parasitica*	Nonself recognition: altered self	AvrRPP5
		RPS4		Resistance against *Pseudomonas syringae*	Nonself recognition: altered self	AvrRps4
	CC-NBS-LRR	***Prf***	*Solanum esculentum*	Resistance against *Pseudomonas syringae*	Nonself recognition: altered self	AvrPto
		Mi		Resistance against *Melodogyne incognita*	Nonself recognition: altered self	
		Gpa2/Rx1	*Solanum tuberosum*	Resistance against *Globodera pallida* and Potato virus X	Nonself recognition: altered self	

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Table 4. Continued

Class	Protein Structure	Gene	Plant	Function	Perception	Putative Ligands
		RPS2	*Arabidopsis thaliana*	Resistance against *Pseudomonas syringae*	Nonself recognition: altered self	AvrRpt2
		RPS5		Resistance against *Pseudomonas syringae*	Nonself recognition: altered self	AvrPphB
		RPM1		Resistance against *Pseudomonas syringae*	Nonself recognition: altered self	AvrRpm1, AvrB
		RPP8/HRT		Resistance against *Peronospora* and Turnip crinkle virus	Nonself recognition: altered self	AvrRPP8
	NBS-LRR	***Bs2***	*Capsicum chacoense*	Resistance against *Xanthomonas campestris*	Nonself recognition: altered self	AvrBs2
		Dm3	*Lactuca serriola*	Resistance against *Bremia lactuca*	Nonself recognition: altered self	Avr3
		I2	*Solanum esculentum*	Resistance against *Fusarium oxyspo-rum*	Nonself recognition: altered self	
		Cre3	*Triticum aestivum*	Resistance against *Heterodera avenae*	Nonself recognition: altered self	
		Xa1	*Oryza sativa*	Resistance against *Xanthomonas oryzae*	Nonself recognition: altered self	AvrXa1
		Pib		Resistance against *Magnaporthe grisea*	Nonself recognition: altered self	
		Pi-ta		Resistance against *Magnaporthe grisea*	Nonself recognition: altered self	AvrPita
		Rp1	*Zea mays*	Resistance against *Puccinia sorghi*	Nonself recognition: altered self	

continued on next page

Table 4. Continued

Class	Protein Structure	Gene	Plant	Function	Perception	Putative Ligands
		Mla	*Hordeum vulgare*	Resistance against *Blumeris graminis*	Nonself recognition: altered self	AvrMla
	TIR-NBS-LRR-NLS-WRKY	***RRS1-R***	*Arabidopsis thaliana*	Resistance against *Ralstonia solanacearum*	Nonself recognition: altered self	PopP2
2	LRR-TM (RLP)	***Cf-2, Cf-4, Cf-5, Cf-9***	*Solanum esculentum*	Resistance against *Cladosporium fulvum*	Nonself recognition: different origins	Avr2, Avr4, Avr5, Avr9
		Ve1, Ve2		Resistance against *Verticillium albo-atrum*, *V. dahliae*	Nonself recognition: different origins	
3	Kinase	***Pto***		Specific resistance to *Pseudomonas syringae* pv *tomato*	Nonself recognition: altered self	AvrPto
		Ptil		Specific resistance to *Pseudomonas syringae* pv *tomato*	Nonself recognition: altered self	
		PBS1	*Arabidopsis thaliana*	Resistance against *Pseudomonas syringae*	Nonself recognition: altered self	AvrPphB
	Kinase-Kinase	***Rpg1***	*Hordeum vulgare*	Resistance against *Puccinia graminis*	Nonself recognition: altered self	AvrB
4	LRR-TM-Kinase (RLK)	***Xa21***	*Oryza sativa*	Resistance against *Xanthomonas oryzae*	Nonself recognition: different origins	
		FLS2	*Arabidopsis thaliana*	Resistance against	Nonself recognition: different origins	
5	Unique	***HS1*** $^{pro-1}$	*Beta vulgaris*	Resistance against *Heterodera schachtii*	Nonself recognition: altered self	
6	Membrane protein	***RPW8***	*Arabidopsis thaliana*	Resistance against *Erysiphe*	Nonself recognition: altered self	
		mlo	*Hordeum vulgare*	Resistance against *Blumeria graminis*	Nonself recognition: altered self	
7	Toxin reductase	***Hm1***	*Zea mays*	Resistance against *Cochliobolus carbonum*	Nonself recognition: altered self	

Table 5. Dual functioning in plant signaling

Component	Dual Function	Reference
BAK1	Is associated with developmental regulation through the plant hormone receptor BRI1, but also has a functional role in PRR-dependant signaling which initiates innate immunity	105, 106
ERECTA	Affects development of aerial organs by controlling organ size and shape and is also involved in disease resistance	142, 143
HAESE/ RLK5	Functions in both developmental processes (abscission) and defense (hypersensitive cell death)	100
LTP1	In wheat, it binds putative receptors for elicitins. In tobacco, it binds jasmonic acid providing protection against *Phytophthora parasitica* similar to that invoked by elicitin	144
LysM type receptors	*CEBiP is a chitin oligosaccharide elicitor binding protein with two LysM motifs with a proposed role in chitin signaling and transcriptional regulation	35
	*NRF1 and NRF5 are two LysM receptor kinases found in *Lotus japonicus*, which are putative receptors for lipochitooligosaccharide Nod-factors	127
	*LYK3, found in *Medicago truncatula*, is involved in specific recognition during later stages of bacterial infection	145
Mi gene product	Confers resistance to nematodes as well as aphids	85
PERK	Up-regulated by wounding and infection in *Brassica* and also plays a role in regulation of growth in *Arabidopsis*	146, 147
Plant gp91^{PHOX} NADPH oxidase	Although involved in the oxidative burst, it also functions in a variety of developmental and physiological processes	103
RIN4	Guardee—interacts with two different R genes, RPM1 and RPS2	92
RPM1	Able to recognize two different Avr effectors	85
RPP8/HRT	Recognizes both viral and oomycete pathogens	85
Rx/Gpa gene	Confer both viral and nematode resistance	85
WAK1	Involved with an epidermal growth factor EGF-like motif linked to plant growth, but also up-regulated in response to pathogen infection and exogenous salicylic acid	139

accessory domains that participate in signal relay. The diversity of P/MAMPs and the identification of the corresponding PRRs, a term describing a functional category, were recently reviewed.[19] Receptors, a term describing a molecular category, which detect microbial patterns can either be surface based or intracellular receptors (Fig. 1). Surface receptors are known to detect primarily microbe-derived elicitors (including P/MAMPs,

if the molecule contains a conserved 'pattern'), as well, in certain cases, avirulence effectors such as Xa21, in which case they are regarded as *R* gene products. The surface receptors include RLKs, RLPs and extracellular binding proteins that may form part of multi-component recognition complexes.[61] The few P/MAMP receptors identified thus far in plants are all surface receptors that physically interact with their cognate ligands.[61] In contrast, interaction between effectors and the intracellular R proteins (which can contain a LRR domain) probably occurs indirectly through a multi-member complex.[62]

Extracellular Sentinels—Receptor-Like Proteins/Kinases (RLP/Ks)

According to the zig-zag model, plants will first be exposed to pathogen-derived elicitors or PAMPs, which upon perception will trigger PTI. Plants are therefore dependent on the initial local recognition of the invader to activate defenses and this is where perception by RLP/Ks can play a determining role.

In *Arabidopsis*, 610 RLK and 56 RLP have been identified, but only a limited number have been functionally characterized and even fewer are reported to act as immune receptors.[63,64] Many *Arabidopsis* genes encoding RLK and RLP were found to be induced upon the amino-terminal fragment of flagellin (flg22) or EF-Tu treatment, suggesting that they may function as immune receptors.[65] Indeed, 27 out of a total 216 LRR-RLK in *Arabidopsis* were found to be transcriptionally induced upon treatment with flg22 or EF-Tu.[61,65] In addition to FLS2, the EFR was identified as a novel PRR and is a LRR-RLK.[28,29,66] Among the up-regulated genes, there also were three encoding RLK containing lysine motifs (LysM) in their extracellular domains, which potentially could recognize microbial carbohydrate structures containing N-acetyl glucosamine (GlcNAc). It appears that P/MAMPs also trigger enhanced expression of their own cognate receptors, as reported for flg22, EF-Tu and chitin.[29,35,65]

The receptor protein kinases (RPKs) can be classified according to different substrate specificities (tyrosine, serine/threonine or histidine) in the kinase domains.[67,68] RLKs form part of the receptor serine/threonine kinase (RSTK) family, also known as the interleukin-1 receptor-associated kinase (IRAK/Pelle) family. The RLKs can be subdivided into transmembrane receptor kinases (TMRKs) and receptor-like cytoplasmic kinases (RLCKs) if an extracellular domain is absent.[63] RLKs are transmembrane proteins with versatile N-terminal extracellular domains and a C-terminal intracellular kinase domain related to the *Drosophila* Pelle kinase.[21,69] They are classified according to their extracellular domains, except for those who do not have a signal peptide and/or a transmembrane region, referred to as RLCKs. TMRKs can be further grouped into arginine and aspartate (RD)-kinases, nonRD kinases and RD-minus kinases.[68] Although only a few RLKs have been shown to play a role in either development, plant defense or even symbiotic interactions, their large number and diversity suggest that they may be able to recognize and respond to a variety of stimuli. Despite the small number of nonRD kinases (10% of the *Arabidopsis* kinome), kinases known or predicted to function in PRR signaling fall into the nonRD class. The reader is referred to Dardick and Ronald,[70] for a review on receptor signaling through nonRD kinases, predicted to function in PRR signaling and thought to be involved in pathogen recognition and innate immunity. RLPs differ from RLKs in that they contain an extracellular domain and a membrane-spanning domain, but they lack an intracellular activation domain. Therefore, they require interaction with adaptor molecule(s) or RLCKs for signal transduction.

The proposed evolutionary relationships between receptor kinase family members arose from an ancient duplication event leading to the divergence of RLKs/*Pelle* from receptor tyrosine kinases (RTKs)/Raf.[21,64] In the case of PTI, the evolutionary history of the plant RLKs indicate that the kinase domains were recruited numerous times by fusion with different extracellular domains to form the subfamilies found in *Arabidopsis*. Subfamilies are assigned based on kinase phylogeny and are shown according to the domain organization of the majority of members in a given subfamily.[21,69]

Diverse sequence motifs are present in the extracellular domains of RLKs and these motifs are potentially responsible for interactions with other proteins, carbohydrates or lipids.[69] The data indicates that RLKs involved in resistance or defense responses may have been duplicated or retained at higher rates in a lineage-specific fashion.[31] The preferential expansion of defense/resistance-related RLKs could be the consequence of strong selection pressure for recognizing pathogens.[31] The large family of plant RLK proteins, therefore, contain distinct protein kinases where each might play a unique role in cellular signalling.[71] These probably comprise receptors for further P/MAMP recognition.[29] In addition, in certain cases plant defense mechanisms seem to exhibit 'multi-tasking', i.e., the use and application of pre-existing biochemical modules or systems to compensate for evolving variables in potential invaders (discussed below).

The members of the RLK family are divided into classes. The S-class/domain RLKs share homology with the self-incompatibility-locus glycoproteins (SLG) from *Brassica*.[72] The extracellular domain has 12 characteristic conserved cysteines, $CX_5CX_5CX_7CXCX_NCX_7CX_NCX_3CX_3CXCX_NC$. Usually, 10 cysteines are absolutely conserved.[73] A conserved PTDT box was observed in 7 different S-domain RLKs.[71] The LRR class contains conserved repeats of leucines, $LX_2LX_2LX_2LXLX_2X_NXLXGXIPX_2$ and the regions are also surrounded by paired cysteines.[74,75] The lectin-like class has an extracellular domain that shares homology with lectin proteins.[63] Some RLKs bind to plant cell-wall components. The extracellular domains of cell wall-associated kinase (WAK)-type RLKs are associated with pectin, a structural component in the middle lamella and primary cell wall.[21,76] WAKs contain an extracellular domain with similarity to epidermal growth factor (EGF)-like domains.[44] The tumor necrosis factor receptor (TNFR) class has a repeat motif that resembles the extracellular domain of the mammalian tumor necrosis factor receptor.[77] The pathogenesis-related (PR) class contains all 16 cysteines in the extracellular domain that are conserved in PR5 antimicrobial proteins.[78] The chitinase-like class has an extracellular domain homologous to both class V tobacco and *Bacillus* WL-12 A chitinases.[79] The cysteine-rich repeat (CRR) class has one or more repeats of the $C\text{-}X_8\text{-}C\text{-}X_2\text{-}C$ motif.[80] RLKs containing lysine motifs in their extracellular domains are characterized as the LysM class. The 'miscellaneous' or 'other' types of RLKs include those with extracellular domains that do not share homology with other known proteins, contain unique motifs and therefore cannot be grouped into the above mentioned classes.

Receptor proteins have also been identified that lack the characteristic RLK kinase domain (i.e., RLPs), or, proteins that are functional kinases that lack extracellular ligand binding domains (RLCKs). In some cases the proteins have an intracellular kinase domain, as well as a transmembrane region, but only have a short extracellular domain. Tables 2 and 3 serve to summarize which RLKs are involved with disease resistance and/or associated with plant-microbe interactions. The role and regulation of RLKs that have been identified in elicitor-initiated defense responses and as dominant *R* genes in race-specific pathogen defense, was recently reviewed.[81]

Intracellular Sentinels—R Proteins

A second class of immune receptors encoded by *R* genes, which occur mainly intracellularly, has the capacity to perceive isolate-specific pathogen effectors encoded by *AVR* genes of the pathogen. This perception can occur either directly or indirectly by sensing the host proteins upon which effectors have acted.[37,61,82,83] Some R proteins structurally resemble RLK and RLP receptors and probably evolved from P/MAMP receptors through the loss of a kinase domain.[70] However, as exceptions, some intracellular R proteins can consist of one (Pto and Fen) or even two kinase domains (RPG1). Similar to *RLKs*, *R* genes subfamilies have evolutionary expansion patterns that show lineage-specific expansions marked by tandem duplicates.[64,70] Some *R* genes are more rapidly evolving as components of the plant immune system, compared to the evolution of P/MAMP receptors.[84]

It has been recognized that plants might not possess enough *R* genes to intercept all potential avirulence determinants, due to the diversity of pathogens and their associated effectors. According to Dangl and Jones,[85] the likelihood of the known *R* genes linked to defense being able to recognize all the possible effector signals, where 'a surprisingly small number of genes mediate recognition of all possible pathogen-encoded effectors' is questionable. For this reason, bacterial effector recognition has likely evolved as an indirect mechanism (within a complex),[62] with a limited repertoire of plant resistance receptors.[14]

The mechanisms of *R* gene-mediated immunity may be explained by the 'gene-for-gene' genetic model or the 'guard hypothesis' molecular model. A 'guard' can refer to a typical R protein, whereas the 'guardee' represents a target of pathogen effectors.[85] Many plant R proteins might be activated indirectly by pathogen-encoded effectors and not by direct recognition.[85] This form of 'guard hypothesis' implies that R proteins are able to indirectly recognize pathogen effectors by monitoring the structural integrity of the host cell targets following effector action. The R proteins in question are, thus, activated as sensors or sentinels of 'pathogen-induced altered-self' molecular patterns and can thereby potentially perceive the presence of more than one effector protein. Plants are able to sense an 'infectious-self', where the host molecules that are normally not available for recognition (but rather are released following microbe detection, wounding or during infection), are recognized as an altered-self.[86] This 'altered/nonself' concept can explain how plants can recognize a diverse set of pathogens and pathogen-specific molecules, using a relatively limited number of pathogen receptors. A recent modification to the model describes 'decoys' that mimic effector targets in the plant in order to trap the pathogen in a recognition event.[87]

Much information has been gained from molecular studies of *R* genes about their organization within genomes and their functional domains. Although polymorphic and divided into several classes, common structural modules found in intracellular plant R proteins are a C-terminal LRR domain, that is believed to sense microbe-derived signals and a central nucleotide-binding (NB) domain (refer to Table 4). The NB domain is part of a larger NB-ARC domain (due to its occurrence in plant R proteins, the apoptotic protease-activating factor, APAF-1 and its *Caenorhabditis elegans* homolog CED-4).[88] These NB-ARC domain proteins belong to the family of STAND (signal transduction ATPases with numerous domains) NTPases. STAND ATPases are modular proteins and display a wide range of fusions to domains involved in protein-protein or protein-DNA interactions, small molecule-binding domains, as well as catalytic domains involved in signal transduction.

These immune sensor proteins are considered to act as regulatory signal transduction switches where the regulatory switch, scaffolding and occasionally, sensory as well as signal-generating moieties are integrated into a single multidomain protein.[89,90] In addition, structurally diverse range of domains was co-opted during evolution and is found on the N-terminal side of the NB domain. These include the coiled coil (CC, formerly referred to as leucine zipper) or TOLL/interleukin-1 receptor (TIR) domains (Fig. 1). In the case of RRS1, a WRKY DNA-binding domain is located at the C-terminus.

Current data points to the existence of the R proteins in auto-repressed conformations in the absence of a cognate pathogen effector. Direct or indirect recognition of effectors by the polymorphic LRR regions initiates conformational changes and ADP/ATP exchange that renders the respective N-terminal effector domains accessible for interactions with downstream targets.[91]

Various studies have shown that the C-terminal part of the LRR domain provides pathogen recognition specificity. Hence, the LRR domain has a dual function; it provides auto-inhibition and it translates pathogen recognition into activation. How exactly the LRR domains recognize a pathogen or pathogen action is unclear. Whereas some R proteins bind effectors directly, others require an intermediary host factor(s). This factor often interacts with the N-terminal domain of the R protein and could represent either the virulence target (thereby acting as a guardee) or a target mimic (thereby acting as a decoy).[87,92] In this situation, the LRR domain is likely involved in sensing the effector-induced perturbations/altered-self of the target.

Many R genes contain nuclear localization signals.[93,94] Recent data indicate that members of the TIR- and CC-type of R protein families function inside the nucleus with nucleocytoplasmic partitioning occurring upon activation. Inside the nucleus, the N-terminal domains of the activated receptor can act as signal relay to transcription factors of the WRKY class.[94] The subgroup of R proteins that have co-opted a WRKY-domain, may exhibit direct DNA-binding capacity. Members of the WRKY transcription factors bind to *cis*-acting regulatory elements called W-boxes and can act as repressors of PAMP-triggered immune responses whilst others act as positive regulators.[95,96]

De-repression of defense genes could thus amplify PAMP-triggered responses and integrate signals generated by defense-associated RLKs and R proteins.[38] It is considered likely that, in addition to interference with WRKY repressors, other potential convergence points between P/MAMP- and R protein-triggered signaling pathways exist.[94] P/MAMP-triggered and mitogen-activated protein kinase (MAPK)-dependent phosphorylation of R proteins can modulate effector-triggered receptor-activity and/or nucleo-cytoplasmic receptor partitioning.[38] This offers an explanation of how perception of nonself structures by RLP/Ks and R proteins can lead to transcriptional activation of defense-responsive genes, thereby linking receptor function to transcriptional reprogramming of the host cells for pathogen defense.[38]

UP-REGULATION OF SURVEILLANCE AND A PRIMED STATE

Perception of general elicitors such as LPS and flagellin from bacteria by plants, resembles recognition based on PAMPs in animals.[97,98] As all types of plant immunity may be considered innate, the response to PAMPs should be considered as an expression of basal resistance. Genes expressed in *Arabidopsis* in response to elicitation by flg22,[99] indicate that a considerable number of the up-regulated genes can be classified as being

involved in signal perception (*RLK* and *R* genes) and signal transduction. This indicates a positive feedback regulation operating in innate immunity with transcriptional activation of the components involved in the perception and signalling.[65] Similar results were found in a transcriptional micro-array analysis of genes expressed in *Arabidopsis* in response to elicitation by LPS (TAIR accession expression set 100808727, Nürnberger T 2006).

Many receptors are transcriptionally activated upon perception of their ligands as well as SA, an effector of SAR.[100] Wang et al[101] provided evidence for a model where the *RPW8* resistance gene from *Arabidopsis* could be induced by invasion of powdery mildew isolates and amplified by a SA-feedback circuit, leading to activation of defense responses, via a conserved basal resistance pathway in a nonrace-specific manner. In the case of LRR-RLK genes, 49 were found to be up-regulated upon either PAMP elicitation and/or pathogen infection.[83]

Recent data indicate the existence of similar and complementary, but independent perception systems, for different PAMPs (e.g., flg22 and elf18), where perception of one PAMP at a binding site induces higher amounts of binding sites for a second PAMP and vice versa. Interestingly, the genes for the RLKs *FLS2* and *EFR*, are also induced by LPS and other PAMPs.[29] Signaling cascades generated by these independent receptors converge to lead to the activation of plant innate immunity systems.[29] If that is generally applicable, plants seem to induce the gene products recognizing the attacking pathogen, thus activating the plant's surveillance system and thereby sensitize or 'prime' the rest of the plant to control the spread of the pathogen. It would also imply sensitization of the innate immune system to perceive and respond to the attacking pathogen, analogous to what constitutes local and systemic induced resistance.[102] The up-regulated expression of *RLK* and *R* genes presumably leads to an enhanced sensitivity of the plant to further stimuli, sensing the presence of invading micro-organisms with other PAMPs or effector signals, i.e., a primed or sensitized state.[1]

DUAL FUNCTIONING IN PLANT SIGNALING

There is currently no conclusive evidence for evolutionary conservation of an ancient P/MAMP detection system,[32,85,103] and independent recruitment of components during evolution is equally plausible. Moreover, there are also various examples where a specific type of biochemical module or protein appears to be used to fulfill a requirement in more than one process, i.e., dual functioning or 'multi-tasking'. Since the pre-existing mechanisms of innate immunity must be specifically utilized to distinguish plant from pathogen, the question arises if it is possible that there might be a sharing of receptors between similar signal molecules, such as a general receptor and/or coreceptor complex for PAMPs with common molecular architectures.

The re-use of highly evolved processes for diverse functions was recently pointed out.[103] It was concluded that a form of the 'guard hypothesis' best explains how plants can potentially recognize a diverse set of pathogens and pathogen-specific molecules, using a relatively limited number of pathogen receptors, but emphasizes that (in addition to PTI), the evolutionary solution in plants to identify pathogens involves surveillance of 'self vs altered-self', whereas the evolutionary solution in the adaptive immune response in vertebrates involves detection of foreign antigens.

The work regarding innate immunity, with specific reference to flagellin, has lead to the perception of a one-to-one specific recognition of pathogen (ligand) by the host (receptor) in plants, as also observed in animal and insect adaptive (and innate) immune

responses.[29,30,99] However, it is not necessarily a specific one-to-one recognition system (such as the Avr-R model). Rather, plant defense mechanisms may follow an adaptation of the guard hypothesis, such as 'one post, multiple guards'.[92] In addition, many R proteins (guards) may perceive the presence of more than one effector protein, whether that protein comes from pathogens with similar or different lifestyles.[85]

An example of independent recruitment of biochemical components for different functions is the LRR motif. LRR domains are found in transmembrane proteins, -kinases and intracellular R proteins. Collectively, LRRs appear to be involved in a range of processes from development to intercellular communication and to disease resistance.[14,104] A number of LRR transmembrane and intracellular proteins act as integral components of ligand perception complexes during ETI.[85] In addition, the LRR motif also plays an important role in PRRs in the evolutionary older PTI.[83]

Is it possible that the same type of receptor could perceive different signals, for example both PAMP signals for defense and MAMP rhizobial signals for symbiosis; although the downstream signaling and the outcome of the plant-microbe interactions are different?[10] An important recent discovery is the role that coreceptors might play in receptor-ligand interactions and it has been suggested that coreceptors might modulate receptor specificity.[105] The brassinosteroid receptor BRI1-associated kinase (BAK1), may be up-regulated and seems to be a crucial component of plant disease resistance and a positive regulator/general signaling adaptor/signal amplifier in signaling and exerts this activity independent of brassinosteroids.[86,106] In addition to BAK1 interacting with FLS2 in a stimulus-dependent manner, it may also have a common role as an adaptor or coreceptor for the regulation of various other receptors. BAK1 is thus not only associated with developmental regulation through the hormone receptor BRI1, but also has a functional role in PRR-dependent signaling which initiates innate immunity.[11,105] Other examples of multifunctional proteins, in addition to BAK1, are compiled in Table 5 and include multiple applications of specific motifs and the re-use of highly evolved processes for diverse functions.

CONCLUSION

The concept of innate immunity centers on the recognition of 'nonself' components, which is accomplished by sentinels. Plants have evolved a unique metabolic plasticity that allows them to perceive pathogens and unleash effective defense strategies, but how the plant can distinguish between itself and pathogens during a defense response has only recently been explored. This highly evolved surveillance system in plants is able to detect a broad range of signals originating from microbes or damaged plant tissues, initiating sophisticated molecular mechanisms that result in defense. Microbe/pathogen-associated molecular pattern molecules, damage-associated molecular pattern molecules, virulence factors, secreted proteins and processed peptides can be recognized directly or indirectly by this surveillance system. Together, receptor-like kinases or receptor like proteins, as membrane bound signaling molecules with an extracellular receptor domain and intracellular nucleotide binding-leucine-rich repeat proteins as receptors of pathogen-secreted effector proteins, provide an early warning system for the presence of potential pathogens and activate protective immune signaling in plants. Much remains to be discovered, e.g., how different perception mechanisms in plants, based on self, damaged-self, altered-self and nonself, are employed for different threats and how those signals are transduced within the inter-connected relay system observed during defense responses.

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CHAPTER 7

HOW DID FLOWERING PLANTS LEARN TO AVOID BLIND DATE MISTAKES?

Self-Incompatibility in Plants and Comparisons with Nonself Rejection in the Immune Response

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Abstract: Self-incompatibility (SI) is a common form of genetically-controlled mate-selection that prevents mating between closely related plants of the same species. SI occurs in about half of all flowering plant species. It has been studied extensively in the Papaveraceae (poppy), Brassicaceae (*Arabidopsis*, cabbage etc), Solanaceae (potato, tomato etc), Plantaginaceae (snapdragon) and Rosaceae (apple, cherry and peach etc). The self-recognition inherent in self-incompatibility has similarities with animal and plant immunity systems giving rise to speculation that the systems are related. Both systems display balancing selection, 'self/nonself' recognition, high polymorphism, high specificity and there are also some similarities in the rejection mechanisms deployed in the two systems. Whether these systems have diverged from a common predecessor is discussed, however similarities may be driven more by biological problems and the available molecular machinery to solve them than by an evolutionary relationship.

INTRODUCTION

When we pick a mate, we may only be thinking about the short-term goal of finding the 'right' match, but in the long-term it's all about genetic fitness—so what are the genetic 'qualities' that we are seeking when discriminating between potential mates? Or, indeed, what are the qualities that plants seek? Darwin's investigations into plant reproduction noted the effects of inbreeding and that in some self-crosses healthy plants were unable

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to produce offspring.[1] Modern studies show that the ability to reject self-pollen enhances genetic diversity in a population. Thus, the qualities selected in individual mating choices have implications for the genetic trajectory of a species. Much research has been undertaken to understand why incompatibilities exist between potential parents and as research continues into the similarities between biological systems that control mate selection we are more able to observe the evolution of mechanisms to stop the 'wrong' mates having offspring. Similarities among these mechanisms could, in principle, indicate evolutionary relatedness across animals, plants and fungi. Animals, for instance, can turn away when the mate isn't suitable, but in plants only the pollen is mobile so selection often operates at the level of biochemical pollen/pistil interactions. Plants, nevertheless, have genetic preferences and have evolved a diverse array of mechanisms to ensure the most suitable pollen fertilizes them. Self-incompatibility systems are mechanisms that have evolved to screen out undesirable matches by preventing mating between individuals of the same or similar genetic makeup. The self-recognition inherent in this system is superficially similar to animal immune systems and this has given rise to speculation that immunity and self-incompatibility are related. Clearly, both systems require 'self' recognition. Whether these systems diverged from a common predecessor is less clear. Similarities between the systems of self-incompatibility and innate immunity have been reviewed by Nasrallah (2005) and Sanabria et al (2008).[2,3]

SELF-INCOMPATIBILITY HELPS PLANTS SCREEN POTENTIAL SUITORS

Plant reproduction is literally a blind date, the first point of contact being when the pollen arrives at the stigma as a result of being carried by an anonymous agent like the wind or an insect pollinator. This pollen may be from many sources, such as closely or distantly related plants or even the same flower. Some of this pollen, frequently self or very closely related pollen, may be unable to fertilize due to incompatibility. Some pollen will 'germinate' with pollen tubes growing unhindered, successfully fertilizing the ovary. In this way, the plant is screening for a viable partner with which to produce offspring.[2] Likewise, animals will often avoid mating with partners that are genetically similar, such as siblings or relatives although the range of mechanisms available to achieve this are completely different.[4] With the evolution of biochemically based reproductive barriers that probe potential partners' genetic makeup and determine compatibility, plants are able to effectively select among the various types of pollen that arrive at the stigma surface.[5-7] These plant barriers to the exchange of genetic information between self and closely related individuals evolved in response to similar genetic pressures as those in animals or any other group of sexually reproductive organisms. For example, inbreeding causes loss of alleles from a population and reduces vigor due to expressed genetic load.[1,8,9]

SELF-INCOMPATIBILITY'S CONTRIBUTION TO THE SUCCESS OF THE ANGIOSPERMS

The angiosperms dominate most terrestrial ecosystems. Today, this group includes 250-300,000 species, a greater number than all other terrestrial plant groups combined.[10] The only other extant seed-plants are the gymnosperms (conifers etc).[11] Angiosperms have not always dominated the land. Palaeobiologists propose a sudden transition from

gymnosperms to angiosperms in the early Cretaceous. This shift generated the majority of extant angiosperms families and implies a major innovation that greatly enhanced diversity and competitive ability.[10,12]

Although both angiosperms and gymnosperms bear seed they have very different reproductive biology. Angiosperm flowers usually contain both male and female reproductive organs. Importantly, the ovules are always completely enclosed. Thus, sperm cells must be conveyed through nonsexual tissue for fertilization to occur. The group's name—angiosperm—refers to this feature of reproduction. 'Angio' referring to the vessel or tube that carries the sperm to the ovule, the pollen tube.[10,12,13] Pollination in angiosperms begins when a pollen grain arrives at the stigma surface.[14] Then, when conditions are right, the pollen will hydrate and initiate pollen tube morphogenesis. An unhindered pollen tube will traverse the pistil via the central transmitting tract until reaching the ovary (see Fig. 1). As the pollen tube grows through the pistil it carries within it two sperm cells. Fertilization occurs when the pollen tube enters an ovule through the micropyle. One sperm cell unites with the egg cell to form the diploid embryo, while the other unites with the two central cells to form the triploid endosperm.[15] As reviewed by de Nettancourt (1997), barriers to inhibit fertilization by self-pollen could, in principle, act at several stages in this process and be found at different stages of a plant's reproductive life cycle.[16-18] However, recognition systems that control pollen tube growth through the pistil offer an important level of control over mating in angiosperms.

Self-incompatibility (SI) is a common form of genetically-controlled mate-selection that prevents mating between closely related individuals of the same species. Thus, some of the angiosperm's success can be attributed to SI, which forms partial to complete barriers during self or related pollen tube growth through the pistil.[8,19,20] In most SI species a single locus controls compatibility and it is usually referred to as the *S*-locus even though different

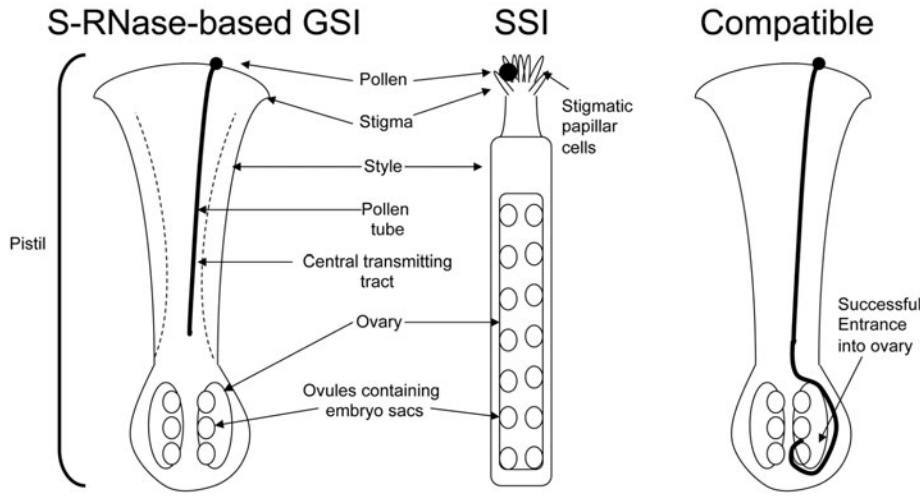

Figure 1. SI affects pollination at different stages, Left, Two types of SI are shown. In S-RNase-based SI incompatible pollen tubes do not penetrate the length of the style. In SSI in *Brassica*, pollen may fail to hydrate or penetrate the stigma. Right, In a compatible pollination pollen hydrates, a pollen tube grows through the stigma and the style to the ovary, penetrates an ovule and releases its two sperm cells to effect fertilization.

species may have entirely different SI mechanisms at the genetic and biochemical levels. Thus, the term *S*-locus merely denotes the locus that controls pollination specificity—the genes encoded by the *S*-locus in different families are often completely different.[21]

SELF-INCOMPATIBILITY ACTS AS A POSTPOLLINATION MATE SELECTION SYSTEM

SI comes in two broad forms: heteromorphic and homomorphic. Heteromorphic SI entails structural barriers to pollination such as the relative position of the pistil to the anthers. The 'pin' and 'thrum' forms of primulas are examples of this that were studied by Darwin.[22] In the pin morph the stigma sits at the mouth of the corolla and the anthers are near the ovary while in the thrum morph these positions are reversed. A single locus controls floral morphology and a simple dominance relationship ensures equal numbers of pin and thrum plants in a population. Darwin noticed that the two morphs deposit pollen on different parts of a bee's body and thus, the pin and thrum morphologies promote between-morph crosses. However, he also discovered that even if this structural barrier is circumvented inter-morph crosses set few seed. We now know that primulas posess both structural and biochemical barriers to self-fertilization.

Homomorphic SI, in contrast, always entails a biochemical interaction that controls compatibility. Compatibility groups do not show alternative floral morphologies. The *S*-locus of homomorphic SI species is often very polymorphic. For example, a recent survey of SI in *Solanum chilense* detected 30 *S*-haplotypes in a population of 34 plants.[23]

Homomorphic SI is further subdivided into two types depending on when and where genetic control of compatibility is expressed. In sporophytic SI, the haploid pollen bears both *S*-specific determinants from the diploid sporophyte that produced the pollen.[24] In simple sporophytic SI systems pollen is rejected if either *S*-haplotype of the pollen parent matches either *S*-haplotype of the pistil parent. Such systems are highly restrictive since not only self-mating but also mating between two individuals sharing even one *S*-haplotype are prevented. The sporophytic SI system in the Brassicaceae (e.g., cauliflower, broccoli and SI species of *Arabidopsis*) has been studied in great detail and reviewed by Takayama et al (2005).[25] In gametophytic SI, pollen is rejected if its single *S*-haplotype is the same as either of the two *S*-haplotypes in the diploid pistil. This makes gametophytic SI less restrictive than a sporophytic system. For example, progeny will normally be cross-compatible with their parents. Gametophytic SI is very widespread among angiosperms; it has been studied extensively in the Papaveraceae (poppy), Solanaceae (potato, tomato etc), Plantaginaceae (snapdragon) and Roseaceae (apple, cherry and peach etc). Reviews of gametophytic SI can be found in Takayama et al (2005), Franklin-Tong et al (1996) and McClure et al (2006).[25-26]

SELF AND NONSELF-REJECTION IN PLANT AND ANIMAL INNATE IMMUNITY

Plants have the capacity to recognize and reject pathogens at various stages of their attempted colonization. A type of passive resistance invokes failure of the plant to elicit a potentially pathogenic response and is referred to as nonhost resistance.[27] Non-specific

rejection often arises as a consequence of the potential pathogen's attempt to breach the first lines of plant defense.

For some, this may be limited to molecular signals released outside the plant cell wall, but for others it includes penetration of the cell wall and the delivery of signal molecules to the plant cytosol. Direct or indirect recognition of these signals triggers host-specific resistance. Our understanding of host-specific resistance and its possible links to nonhost-specific resistance has advanced significantly as more is discovered about the nature and function of the molecules underpinning both kinds of resistance.[27] Furthermore, the ability to recognize 'self' and 'nonself' forms the basis of the immune response observed in many living organisms.[28]

The self-recognition inherent in SI is familiar to immunologists. Highly diverse receptors and ligands that ensure specific recognition are common to both SI and innate immunity in both plants and animals. The plant innate immune response, reviewed by Jones et al (2006), operates by a two-phase pathogen recognition mechanism.[27] The first phase recognizes and responds to common pathogens, whereas the second phase responds to specific virulence factors. Pathogens use a diverse range of mechanisms to infiltrate the plant, thus, plants need to respond to the invasion in similarly diverse ways.[29] Compared to mammalian systems, the plant innate immune system is more localized in its response, nevertheless, it is extremely effective. As an initial recognition phase both plant and animal innate immunity systems often use trans-membrane pattern recognition receptors (PRRs) to respond to conserved PAMPs/MAMPS (pathogen/microbe associated molecular patterns) present in diverse pathogens. PAMPs are produced only by pathogens, not the host. Thus, allowing 'self' and 'nonself' recognition.[30] Mammalian PAMP recognition is via a PRR; a family of toll-like-receptors (TLR) that recognize the 'molecular signatures' of a pathogen. TLRs are transmembrane receptors that recognize the PAMP infection and initiate signal transduction that activates immune cells.[31] Upon activation immune cells can respond in various ways. For example, bacterial pathogens may be phagocytosed and digested, while viruses may initiate a programmed cell death response. Mammals also have mobile defender cells that respond to pathogen effectors by converging on the infected region. Plants lack mobile defender cells and innate immunity acts at the individual cell level, thus ensuing a localized defensive response in areas of infection. For a review see Ausubel (2005).[32]

Flagellin is an extremely abundant protein component in the bacterial flagellum and it is a classic example of a PAMP. Animals and plants both have specific innate immune responses to flagellin. In animals the response involves receptor TLR5.[33] In *Arabidopsis thaliana* perception of flagellin is via FLS2 (flagellin-sensitive-2) a receptor-like kinase that activates defense mechanisms upon positive recognition of a conserved amino acid sequence at the flagellin N-terminus.[34] FLS2 signaling may be initiated by a 22 residue flagellin peptide leading to a production of a range of pathogen response (PR) proteins and reactive oxygen species production that limit pathogen growth.[35]

A second type of plant pathogen response is associated with nucleotide-binding-LRR (NB-LRR) proteins that provide resistance (R) to specific pathogens.[31] Pathogens from diverse kingdoms express effectors described as 'avirulence' (*avr*) genes.[32] The recognition systems between plant NB-LRR *R* genes and their cognate pathogen *avr* genes are collectively referred to as gene-for-gene systems.[32]

MOLECULAR BASIS OF SELF-RECOGNITION IN SELF-INCOMPATIBILITY

The molecular interactions that give a plant the ability to recognize, accept or reject self-pollen are beginning to be understood.[36-38] As previously mentioned two major homomorphic SI systems are known: gametophytic and sporophytic (GSI and SSI). SI systems may differ in the timing of the response. As shown in Figures 1 and 2, pollen tubes are rejected during growth in the style in gametophytic S-RNase-based systems while rejection occurs on the stigma in the *Brassica* SSI system.[39] Stigmatic rejection is also a characteristic of SI in poppy, which displays a gametophytic SI system.[40-42] However, in the *Brassica* system recognition occurs in the stigmatic papillar cells while in the poppy recognition occurs in the pollen tube (i.e., the gametophyte).[40,43]

Two forms of GSI, reviewed by McClure et al (2006), are being actively researched: Papaveraceae-type GSI and Solanaceae-type GSI.[26] GSI in the Papaveraceae is well understood at the physiological level although much remains to be learnt about signaling. In *Papaver rhoeas* S-proteins (PrsS) are secreted by the cells of the stigma and initiate a signal transduction cascade in self pollen tubes. Rejection entails very rapid increases of cytosolic calcium (Ca^{2+}) and changes in the actin cytoskeleton that cause an almost immediate cessation of pollen tube growth.[44] Further signaling triggers long-term irreversible responses that eventually result in programmed cell death in self-pollen.[45] The corresponding pollen S-determinant, *PrpS*, has been cloned and encodes a 20kDa transmembrane protein.[46] It is not yet known how interaction of PrsS and *PrpS* initiate these signaling events.

In contrast to GSI, pollen compatibility in SSI (Fig. 1) systems is determined by the haplotype of the pollen-producing parent, rather than the *S*-haplotype of the haploid gametophyte itself. The critical interaction occurs between proteins located in the pollen coat and papillar cells on the stigma surface.[47] The *S*-locus encodes two highly polymorphic proteins—the S-receptor kinase (SRK) expressed in the stigma papillar cell's plasma membrane and S-locus Cys-rich (SCR or SP11) protein deposited on the pollen coat. These two proteins are the specificity determinants for pollen-pistil recognition.[48] When pollen arrives on the stigma surface SCR proteins from the pollen coat bind to SRK signaling the papillar cell to interfere with pollen hydration, pollen tube emergence and growth.[49]

CASE STUDY: S-RNASE-BASED GAMETOPHYTIC SELF-INCOMPATIBILITY

S-RNase-based SI occurs in the Solanaceae (Fig. 2), Plantaginaceae and Rosaceaea.[50] Many of the proteins involved in S-RNase-based SI have been identified and the outlines of the physiological process of pollen rejection are understood. However, much remains to be done to connect specific biochemical interactions to the overall physiology. In this case study we will summarize what is known about self-recognition and its consequences in this system.

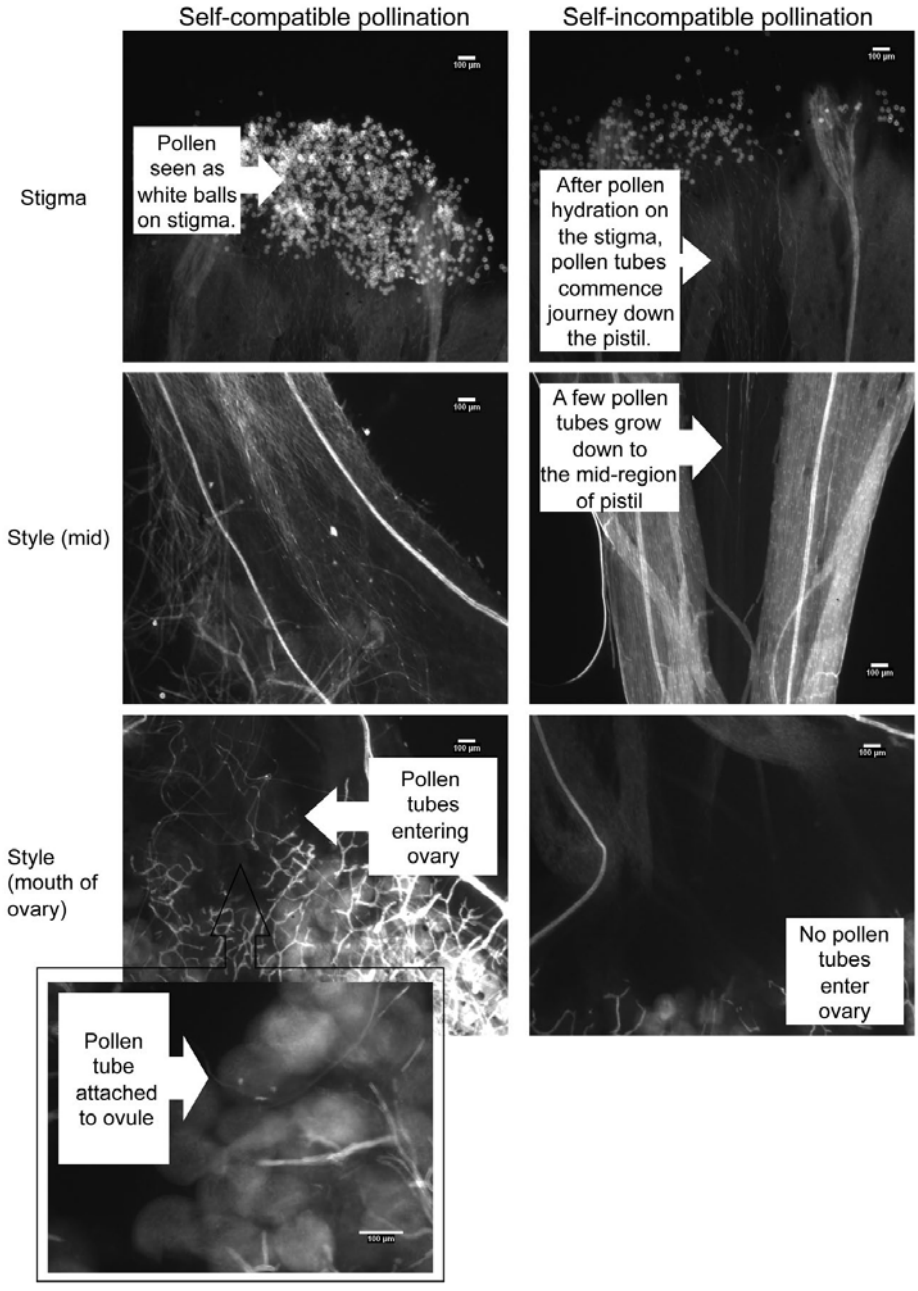

Figure 2. Gametophytic self-incompatible and self-compatible pollinations in *Solanum goniocalyx* (cultivated potato). Images taken at different areas of the pistil: stigma, mid-style and at the mouth of the ovary. Pistils were fixed 48 hours after self-pollination and stained in decolorized aniline blue.

S-RNases—the Female Determinants

Bredimeijer et al (1981) were the first to correctly identify pistil proteins in *Nictotiana alata* that cosegregate with the ability to recognize and reject pollen with specific *S*-haplotypes.[51] Anderson et al (1986) cloned a cDNA encoding one of these proteins enabling a series of studies that established many of the basic facts of S-RNase-based SI.[52] The pistil *S*-specificity determinants are expressed at very high levels in the pistil transmitting tract and are secreted into the extra-cellular matrix (ECM).[53,54] They possess variable amounts of N-linked glycan, although this is not required for function in SI.[55,56]

The discovery that the pistil determinants of *S*-specificity are ribonucleases, S-RNases, laid the foundation for the cytotoxic model.[36,57,58] In this model S-RNases are taken to act as both recognition proteins and as highly specific cytotoxins. Each *S*-haplotype encodes a unique S-RNase that is expressed in the pistil and deposited in the ECM. Incompatible pollen with a matching *S*-haplotype (e.g., S_1-pollen on an S_1S_2 pistil) is inhibited by the action of S-RNase. Degradation of RNA in incompatible pollen tubes would interfere with translation and, thus, eventually cause growth to cease (Fig. 2).[53,54] All other pollen-*S* genotypes are compatible and evade this cytotoxic activity of S-RNase (e.g., S_x-pollen on an S_1S_2 pistil where $S_x \neq S_1$ or S_2). Thus, it is thought that the *S*-specificity determinant in pollen functions to prevent the action of nonself-S-RNase.

Structural Features of the S-RNases

Figure 3 shows the pattern of conserved and variable sequences in S-RNases. Five conserved regions (C1 to C5) account for about 40 of the approximately 200 amino acids in a typical S-RNase. Conserved regions C2 and C3 each contain a histidine residue implicated in catalysis.[59] The three dimensional structure of S_3-RNase from *Pyrus* shows the conserved sequences contributing to the core of the protein and the catalytic histidine side chains extend into the active site grove.[60] Sequence variability between allelic S-RNases can be very high and, of course, the variable residues are important for recognition. Zurek et al (1997) exchanged sequences between S_{A2}—and S_{C10}-RNase and concluded that all regions outside the conserved regions C1 to C5 contribute to recognition of this pair of S-RNases.[61] In contrast, Matton et al (1997) found that by exchanging just four residues in HVa and HVb between *Solanum chacoense* S_{11}—and S_{13}—RNase could switch the *S*-specificity.[62] Thus the S_{11}- chimeric gene, with the four substituted codons from S_{13}-, was transformed in to $S_{12}S_{14}$ plants and transformants gained the ability to reject S_{13}-pollen.[63] The apparent contradiction between these two studies is explained by the choice of experimental system. The S-RNases examined by Zurek et al (1997) are typical of those in many species and display about 40% identity while the pair examined by Matton et al (1997) were unusually similar, differing by only eleven residues.[61,63] Overall, the data show that *S*-specific recognition is a function of the active S-RNase protein. Residues needed to distinguish any particular pair of S-RNases may be scattered over the entire surface of the protein. HVa and HVb are the most variable regions but they are not unique in determining self-recognition. S–RNase is the only factor that directly determines pistil specificity, however modifiers such as HT-B are required for S-RNase function.[64,65]

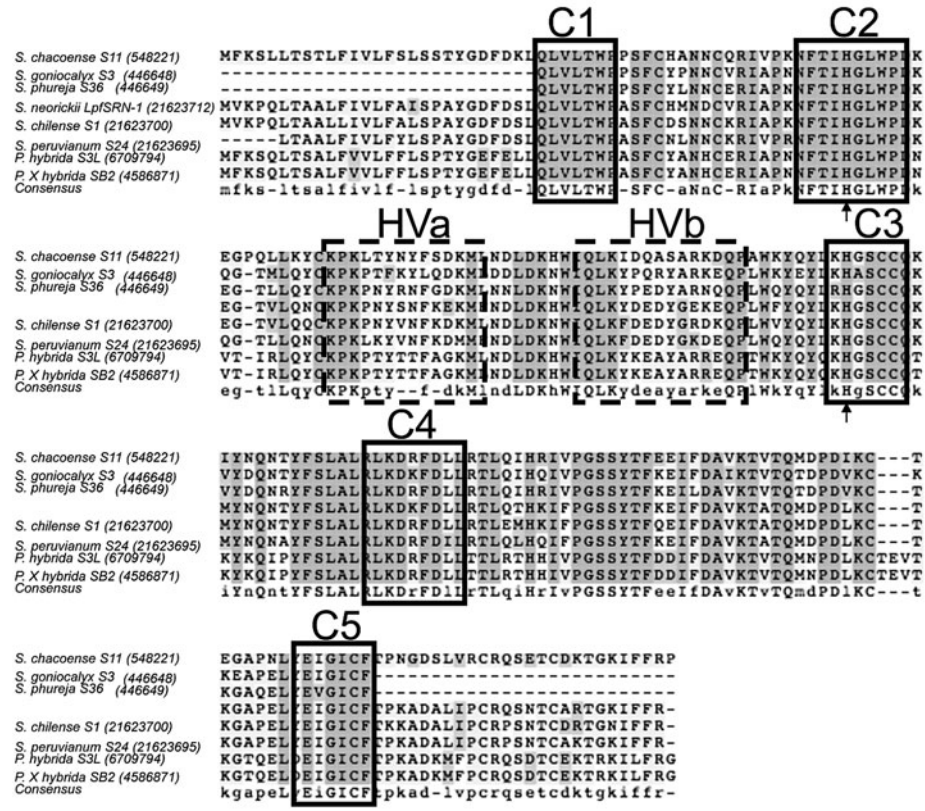

Figure 3. Structural features of S-RNases. S-RNase conserved regions C1 to C5 are shown.59 Arrows, histidine residues implicated in ribonuclease activity. All other sequences are potentially variable when large numbers of S-RNase sequences are compared. The regions that show greatest variability, HVa and HVb, are boxed. Conserved S-RNase sequences contribute to functions common to all S-RNases. Variable regions contribute to *S*-specificity recognition. Shading is reflective of the similarity between all sequences. Darker shades reflect greater similarity.

S-RNase Modifiers

Modifier genes contribute to non*S*-specific functions in SI and their characterization can, therefore, reveal different aspects of the recognition reaction. The first non*S*-specific factor was identified through experiments comparing the pollination behavior of SC *Nicotiana plumbaginifolia* and SC *Nicotiana alata.* Previous genetic experiments demonstrated that SC *N. alata* expressed all the pistil-side factors needed for SI apart from the *S*-specificity determinant, S-RNase.[61] A sequence now named *HT-B* hybridized strongly to RNA from SC *N. alata* pistils but not to RNA from styles of *N. plumbaginifolia.* It was noted that *HT-B* transcript accumulation correlated exactly with the developmental onset of pollen rejection.[63] The precise correlation of *HT-B* expression and *S*-specific pollen rejection provided circumstantial evidence for a role in SI. Antisense experiments provided direct evidence. Plants with suppressed HT-B protein levels did not show *S*-specific pollen rejection even though they expressed

near normal amounts of S-RNase. Similar experiments in *Solanum chacoense* also demonstrate a role for HT-B in self-pollen recognition.[66]

The precise biochemical role of HT-B is still unknown, however sequence analysis shows that HT-B proteins possess an unusual C-terminal segment, usually 20-22 amino acids, composed entirely of asparagine and aspartic acid residues.[67] This 'ND-domain' is flanked by highly conserved cysteine motifs, CXXCXC and CXXXCC.[68] The functional significance of these sequences is unknown. The only biochemical clues to the function of HT-B are that a portion of the protein is associated with the membrane and that it is preferentially degraded in compatible pollinations.[82] This may be significant because alterations in the pollen tube endomembrane system are associated with SI and the *S*-specific degradation of HT-B protein in compatible pollen tubes is one of the only known consequences of SI, apart from degradation of pollen RNA in incompatible pollen tubes.

The 120kDa glycoprotein, 120K, is another factor required for SI on the pistil-side and was first identified as an abundant component of the pistil ECM.[69] It is a highly basic arabinogalacton protein (AGP) comprised of about 40% carbohydrate. Later studies showed that 120K, along with certain other abundant AGPs, is an S-RNase binding protein.[70] Under one hypothesis, S-RNase forms complexes with these AGPs in the ECM and this represents the form of S-RNase that interacts productively with pollen tubes. However, only 120K appears to be required for SI. Suppressing 120K expression by RNAi caused loss of *S*-specific pollen rejection.[78] Unfortunately, like HT-B, the precise role of 120K is not known. However, immunolocalization experiments show that 120K is taken up by growing pollen tubes.[71,82]

SLF/SFB—Pollen S-Specificity Determinants

The male determinant of *S*-specificity (pollen-*S*) is less abundant than S-RNase and different approaches were required to identify it. Genomic sequencing of the region surrounding the *S-RNase* gene identified the first pollen-*S* gene in a member of the Plantaginaceae, *Antirrhinum hispanicum*.[70,72] Sequence analysis revealed an F-box protein gene referred to as *AhSLF-*S_2 (*S*-locus-F-box from *A. hispanicum*).[73] Transformation experiments in *Petunia inflata* proved that *SLF* genes determine pollen-part *S*-specificity.[74] An *S*-specific effect consistent with the expected ability of the pollen-*S* gene to confer resistance to nonself-S-RNase came from transforming SI S_1S_3 plants with the *PiSLF*$_2$ gene. As expected, these transformed plants became self-compatible. SLF proteins have been shown to bind S-RNase in vitro, however, this biochemical interaction in not *S*-specific.[72,75] Thus, although the genetic function of *SLF* is established its biochemical function is not fully understood.

F-box proteins are best known for their function in ubiquitin-mediated protein degradation mediated by SCF (Skp-1, Cullin, F-box) complexes.[76] Experiments in petunia and snapdragon identified SLF binding proteins, but the exact nature and function of the complex in pollen are not yet clear. The *Antirrhinum* SLF proteins appear to interact with Skp-l-like proteins, AhSSK1, as expected.[77] These may further associate with cullin to form an SCF^{SLF}- like complex that functions as an E3 ubiquitin ligase. In contrast, experiments in petunia provide no evidence for binding to a Skp1-like protein and alternative SLF-binding proteins have been suggested to form E3 ubiquitin ligase complexes that function in SI.[78,79]

Models for Self-Recognition in S-RNase-Based SI

Two models have been advanced to explain S-RNase based SI. The basic facts are that S-RNase and SLF determine *S*-specificity on the pistil and pollen-sides, respectively.[65,80] Furthermore, HT-B and 120K are required for pistil-side function.[81,82] Finally, all current models propose that S-RNase causes a cytotoxic degradation of pollen RNA in incompatible pollen tubes and that compatibility involves preventing S-RNase cytotoxicity.[53,54] It is not known when and where S-RNase and SLF interact, nor are the direct consequences of this interaction known. One model proposes that a compatible interaction leads to ubiquitylation and degradation of nonself-S-RNase.[76,80,83] In support of this model, Hua et al (2006) provide evidence for three S-RNase binding domains in petunia SLF.[75] Nonself-S-RNase is thought to bind to a common SLF domain, FD2, leading to ubiquitylation and subsequent degradation. A self-S-RNase is thought to interact with two *S*-specificity domains, FD1 and FD3, that interfere with ubiquitylation.[75] Thus, self-S-RNase is not degraded and its cytotoxic ribonuclease activity is expressed. This type of model explains the differential cytotoxic effects in SI as well as certain other effects, but it does not have a role for HT-B or 120K.[84] An alternative model emphasizes S-RNase compartmentalization as the fundamental mechanism that allows compatible pollen tubes to evade S-RNase cytotoxicity.[26] Goldraij et al (2006) showed that S-RNase is taken up and compartmentalized in vacuoles in both compatible and incompatible pollen tubes.[85] However, in incompatible pollen tubes the vacuoles become disrupted. This could release S-RNase into the cytoplasm and cause rejection of self pollen tubes. Significantly, Goldraij et al (2006) were unable to detect degradation of nonself-S-RNase in compatible pollen tubes, as predicted in the S-RNase degradation model.[85] However, HT-B protein is degraded preferentially, albeit not exclusively, in compatible pollen tubes. Since HT-B protein is known to be required for self-pollen rejection, its degradation in compatible pollen tubes could contribute to compatibility, but this cannot be a direct effect. Goldraij et al (2006) suggest that a hypothetical pollen protein targets HT-B for degradation and that the self-S-RNase/SLF interaction interferes with this process, thus effectively stabilizing HT-B and contributing to rejection of self-pollen.[85] Although the compartmentalization model explains features of SI that the S-RNase degradation model does not, it is not clear where or how S-RNase and SLF interact. It is possible that both S-RNase compartmentalization and S-RNase degradation contribute to SI.[26]

COULD PARALLELS BETWEEN IMMUNITY AND SELF-INCOMPATIBILITY SUGGEST AN EVOLUTIONARY RELATIONSHIP?

There are similarities and differences between SI and immune systems. Both are, in a sense, forms of protection or defense against undesirable invaders. However, the function of SI is to recognize and reject self while immune systems recognize and reject nonself. SI functions to maintain diversity by favoring nonself matings while immunity systems protect a host from attack. At the molecular level, both systems require a mechanism for specific recognition against a diverse background. In SI this is accomplished by *S*-specific recognition of haplotype-specific proteins expressed in pollen and pistil. Since mating types are under frequency-dependant selection diversity is favored but generating new types is constrained by strict requirements for co-evolution of pollen and pistil factors.

Similarly, pathogens can increase their success by diversifying to avoid recognition. Immune systems also must co-evolve to maintain specific recognition such that pathogens trigger rejection but self-factors do not. Finally, both SI and immunity entail rejection and while there are a variety of potential mechanisms to reject undesirable cells, whether self or nonself, it is not an infinite variety. Thus, it is not surprising to encounter common pathways such as programmed cell death or RNase-based cytotoxicity in both systems.[27,45,86] Notwithstanding the finite number of rejection mechanisms for self or nonself cells some speculate that SI and immunity share common ancestry. This begs the question of whether SI and immunity are related. Could an immunity protein active in self-recognition have been adapted for self-rejection in plant reproduction?[2,3] Answering this question would help researchers understand the origins of SI, however, conclusive evidence is lacking. Yet, within the terrestrial plants plausible cases for common ancestry exist. The wheat leaf rust kinase (WLRK) is a possible example of divergence, as it is similar to the SRK deployed in the *Brassica* SSI system described earlier.[87] Further, in animals, the Major Histocompatibility Complex (MHC) genes, share some parallels with SI genes because they both have ancient allelic lineages, a high allelic diversity, sequence divergence and display balancing selection.[28,88] Also, resemblances can be found between the SSI pollen-determinant SCR and defensins. Defensins are involved in innate immunity in animals, plants, fungi and bacteria.[89,90] SCR is structurally similar to defensins, but there is little or no primary sequence conservation.[91] Nasrallah (2005) speculated that a family of ancient defense molecules, involved in nonself recognition may have been adopted for both self-recognition systems and cell-to-cell signaling and that regardless of any evolutionary relationship, natural environmental pressures, such as frequent exposure to diverse pathogens or unsuitable pollen, will have caused SI and immune systems to evolve similar mechanisms to defend themselves.[2] Thus, similarities between SI and immunity may reflect convergent evolution driven by similarities in the biological problems and the molecular machinery available to solve them. Therefore, the dissimilarities between SI and immunity are, perhaps, more noteworthy than the similarities. Fundamentally, the biochemistry of recognition and defense differ markedly.

CONCLUSION

Homomorphic SI provides reproductive barriers that prevent self-pollen from fertilizing ovules and producing offspring. This barrier has contributed to the success of the angiosperms and their colonization of niches on every continent. Homomorphic SI entails molecular recognition and, thus, bears some superficial resemblance to immune recognition. Recognition between SRK and SCR in sporophytic SI has clear similarities with transmembrane TLR recognition of PAMPs in innate immunity. Upon positive recognition both SI and immunity systems initiate defensive mechanisms that may include common mechanisms such as the programmed cell death response in *Papaver* GSI.

The parallels between SI and immunity have drawn some researchers to hypothesize that they diverged from a common ancestral recognition system. Whereas plants, fungi and animals use immunity systems to identify and reject nonself organisms, SI systems actively reject pollen tubes from self or closely related potential mates. As in immunity, the genes controlling SI are often very polymorphic and recognition consists of interactions mediated by specific proteins. The outcome of recognition is expressed as a form of compatibility in each case. In pollination, compatibility means fertilization is allowed.

In immunity compatibility may lead to disease or resistance depending on nomenclature, but specific recognition occurs in either case. Whether these two mechanisms are descended from a common mechanism is uncertain and presently the overriding fact is that they are very different systems that display superficial similarities. However, this does not necessarily mean that there is no relationship. Indeed, the small similarities may be the keys to elucidate an evolutionary relationship. Nevertheless, a putative evolutionary transition from immunity to SI will be difficult to prove because both systems require diversifying selection.

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CHAPTER 8

SIGNALING PATHWAYS THAT REGULATE LIFE AND CELL DEATH:

Evolution of Apoptosis in the Context of Self-Defense

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Abstract: Programmed Cell Death is essential for the life cycle of many organisms. Cell death in multicellular organisms can occur as a consequence of massive damage (necrosis) or in a controlled form, through engagement of diverse biochemical programs. The best well known form of programmed cell death is apoptosis. Apoptosis occurs in animals as a consequence of a variety of stimuli including stress and social signals and it plays essential roles in morphogenesis and immune defense. The machinery of apoptosis is well conserved among animals and it is composed of caspases (the proteases which execute cell death), adapter proteins (caspase activators), Bcl-2 family proteins and Inhibitor of Apoptosis Proteins (IAPs). We will describe in this chapter the main apoptotic pathways in animals: the extrinsic (death receptor-mediated), the intrinsic/mitochondrial and the Granzyme B pathway. Other forms of non-apoptotic Programmed Cell Death which occur in animals will also be discussed. We will summarize the current knowledge about apoptotic-like and other forms of cell death in other organisms such as plants and protists.

Additionally, we will discuss the hypothesis that apoptosis originated as part of a host defense mechanism. We will explore the similarities between the protein complexes which mediate apoptosis (apoptosomes) and complexes involved in immunity: inflammasomes. Additional functions of apoptotic proteins related to immune function will be summarized, in an effort to explore the evolutionary origins of cell death.

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INTRODUCTION: PROGRAMMED CELL DEATH

Cell death regulated by a genetic program, or Programmed Cell Death (PCD), is a fundamental mechanism of homeostasis of tissues. For this reason, it is a critical process for many multicellular organisms, which have developed different programs of programmed cell death such as apoptosis. Programmed cell death, however, does not only occur in multicellular organisms: it has also been shown to occur in yeast, protozoans, bacteria and unicellular algae. This suggests that a program designed for self killing of individual cells is in the long-term beneficial for the colony or 'group of individuals'.

Programmed Cell Death (PCD) has been studied thoroughly in animals. In response to stress due to starvation or damage, animal cells engage a biochemical pahtway to destruct themselves. Moreover, upon detection of a virally infected cell, cytotoxic cells kill other cells from the same organism by inducing their suicide. Cell death is not only employed to remove damaged or infected cells, but it is also important for sculpting tissues. During embryonic development, animals produce many cells which are no longer needed in the adult animal and which therefore undergo PCD to eliminate themselves. Classical examples of this are the removal of the tadpole of frogs during metamorphosis or the disappearance of interdigital tissues during mammalian development to sculpt fingers. In the adult animal, unwanted cells are eliminated through PCD when they are no longer needed. For instance, during an immune response there is a rapid production of lymphocytes which respond to a specific pathogen. These lymphocytes have to be eliminated after the pathogen has been cleared. For these reason, these cells are 'programmed' to eliminate themselves after a few days of life.

Programmed cell death can occur through different biochemical programs. Apoptosis is the best known of these programs and it is the major form of cell death in animals. This process occurs through a coordinated dismantling of a cell in a matter of hours. Cells shrink, detach and end up forming small pieces or 'apoptotic bodies', which are immediately cleared by phagocytes and are thus removed from the body in a silent way. During apoptosis, cells maintain integrity of their plasma membrane, which helps avoid inflammation. In contrast, cell death by 'necrosis', which occurs in situations of uncontrolled tissue damage (for instance after traumatic injury due to heat shock or radiation) is a 'passive' form of cell death which triggers inflammation.

Apoptosis requires a number of genes that are well conserved across animal evolution. In the past few years, multiple genetic and cell biology experiments have shown that apoptosis is executed through similar biochemical pathways in vertebrates, Drosophila and *C. elegans*. These biochemical pathways lead to activation of proteins termed 'caspases'. What distinguishes apoptosis from other forms of cell death is a number of morphological and biochemical features which are consequence of the activity of caspases.

APOPTOSIS IS EXECUTED THROUGH ACTIVATION OF CASPASE PROTEASES

Caspases are cysteine proteases (they have a cysteine in the active site) which achieve apoptotic cell death through the cleavage of several substrates. A few hundred caspase substrates have been described in human cells. Roughly, we can say that during apoptosis, caspases cleave two different subsets of proteins. The first group comprises proteins that are necessary for maintenance of cellular structures (cytoskeletal components, organelle

proteins, etc.) and proteins whose cleavage induces activation of proteins that destroy the cells, such as nucleases. Cleavage of these substrates produces the morphological changes associated with apoptosis. The second group of apoptotic caspase substrates comprises proteins that are involved in what we could define as "life support" functions (transcription and translation, metabolism, growth promoting signaling molecules etc.).[1] By cleaving these substrates, caspases ensure the termination of the life of the cell.

Caspases are normally present in the cytosol. Since killer proteases are obviously dangerous, they are kept in check by a number of safety mechanisms. The most important is that they are inactive until a 'deadly' stimulus activates them by promoting their oligomerization and subsequent cleavage. Cleavage of the caspase precursors, which we call the 'procaspases', allows the formation of the mature proteases. When activated, caspases can cleave and activate other caspase molecules and this leads to an irreversible proteolytic cascade that ends up killing the cell. It should be noted that not all caspases are involved in apoptosis; there is a group of caspases which includes caspase-1 which play roles in inflammation but not in cell death, as will be discussed in more detail later. Caspases of this group are called 'inflammatory caspases' as opposed to the rest of caspases, which are apoptotic caspases. We can classify apoptotic caspases in two groups based on their sequence homology and their role in the proteolytic cascade: initiator (apical) and executioner (effector) caspases. Initiator caspases (caspase-8 and -9 in mammals) have a long domain in the N-terminus of the protein: the pro-domain. Pro-domains are responsible for interactions between initiator caspases and the molecules which activate them. These domains are mainly of two types: CARD (Caspase Recruitment Domain), present in caspase-9 and DED (Death Effector Domain), present in caspase-8. These domains are protein interaction modules composed of six alpha-helical bundles. Upon an apoptotic stimulus, homotypic CARD-CARD or DED-DED interactions bring two molecules of initiator caspases in close proximity to each other and this event leads to their activation and subsequent inter-molecule cleavage.

The so-called 'executioner' or 'effector caspases' (caspase-3 and -7 in mammals) are the ones that cleave the substrates required for death of the cell. These proteins are inactive until they are cleaved by 'initiator' caspases. Since activation of executioner caspases require previous activation of initiator caspases, the first steps leading to initiator caspase activation are highly regulated events which determine the onset of apoptosis.

In vertebrates there are several biochemical pathways that can activate caspases. Notably, these pathways are quite similar to apoptotic pathways in other species such as *C. elegans* and *D. melanogaster* (Fig. 1). As a summary, a stimulus (coming either from within the cell or from the outside) triggers the formation of a multi-protein complex which recruits and activates an initiator caspase. Activation of these caspases occurs when several molecules are recruited to these 'deadly complexes' by oligomerization with the so-called 'adapter molecules' or caspase activators. Initiator caspases then cleave and activate effector caspases and this triggers death of the cell.

CONSERVED APOPTOTIC REGULATORS IN VERTEBRATES, FLIES AND NEMATODES: CASPASES, IAPS, ADAPTER MOLECULES AND BCL-2 FAMILY PROTEINS

Stress and developmental or social cues induce apoptosis through a biochemical pathway which is very similar in mammals, flies and nematodes. The caspase activation

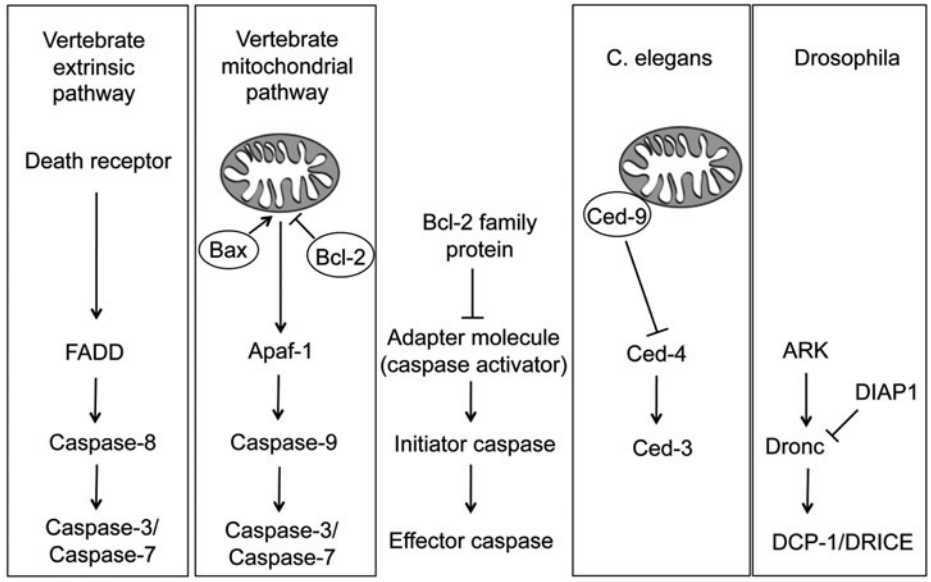

Figure 1. Apoptosis in animal models. The apoptotic machinery in insects (*D. melanogaster*), nematodes (*C. elegans*) and vertebrates is very similar. Adapter proteins such as Apaf-1 or FADD recruit activator caspases, which cleave and activate effector caspases. In vertebrates and *C. elegans*, Bcl-2 proteins control the pathway either directly by inhibiting the Apaf-1-like molecule (in *C. elegans*) or indirectly by controlling mitochondrial permeabilization, which in vertebrates is required for formation of the Apaf-1/caspase-9 complex (apoptosome).

cascade begins when several molecules of initiator caspases are activated through oligomerization in a complex termed 'apoptosome'. In mammalian cells, the apoptosome is nucleated by the adapter molecule Apaf-1, which recruits the initiator caspase-9 through interactions between the CARD domain present both in Apaf-1 and caspase-9 (Fig. 2). Caspase-9 bound to the apoptosome can cleave and activate effector caspases-3 and -7, which are responsible for the death of the cell.

In the nematode *C. elegans*, caspases are also activated through the formation of apoptosomes around Apaf-1-like molecules. In fact, the homolog of Apaf-1 in nematodes, Ced-4, was discovered before Apaf-1. *C. elegans* has been an invaluable tool to identify the proteins which participate in apoptosis. The reason is that adult animals all have exactly the same number of cells. During development, a number of cells that are no longer needed in the adult animal die by apoptosis. This way, it is easy to identify mutants with 'extra' cells (mutated in genes essential for apoptosis) or with an excess in cell death (possibly due to a mutation in a gene which inhibits apoptosis). This system led to the identification of the essential apoptotic genes. Caspases, adapter molecules (Apaf-1-like) and Bcl-2 family proteins were first identified as key components of the apoptotic cell death machinery in the nematode and their homologs are also key for apoptosis in mammals.

The apoptotic pathway in *C. elegans* is relatively simple (Fig. 1). It comprises three proteins: a protein located in the mitochondrial membrane, Ced-9 (homolog of human Bcl-2 family proteins), a caspase activator, Ced-4 (Apaf-1 homolog) and a caspase, Ced-3.[2] Ced-9 holds the caspase activator Ced-4 inactive. At a point during development of the

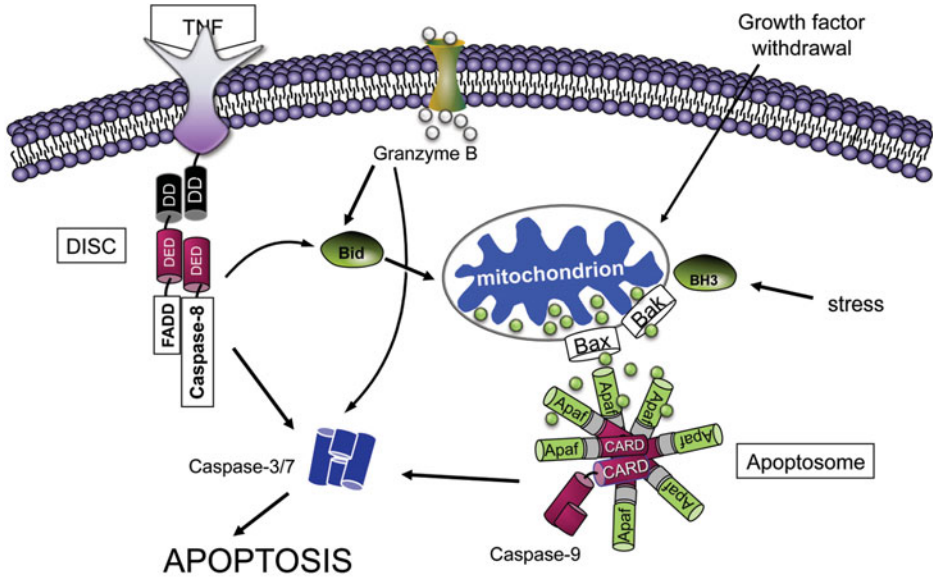

Figure 2. Two main pathways of apoptosis in vertebrates: the extrinsic and the mitochondrial pathway. In the extrinsic pathway (left), a death ligand such as TNF, TRAIL or Fas Ligand activates a death receptor, which recruits FADD through its Death Domain (DD). FADD recruits Caspase-8 through Death Effector Domain (DED) interactions. Caspase-8 can directly cleave and activate effectors caspases such as -3 and -7, or cleave Bid to induce Bax/Bak activation. In the mitochondrial pathway, a BH3-only protein (labeled "BH3") such as Bid, Puma, Noxa etc. activates Bax and/or Bak which permeabilize mitochondria triggering cytochrome c release (depicted as small spheres). Cytochrome c triggers the oligomerization of Apaf-1 and formation of the apoptosome, where Caspase-9 is activated. Caspase-9 cleaves and activates effector caspases.

animal, an inhibitor of Ced-9 is synthesized in some cells and this promotes the release of Ced-4. Ced-4 then forms an apoptosome that recruits and activates the caspase Ced-3 through CARD-CARD interactions and this triggers death of the cell. This pathway is simpler than in mammals, since no initiator caspases are needed: the caspase Ced-3 functions as both activator and effector caspase and executes death upon activation.

The apoptotic process has been also characterized in detail in the fly *Drosophila melanogaster*. Caspase activation pathways are also quite similar, with caspases being activated through recruitment to an apoptosome, which is nucleated by a protein very similar to Ced-4 and Apaf-1: ARK. In Drosophila, however, activation of the apoptosome is triggered in a different form: during development, several proteins are synthesized that inactivate a caspase inhibitor: DIAP1. DIAP1 is holding the initiator caspase-9 homolog, DRONC, inactive. Degradation of DIAP1 is sufficient to trigger apoptosome formation and apoptosis. DIAP1 belongs to a family of proteins whose homologs have anti-apoptotic functions in mammals. These proteins, named IAPs (Inhibitor of Apoptosis Protein) bind and inactivate caspases directly or promote their ubiquitination and proteasomal degradation.

The genes encoding proteins of these four groups of apoptosis-related proteins (Bcl-2 homologs, Apaf-1 homologs, IAPs and caspases) exist in all animals studied.[3,4] This suggests that the apoptotic process existed in the precursor of animals (Table 1). To summarize,

Table 1. Summary of presence of apoptotic proteins in animals

	Ecdysozoa		Cnidaria	Deuterostomia		
Phyla	Arthropoda (insects and others)	Nematoda (roundworms and others)	(jellyfish, Hydra)	Echinodermata (starfish, sea urchins)	Chordata: Vertebrata (fish, mammals and others)	Chordata: Urochordata (tunicates)
Caspases	7 in Drosophila	+	+	+	+	+
Death receptors/ligands	TNFR-like without DD	–	?	Several genes	Several proteins	?
Bcl-2-like proteins	+	+	+	+	+	+
Apaf-1	+	+	+	+	+	not detected
NOD-like proteins, non-Apaf	+	+	?	+	+	?
IAPs or BIR-containing proteins	Several IAP genes	BIR-containing proteins	Several genes	Several genes	Several IAP genes	BIR-containing proteins

Caspases are the proteases responsible for cell death and they exist in all animals studied. Death receptors and their ligands activate the extrinsic apoptotic pathway in vertebrates and they are involved in immune responses in insects and mammals. Bcl-2 family proteins regulate apoptosis in vertebrates and *C. elegans*, but their role in other organisms is unclear. IAPs (Inhibitors of apoptosis proteins) inhibit apoptosis in Drosophila and mammals. BIR-containing proteins are homologs of IAPs which contain only the BIR domains present in IAPs, but not other domains. These proteins do not necessarily play roles in apoptosis.

we can conclude that apoptotic pathways in animals from three different animal phyla are similar, but they differ in the form in which initiator caspases are activated. In Drosophila and *C. elegans*, apoptosomes are held constitutively inactive due to the presence of an inhibitor and they are activated upon the synthesis of a molecule that neutralizes this inhibitor. In Drosophila, this inhibitor is a caspase inhibitor (DIAP1). In *C. elegans*, the inhibitor is a Bcl-2 family protein, Ced-9. On the contrary, in mammals, the apoptosome is formed upon the presence of an activator. This activator is cytochrome c, which is a molecule which sits in the mitochondria in healthy cells and during apoptosis it is released to the cytosol where it activates the apoptosome (Fig. 2).

CELL SUICIDE: MITOCHONDRIA AND BCL-2 FAMILY PROTEINS REGULATE "SELF-INDUCED" CELL DEATH IN MAMMALS

In vertebrates, including Zebrafish and mammals, cell death is controlled by a group of proteins that belong to the family of the oncogene Bcl-2.[5] As discussed above, in *C. elegans*, a Bcl-2 family protein (Ced-9) controls apoptosis in a direct manner by holding

the apoptosome-forming protein Ced-4 inactive. In vertebrates the situation is different. Bcl-2 proteins control caspase activation indirectly, through controlling mitochondrial permeabilization. Mitochondria are organelles which provide energy and metabolites to the cell. But these organelles also participate in apoptosis in several organisms, because they contain proteins which can activate caspases when the outer membrane is permeabilized. As discussed above, apoptosis is initiated upon the formation of the apoptosome, a cytosolic complex formed by multimers of Apaf-1, similar to the complex which initiates apoptosis in flies and nematodes.[6] This complex is formed by seven molecules of Apaf-1 which recruit and activate Caspase-9 (Fig. 2). The stimulus which triggers the formation of the mammalian apoptosome is the binding of cytochrome c to Apaf-1. The discovery of the requirement of cytochrome c for apoptosis was surprising, since this is a protein of the mitochondrial respiratory chain. Cytochrome c is normally playing a role in cell metabolism, inside the mitochondria. If so, how does cytochrome c locate to the cytosol during apoptosis? A number of models have been proposed, including the formation of putative channels that would provoke mitochondrial swelling, leading to rupture of the outer mitocondrial membrane. However, the current model implies that two proteins of the Bcl-2 family, Bax and Bak, integrate in the mitochondria during apoptosis and form pores that allow the passage of intermembrane space proteins, including cytochrome c.[7] Bax and Bak are required and perhaps sufficient to form pores in the outer mitochondrial membrane. These proteins, when activated, can form pores in liposomes.[8]

Bax and Bak are part of the Bcl-2 family of proteins. This family is divided in three groups. The first group is comprised of homologs of the mammalian protein Bcl-2 which have an antiapoptotic function, including Bcl-2 itself. Members of this family such as Mcl-1 and Bcl-xL, like Bcl-2, are overexpressed in many human tumors and protect tumor cells from apoptosis. The proapoptotic proteins Bax and Bak are part of the second subset of Bcl-2 family proteins: these proteins are called 'multidomain' proapoptotic Bcl-2 proteins because they share a number of domains with the antiapoptotic Bcl-2 homologs. In fact, the tridimensional structure of the anti-apoptotic and the pro-apoptotic multidomain Bcl-2 proteins is remarkably similar, although they have opposite functions. Bax and Bak form pores in the mitochondrial membranes, Bcl-2, Bcl-xL and Mcl-1 inhibit the formation of these pores and the release of cytochrome c.

The third group of Bcl-2 family proteins comprises pro-apoptotic proteins such as Bid, Bim, Bad, Puma and Noxa. These proteins are less similar to Bcl-2 than Bax and Bak: they only share the BH3 domain, which is a short motive of around 25 amino acids. For these reason, these proteins are called 'BH3-only' proteins. The BH3 domain is required for these proteins to bind to the multidomain Bcl-2 proteins and exert their proapoptotic function. BH3 proteins can directly bind and activate Bax and Bak, while they also inactivate antiapoptotic Bcl-2 proteins. Apoptosis is initiated when a sufficient number of BH3-only molecules inactivates the antiapoptotic proteins and activates the proapoptotic ones.[9] Some BH3-only proteins are specifically induced after some types of stress. For instance, Puma is induced in a p53-dependent manner after genotoxic stress and Bim is induced after endoplasmic reticulum stress. Other BH3-only proteins are constantly present in some cell types but they are kept inactive through posttranslational mechanisms such as phosphorylation, and certain stresses activate them through removal of these modifications. This way, BH3-only proteins function as 'stress sensors' that determine whether a cell will die.

Social signals such as growth factor limitation or loss of cell-to-cell contact also induce apoptosis through activation of BH3-only proteins and induction of the mitochondrial

pathway. As discussed above, in multicellular organisms, cell death contributes to maintenance of tissue homeostasis. When cells lack proliferation factors due, for instance, to tissue overgrowth, they stop proliferating and in many cases, they die by apoptosis. This is critical for the maintenance of cell numbers in tissues with high cell turnover, particularly the immune system. Some immune cells are "addicted" to cytokines and when they are deprived of cytokines they stop growing and undergo apoptosis mediated by the activation of BH3-only proteins.[10] Indeed, mutations of several components of the mitochondrial pathway produce a number of phenotypes related with hyperproliferation of immune cells in mice and mutations in BH3-only proteins are associated with human immune diseases.[11]

IS THERE A ROLE OF THE MITOCHONDRIA IN APOPTOSIS OF INVERTEBRATES?

Apoptotic pathways are fairly similar in nematodes, flies and vertebrates. Caspases are required for apoptosis in all animals studied and so are their activators (Apaf-1-like molecules). In mammals, the mitochondrial pathway is of great relevance, because it is activated in response to multiple stimuli. Mitochondria (and cytochrome c) are required for apoptosome-mediated caspase activation in response to a diversity of stimuli, including developmental cues, growth factor withdrawal, heat shock, nutrient deprivation, endoplasmic reticulum stress and DNA damage. As discussed above, during apoptosis mitochondria release cytochrome c upon formation of pores composed of Bax and/or Bak. Cytochrome c then binds Apaf-1 and the subsequent conformational change of Apaf-1 attracts Caspase-9, leading to the formation of the apoptosome.

But when did this connection between mitochondria and apoptosis arose? A role for cytochrome c has not yet been found in animals other than mammals. Our other two main animal models to study apoptosis, *C. elegans* and *D. melanogaster*, do not seem to require neither homologs of Bax/Bak proteins acting on mitochondria, nor mitochondrial permeabilization or the release of cytochrome c. In *C. elegans*, the Bcl-2 homolog Ced-9, which is a mitochondrial protein, controls apoptosis by keeping the Apaf-1 homolog Ced-4 inactive and not by releasing cytochrome c. Indeed, cytochrome c could not possibly activate Ced-4 in the same manner in which cytochrome c activates Apaf-1, because Ced-4 does not contain the domain which is responsible for Apaf-1 binding to cytochrome c: the WD40 domain. It is intriguing, though, that *C. elegans* Ced-9, which controls apoptosis, is a mitochondrial protein, like mammalian Bcl-2 proteins. This suggests an ancestral link between mitochondrial permeabilization and apoptosis that may have not been maintained in nematodes.[12]

Drosophila apoptosome does not require cytochrome c either. The Apaf-1 homolog, ARK, does contain the WD repeats which are responsible for the interaction of Apaf-1 with cytochrome c. It is possible that during evolution of insects, an ancestral role for cytochrome c was lost in favor of a more direct way to control apoptosis: the synthesis of DIAP inhibitors. We definitively need to expand our knowledge of how apoptosis proceeds in other metazoan phyla in order to understand how the mitochondria-caspase connection arose. It is possible that it appeared early during evolution and that it was lost in nematodes and insects, which are relatively close groups in evolutionary terms. These organisms would have simplified the apoptotic pathways by eliminating the mitochondrial control, reducing this way the number of molecules implicated in induction of apoptosis.

This hypothesis is supported by the fact that cnidarians and equinoderms contain numerous Apaf-1 homologs, some of which can potentially interact with cytochrome c through their WD repeats.[3] However, the alternative possibility, that mitochondrial control of apoptosis appeared during evolution of vertebrates, cannot be discarded at this point.

CELL DEATH BY SUICIDE INDUCTION: THE DEATH RECEPTOR (EXTRINSIC) APOPTOTIC PATHWAY

In vertebrates, the mitochondrial pathway is responsible for induction of apoptosis upon stress, or upon loss of survival signals such as cytokines or growth factors. As discussed, this pathway is regulated by Bcl-2 family proteins and it is mediated by cytochrome c release from mitochondria, apoptosome formation and activation of caspase-9, which is the apical or initiator caspase in this pathway. While the mitochondrial pathway is of vital importance for tissue homeostasis and stress responses, there is a second apoptotic pathway with important roles in immune response and tumorogenesis: the extrinsic pathway.

The extrinsic or death receptor-mediated pathway is induced upon activation of receptors related to Tumor Necrosis Factor (TNF) receptor. TNF-α is a cytokine which participates in immune responses through the activation of NF-kappaB. But under some circumstances, the outcome of stimulating a cell with TNF is death of the cell instead of NF-kappaB activation. TNF-α and its receptors belong to a family of proteins (the TNF superfamily) which comprises a few dozen proteins with roles in inflammation and immunity. A subset of the TNF-related proteins can induce cell death and are named death ligands. These proteins are TNF-α, Fas ligand (CD95L) and TRAIL (TNF-related apoptosis-inducing ligand). Death ligands are secreted proteins which behave as cytokines that regulate inflammation and other immune-related processes. They can also be expressed in a membrane-bound form by lymphocytes and Natural Killer cells, which use them to kill infected or antigenic tumor cells through the induction of apoptosis in their targets.

The extrinsic pathway is very similar in mammals and fish.[13] This form of apoptosis requires a few molecules that couple the signal from the ligand to caspase activation (Fig. 2). Death ligands induce oligomerization and conformational change of their receptors, the so-called 'death receptors'. These receptors contain in the intracellular portion a 'Death Domain' (DD), which is evolutionarily related to the CARD domain present in caspase-9 and other caspases. Upon oligomerization, the receptor recruits through a homotypic interaction an adapter molecule that also contains a Death Domain. In most models, this molecule is FADD. The other portion of FADD comprises a Death Effector Domain (DED). This domain, which is also structurally and evolutionarily related to CARD domains, is present in the initiator caspase-8. When FADD aggregates in death receptor complexes, caspase-8 is recruited to the complex through homotypic DED-DED interactions, in a manner that resembles CARD-CARD interactions to form the apoptosome. The complex that contains the death receptor, FADD and caspase-8 is called the Death Receptor Signaling Complex (DISC).

The activation of caspase-8 at the DISC is not sufficient per se to kill the cell. This protease, upon activation, cleaves and activates effector caspases such as caspase-3 and this leads to death of the cell. In some cell lines, however, activation of caspase-8 is not sufficient to activate caspase-3 directly. In these cells, caspase-8 needs an amplification signal to induce effector caspase activation: it cleaves the BH3-only protein Bid. Bid then acts on Bax and/or Bak on the mitochondrial membrane to trigger the release of

cytochrome c and the formation of the apoptosome. For this reason, in some cell types Bcl-2 or Bcl-xL overexpression blocks death receptor-induced cell death.[14]

This apoptotic pathway seems to only be present in vertebrates. No death receptors have been found in *C. elegans*. In Drosophila, the TNF-receptor homolog, Wengen, can induce cell death, but as we will discuss later, this form of cell death is not classical apoptosis and it does not require caspase-8.[15]

CELL DEATH BY MURDER: THE GRANZYME PATHWAY

Cytotoxic lymphocytes (CTL) and Natural Killer (NK) cells owe their names to their ability to induce death of their target cells. In order to avoid propagation of a virus, cytotoxic cells attack and kill the infected cells and they use several effector mechanisms in order to do so. One mechanism is the induction of apoptosis in the target cell through the extrinsic pahtway. CTL and NK cells can express Fas Ligand in their surface. This death ligand, as discussed above, can activate the death receptor Fas in the target cell and thus induce its suicide through caspase-8 activation. Moreover, cytotoxic cells deliver the content of toxic granules towards the target cell. These granules contain, among other components, TNF, which in a manner similar to Fas Ligand can interact with its receptor, leading to activation of the TNF-receptor mediated extrinsic pathway and subsequent caspase-8-mediated death. Additionally, granules contain perforin, a protein that forms transmembrane channels and facilitates intracellular delivery of the protease Granzyme B, another component of cytotoxic granules.

Granzyme B can induce apoptosis through directly activating caspases.[16] This protease is able to activate caspases through its proteolysis, in a similar manner than initiator caspases activate other caspases. Several caspases have shown to be activated after treatment with a combination of Granzyme B and perforin, including caspase-8 and the effector caspases -3 and -7. Cleavage and activation of caspase-3 can lead directly to 'classical' apoptosis. However, Granzyme B can also use a mitochondrial pathway to induce apoptosis, by cleaving the BH3-only protein Bid (Fig. 2). Bid cleavage activates the Bax/Bak mediated mitochondrial apoptotic pathway, with subsequent activation of the apoptosome and the caspase-9 mediated pathway. Granzyme B-mediated killing can be inhibited by overexpression of Bcl-2 in human cells, indicating that the mitochondrial pathway is more relevant than direct activation of effector caspases.

APOPTOSIS IS NOT THE ONLY WAY TO DIE: NON-APOPTOTIC FORMS OF PROGRAMMED CELL DEATH IN METAZOANS

Apoptosis is the main form of cell death in animals. However, it is not the only form of programmed cell death which has been observed in animals. Cells can die by pathways that do not involve caspase activation and the morphology of dying cells can be completely different from the classical morphology of apoptotic cells.[17] It should be noted that there is still a good level of disagreement regarding the definition and the physiological roles of some of these alternative forms of cell death. Traditionally, before we had been able to start the molecular characterization of apoptosis (which started in the nineties), cell death had been classified in three different types according to morphology: apoptotic (Type I), autophagic (Type II) and necrotic (Type III). This classification was

based on microscopic analysis of developing animals and has long been abandoned by researchers in the field. After the discovery of the molecular pathways of apoptosis, we could characterize apoptosis more precisely than if we regarded morphology only. We now define apoptosis as caspase-dependent cell death, which is usually accompanied by morphological changes which are consequences of caspase activation, including chromatin condensation and cell shrinkage.

After the beginning of the molecular characterization of apoptosis, for many years, other forms of cell death used to be classified by default as 'necrosis', or accidental, nonregulated death. However, in recent years, it has been acknowledged that non-apoptotic cell death can occur in a regulated fashion and some molecules that regulate non-apoptotic cell death have been identified. At present, we still use the term 'necrotic cell death' to define death which proceeds without signs of apoptosis. But this includes true "accidental", sudden, uncontrolled cell death (for instance, after severe heat shock, ischemia or mechanical rupture) but also other forms of non-apoptotic cell death such as necroptosis, which is regulated by a number of proteins which we are beginning to identify (Table 2).

Necroptosis (or death receptor-induced necrosis) is a form of cell death induced by death ligands and mediated by the protein RIP1.[18,19] RIP (RIPK1) is a kinase which associates with death receptor/NF-kappaB-activating complexes. As discussed above, activation of death receptors usually kills cells through caspase-8 activation and the extrinsic pathway. However, in some cell types, ligation of death receptors –in particular, TNF-receptors- induces a form of cell death which is not mediated by caspases. Not only cells die in a caspase-independent manner, but indeed, caspase inhibition can enhance necroptotic cell death. Necroptosis is mediated by production of Reactive Oxygen Species (ROS) and by the JNK kinase. This form of cell death, although only described in mammals so far, resembles the way by which Drosophila TNF kills cells: in Drosophila, overexpression of Wengen (the TNF receptor homolog) or Eiger (its ligand) kill in an ROS and JNK-dependent manner, independently of caspase-8.[15] The involvement of the apoptosome and caspases in Eiger-induced cell death is unclear, since several reports show contradictory results. The resemblance between Eiger-induced cell death and necroptosis is strengthened by the fact that a similar group of proteins (such as RIP1, JNK, CYLD and TRAFs) regulate necroptosis in mammals, as well as Eiger-induced cell death in Drosophila.[20] This suggests a possible conservation of a non-apoptotic cell death pathway which role seems to be related to immune defense, since necroptosis has been shown to occur after treatment of virally infected cells with TNF and upon ligation of Toll-like receptors.

'Autophagic cell death' is a form of cell death observed during development of salivary glands in Drosophila.[21] By morphological criteria, this form of cell death is different from apoptosis, since it is associated with massive vacuolization due to the presence of a great number of autophagic vesicles. These vesicles are doubled membrane vesicles which engulf organelles or cytoplasm to target them to lysosomes for degradation. Autophagic cell death is induced in salivary glands or the midgut of Drosophila upon developmental cues. Although this form of cell death has been shown to exist in the fly, an enormous debate has been generated in the past few years regarding whether autophagic cell death exists in mammals. In general, autophagy is a cytoprotective process. In some cases, autophagic vesicles are observed in cells that undergo apoptosis and in other cases, autophagic proteins have been shown to promote caspase activation and apoptosis. This had lead to the erroneous conclusion that cell death under some circumstances is "autophagic". To complicate the issue, 'autophagic cell death' has been

Table 2. Types of Programmed Cell Death

Type of Programmed Cell Death	Organism	Function	Key Molecules	Morphological Characteristics
Apoptosis—extrinsic pathway	Vertebrates	Immune homeostasis	Death receptors, death ligands, caspases	Chromatin condensation, cell shrinkage, phosphatidylserine exposure, DNA degradation
Apoptosis—intrinsic/mitochondrial pathway	Vertebrates, *C. elegans*	Development, elimination of damaged cells	Bcl-2 family proteins, caspases	Same as above
Apoptosis	Drosophila	Development	DIAP1, caspases	Same as above
Necroptosis	Mammals	Possibly, immune defense	RIPK1, ROS, JNK	Plasma membrane rupture
Necrosis	All organisms	Probably, no function	unknown	Cytoplasmic swelling, plasma membrane rupture
Hypersensitive Response	Plants	Immune response	ROS, proteases, resistance (R) gene products	Large cytoplasmic vesicles, release of hydrolytic enzymes into the cytoplasm
Viral, aging or stress-induced cell death	Yeast	Possibly, maintenance of fittest cells	ROS, proteases	Chromatin condensation, shrinkage
Autophagic cell death	Dictiostelium	Development	Autophagy genes	Massive vacuolization of the cytoplasm
Autophagic cell death	Drosophila	Organ involution during development	Autophagy (Atg) genes, caspases	Massive vacuolization of the cytoplasm (autophagic vesicles)

described in mammalian cells in culture upon treatment with caspase inhibitors and it has been shown to be mediated by JNK and RIP1; all of these are features of necroptosis. This suggests that autophagic cell death and necroptosis are the same form of cell death. The involvement of autophagy –but not caspases– in a physiologically relevant cell death process in mammals remains to be proven.

A process involving programmed cell death which we are all familiar with is death of skin cells. The skin is constantly being renewed and this renovation involves death of cells in outer layers of the skin. These cells do not die by apoptosis, but by a process called ‘cornification’ or ‘keratinization’ of the outer layers of the epidermis. Keratinization involves caspases such as caspase-14 which do not play roles in apoptosis. Besides

keratinization, there is another form of cell death which involves non-apoptotic caspases. Pyroptosis requires the activation of caspase-1, which is an inflammatory caspase that does not play a role in apoptosis. Pyroptosis, also called 'caspase-1 dependent necrosis', has been observed in macrophages upon infection or treatment with lipopolysaccharide. This form of death does not require effector caspases. Dying cells display mixed morphological features of apoptosis and necrosis. Because this form of death occurs upon infection and it is pro-inflammatory, it is likely that the purpose of this form of cell death is to awaken the immune system.

CELL DEATH IN PLANTS, FUNGI AND PROTISTS

Apoptosis is not the only form of cell death in animals, which indicates that a number of alternative cell death programs exist in nature. Programmed cell death has been observed during development or in response to infection in a number of organisms (Table 2).

In plants, cell death is part of the normal cycle of the organism. Developmental cell death is observed in many plant tissues, being crucial for instance for senescence of leaves and flowers, formation of polen and seeds and terminal differentiation of vascular elements. Moreover, plant cells undergo Programmed Cell Death in response to infection. When attacked by a pathogen, plants engage in a form of immune response which involves death of the tissue which surrounds the infected site. This phenomenon was named the 'hypersensitive response' because the plants which could respond and resist a pathogenic infection seemed to be "hypersensitive", in the sense that abnormal cell death was observed in the sites of infection.[22] Cell death is mediated by early massive production of Reactive Oxygen Species and by proteases. The morphology of dying cells in these tissues shares some characteristics of apoptosis such as chromatin condensation and protoplast retraction (shrinkage). However, the morphology of dying plant cells is in general more similar to necrosis of mammalian cells, with massive vacuolization. Intriguingly, caspase-like protease activities have been readily detected during several forms of cell death in plants and cell death can frequently be attenuated by the use of peptidic inhibitors of mammalian caspases. This has led to the proposal that cell death in plants can occur by apoptosis. Since there are no caspases in plants, it was proposed that their distant relatives, metacaspases, participate in apoptotic-like cell death of plants and unicellular organisms, but this is still under debate. Metacaspases differ from caspases in their cleavage specificity and probably in their physiological substrates.[23] Moreover, it is unclear why plants would undergo apoptotic-like cell death, whose main purpose is, in opposition to other forms of cell death, to eliminate cells in a "clean" manner without rupture of the plasma membrane and without inflammation. In animals, apoptotic bodies are rapidly eliminated by phagocytosis. However, the cell wall in plant cells would preclude any possible phagocytosis of dying cells.

Yeast are unicellular fungi. At first sight, the existence of programmed cell death in unicellular organisms does not make much sense. However, yeast undergo a form of programmed cell death which resembles apoptosis. A number of stress-inducing agents such as UV radiation, acetic acid or antimicrobial peptides trigger apoptotic-like cell death which can be inhibited by inactivation of certain genes, including the yeast metacaspase Yca1. Moreover, apoptotic-like cell death has been shown to occur as a consequence of chronological aging. The "age" of the individual is determined, in

budding yeast, by the number of daughter cells that a mother cell produces. In strains which do not undergo asymmetric divisions, the concept of age is determined by the lifespan of postmitotic cells in culture media which is not replenished. It is believed that programmed cell death in this unicellular organism is beneficial for the colony because it saves limited nutrients for the healthy cells. Additionally, yeast cells infected with certain viruses secrete toxins which can induce necrotic-like or apoptotic-like features in neighboring non-infected cells. It can be hypothesized that programmed cell death contributes to limit the infection by isolating the virus, which would not be able to reproduce in neighboring dying cells. A number of apoptotic markers have been observed in dying yeast, including chromatin condensation, DNA fragmentation and phosphatidyl-serine exposure in the outer membrane, which is a feature of apoptosis which helps macrophages recognize and engulf apoptotic bodies. As in the case of plants, it is unclear why yeast would undergo an apoptotic-like form of programmed cell death, since they cannot phagocyte neighboring cells. And again, as with plants, it remains unclear whether the metacaspase present in S. cerevisiae is responsible for the caspase-like protease activities detected during yeast cell death.

Human unicellular eukaryotic parasites have also been shown to undergo cell death with apoptotic features.[24] Cell death has been extensively studied in the flagellated parasites Trypanosoma and Leishmania and it is frequently associated with production of Reactive Oxygen Species. The genes responsible for death, as well as the role in vivo of cell death still need characterization; for this reason, it might be a bit premature to assert that these organisms undergo programmed cell death. Cell death can frequently be inhibited by peptidic caspase inhibitors, but it has been shown in many cases that the metacaspases present in these organisms do not play a role in cell death in protozoans. As discussed, metacaspases have different substrate affinity than caspases and their physiological role in trypanosomes and Leishmania is linked to cell cycle progression.[25] The putative role of a programmed form of cell death in unicellular parasites is unclear. Dying parasites have been detected inside infected macrophages and in the midgut of the insect vector which transmits the parasite to humans. It has been suggested that programmed cell death would contribute to eliminate the least fit (less infective) organisms in the insect. It could also contribute to limit parasite infection intensity in the human host, in order to reduce the risk of death of the host cell, which could boost an immune response.

Dictyostelium discoideum, or slime mold, is one of the non-animal organisms in which programmed cell death has been studied in more detail. Dictyostelium belongs to a group of eukaryotes named Amoebozoa. This organism is particularly interesting from an evolutionary point of view because it transitions from a unicellular to a pluricellular stage during its life cycle. Upon starvation, Dictyostelium individuals aggregate and form a fruiting body: a fungus-like structure with a stalk composed of dead cells. Massive autophagy is detected in dying cells and death has been shown to depend on autophagic genes but not on caspase-like activity. Dictyostelium is thus, with Drosophila and possibly some plant tissues, another organism whose cells undergo autophagic cell death during development.[26]

We can conclude that there are multiple programmed cell death programs in nature. Interestingly, cell death in some non-animal organisms is accompanied by features which resemble animal apoptosis. This suggests that an apoptotic-like program may have originated in primitive unicellular eukaryotes.

HOST DEFENSE AND THE ORIGINS OF APOPTOSIS. PATHOGEN-SENSING COMPLEXES AND APOPTOSOMES ARE STRUCTURALLY SIMILAR

Apoptosis serves three main purposes in animals. The first one is the removal of cells which are no longer necessary, such as cells which played a role during development but are not needed in the adult, or immune cells after the pathogen has been cleared. The second purpose is the elimination of damaged cells, which may not be able to perform their function properly, or are potentially dangerous to the organism (for instance, cells with DNA damage which may lead to mutations). The third main function is the elimination of infected cells. Finding out which of these functions is older in evolutionary terms may help elucidate the origins of cell death. So, which one was the most ancestral function of apoptosis? Several pieces of evidence suggest that apoptosis originated as a means to eliminate infected cells and that this mechanism could have arisen in primitive organisms such as unicellular precursors of metazoans.

In 2002, James and Green[27] proposed a theory to explain how a suicidal program could have originated in unicellular organisms: this would have occurred in the context of infection and self defense. The origins of a suicidal program in single-celled organisms are hard to understand, because the cells that acquired the ability to kill themselves would likely have a disadvantage and the emergence of "cheaters" with mutant genes would impair the maintenance of such a program. However, the emergence of such a process could be explained in the context of an infection. We can envision the situation as follows. In certain unicellular organisms, a program to detect a parasite engaged proteases, perhaps as a means to degrade pathogens upon infection. At certain point during evolution, these proteases acquired the ability to kill the infected cell upon detection of the pathogen. Engagement in such a self-killing program would be beneficial for the kin or group of genetically identical organisms, because this would limit the spread of the infection. And if the infected cell "cheated" or it contained a mutated version of the killer gene, it would die anyway from the infection. Therefore, in the long-term, the acquisition of this suicidal program would be beneficial for the colony. Moreover, this program would be maintained in multicellular organisms, in which the machinery could be "recycled" and used for more diverse funcions such as body sculpture. Let's review some of the multiple links between the apoptotic machinery and immune responses which offer support to this hypothesis.

Perhaps the best piece of evidence in favor of an origin of apoptosis in the context of host defense is the fact that apoptosis-initiating complexes such as the apoptosome and immunity-activating complexes such as the inflammasomes are very similar to each other. Apaf-1, the nucleating component of the apoptosome, is very similar to a family of proteins that play immune roles: the NOD-like receptors. These proteins recognize intracellular pathogens and activate immune responses. Upon recognition of a pathogen or a danger signal, NOD-like receptors oligomerize and nucleate multiprotein complexes which then recruit caspase-1, which is a non-apoptotic caspase responsible for formation of the mature form of the cytokine interleukin-1. The function of inflammasomes is to promote NF-kappaB activation and/or formation of the proinflammatory cytokine interleukin-1.

NOD-like receptors are grouped in this family due to the presence of a conserved nucleotide-binding domain in the central regions of the molecule: a NOD domain, which is also present in Apaf-1. This domain is responsible for oligomerization of the molecule

and it is essential for the formation of oligomeric complexes such as the inflammasomes and apoptosomes. Apart from the NOD domain, the other portions of NOD-like receptors contain a variety of different domains which include CARDs and Death Domains, which are domains present in apoptotic proteins. The structure of some NOD-like receptors resembles enormously the structure of Apaf-1, which contains a c-terminal WD40 domain (involved in the binding to cytochrome c), a NOD domain and an n-terminal CARD domain, responsible for recruitment of caspase-9 to the apoptosome.

In humans we find over a dozen of NOD-containing proteins, most of which contain a Leucine Rich Repeat in the carboxyl termini, which is a ligand-recognition domain.[28] Apaf-1 differs from some human NOD-containing proteins because it contains the cytochrome c-binding WD40 domain instead of the Leucine Rich Repeat. This suggests an intriguing possibility. Is it possible that Apaf-1, like other NOD-containing proteins, was originally a molecule that recognized a bacterial component and triggered an immune response? This component would have been cytochrome c, which was a protein present in the—first pathogenic, later endosymbiotic—bacteria. Recent phylogenetic analyses offer more pieces of evidence that point towards an immune origin of the Apaf-1-like proteins: some Apaf-1 homologs in cnidarians contain TIR domains. These domains are found in Toll-like receptors, which are proteins involved in recognition of extracellular pathogens.[3] Moreover, the NOD domain in Apaf-1 is strikingly homologous to the NOD domain present in certain plant proteins which are involved in the Hypersensitive Response. These proteins, the R gene products, have Apaf-1 like NOD domains and also leucine rich repeats like mammalian NOD-like receptors. R gene products, upon pathogen detection, trigger an immune response that involves cell death, as discussed above. Given the similarity between all these proteins, it is very likely that Apaf-1 molecules were originally pathogen sensors.

As proposed by James and Green,[27] molecules present in the surface of the mitochondrial endosymbiont such as cytochrome c may have originally triggered an immune response in the unicellular host, which was later suppressed when the association became mutually beneficial. Then, a remnant of this immune response was maintained in animals. At a certain point the cell "learnt" to control cytochrome c release, which perhaps became a signal associated with mitochondrial damage. This would then trigger a caspase cascade that would lead to cell death instead of an immune response. Cytochrome c release then became a central point of control of what became an essential mechanism to eliminate unnecessary cells: apoptosis.

NON-APOPTOTIC FUNCTIONS OF APOPTOTIC PROTEINS ARE RELATED TO IMMUNITY

As discussed above, there are four main groups of apoptotic proteins: caspases, Bcl-2 proteins, IAPs and Apaf-1- or FADD-like adapter molecules. Many of these proteins play roles in the immune response. Among the caspases, both the apoptotic caspases and the inflammatory caspases play roles in the immune system. The group of inflammatory caspases comprises caspase-1, -4, -5 and -12. These caspases are activated in inflammasomes, which are very similar to apoptosomes, as described earlier. They are activated when a pathogen or a danger signal is detected. Their proteolytic activity is linked to production of awareness signals to alert the immune system. However, these are not the only caspases with roles in immunity. Caspase-8, the apical caspase in the

apoptotic death receptor pathway, is a bona fide apoptotic caspases which also plays roles in proliferation and immunity. As discussed above, pro-apoptotic activation of caspase-8 usually occurs in response to a death ligand such as TNF. In most cells, however, the response to TNF-alpha (and to a lesser degree, to other death ligands) is not the induction of cell death but the activation of NF-kappaB, which occurs in a caspase-8-dependent manner. Caspase-8 is also required for proliferation of lymphocytes and activation of NF-kappaB after ligation of the T-cell receptor.[29]

In drosophila, caspase-8 is not an apoptotic caspase. In flies we do not find an apoptotic pathway similar to the mammalian extrinsic pathway, in which caspase-8 is the initiator caspase. The main role of the fly caspase-8 homolog Dredd seems to be the activation of the immune response against gram-negative bacteria, which is mediated through the Immune Deficiency (IMD) signaling cascade.[30] IMD is a protein which is activated after infection. Similar to death receptors, IMD contains a Death Domain and it presumably forms a DISC-like complex. IMD recruits Drosophila FADD (dFADD) and this leads to the activation of Dredd. The IMD pathway is thus very similar to the mammalian TNF-alpha signaling pathway as it involves Drosophila Caspase-8 (Dredd) and the FADD homolog. Similarly to what occurs in the human non-apoptotic TNF signaling pathway, the IMD pathway promotes the activation of NF-kappaB and this requires the caspase Dredd. The IMD pathway thus presents similarities with the TNF/apoptotic extrinsic pathway but also with the inflammasome, since it is executed through interaction between caspases and adapter molecules in a complex with proteins that recognize pathogens. Thus, both caspases and the adapter protein FADD, which are apoptotic in humans, play roles in the immune response both in humans and insects.

IAPs (Inhibitors of Apoptosis Proteins) were originally identified by virtue of their homology with a protein from a baculovirus which inhibits caspases. The well studied IAP from Drosophila DIAP1 is a caspase inhibitor which is critical for regulation of apoptosis during embryonic development. Its downregulation is enough to activate the initiator caspase DRONC and trigger apoptosis. In mammals, however, loss of IAPs per se usually does not lead to caspase activation and cell death. The main role of IAPs is related to immunity and activation of NF-kappaB. It has recently been shown that only one of the eight IAP proteins present in humans, XIAP, is a direct caspase inhibitor. cIAP1 and cIAP2 are evolutionarily related to XIAP and they inhibit apoptosis, although they do not inhibit caspase activity. These proteins have a similar structure to XIAP but they lack aminoacid residues involved in direct caspase activation, which suggests two possible scenarios: either some of these proteins lost the capability to directly inhibit caspases, or this function was acquired late during evolution.[31] If this second scenario was true, what where their other, more ancient functions? In human cells, cIAPs participate in TNF receptor signaling. They are recruited to the DISC through indirect interactions with TNF receptors and they participate in TNF-mediated induction of NF-kappaB. cIAPs and paradoxically, also the sudden loss of cIAPs through chemical inhibition, activate both canonical and noncanonical NF-kappaB activation pathways.[32]

Activation of NF-κB seems to be a conserved function of IAP proteins even in invertebrates. DIAP1 is essential for apoptosis in Drosophila. It was recently acknowledged that another member of the IAP family, Drosophila IAP2, unlike DIAP1, is dispensable for development of the fly but it is necessary for the innate immune response against gram-negative bacteria. DIAP2 mediates NF-κB activation in the IMD pathway.[33] As mentioned before, the IMD pathway is activated in response to bacteria and it may involve the formation of a DISC-like complex with Drosophila FADD and Caspase-8 homologs.

NAIP is another human IAP protein which does not play a role in apoptosis: it participates in the formation of the inflammasome in response to intracellular bacteria.[34] Lastly, mammalian XIAP, which is a *bona fide* caspase inhibitor, has also been shown to participate in NF-kappaB signaling after the detection of intracellular pathogens. Thus, IAPs and caspase-8, like other caspases and NOD-containing proteins, play major roles in immune signaling.

CONCLUSION AND PERSPECTIVES

Cell death is vital to life of multicellular organisms and it plays a role in maintenance of homeostasis of populations of unicellular organisms. It is unclear how cell death originated and it is possible that this process evolved independently in several lineages. The diversity of cell death programs described, which differ both in morphology of the dying cell and in the biochemical pathways responsible for their execution, suggests that there is not a common origin of all cell death programs. However, pathogen-recognition pathways are linked to cell death at least in animals and plants, which suggest that the origins of at least some forms of cell death are related to the ability of an organism to kill its own infected cells.

Apoptotic proteins have several alternative roles besides cell death. Some evidence suggests that these alternative roles were actually the ancestral functions of these proteins and that the currently 'apoptotic' proteins got recruited to the apoptotic machinery at different points during evolution. The exploration of these alternative roles would probably yield important information to solve the puzzle of the evolutionary origins of cell death.

Many questions remain to be solved. For instance, which are the most ancestral functions of caspases? Caspases do not only participate in immune function, but they also play roles in cell proliferation and differentiation.[35] What are the roles of metacaspases? The proteases implicated in cell death of non-animal organisms need to be identified. More intriguing is the issue of when Bcl-2 family proteins got recruited to the apoptotic machinery. Despite extensive searches, no Bcl-2 homolog has been found outside the animal kingdom. These proteins seem to have other conserved roles besides regulation of apoptosis; amongst these, the most relevant seem to be the regulation of mitochondrial dynamics and calcium homeostasis.[36,37] Moreover, the role of these proteins in cell death has only been proven so far in *C. elegans* and vertebrates. Much work needs to be done in animals from other animal phyla to determine the ancestral role of these proteins, which are extremely relevant in human pathologies such as cancer. Maybe one day we will be able to explain how cell suicide, the most altruistic behavior possible, arose and was maintained during evolution.

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CHAPTER 9

SENSING NECROTIC CELLS

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Abstract: Multicellular organisms have developed ways to recognize potentially life-threatening events (danger signals). Classically, danger signals have been defined as exogenous, pathogen-associated molecular patterns (PAMPs) such as bacterial cell wall components (e.g., lipopolysaccharide and peptideglycan) or viral DNA/RNA. PAMPs interact with dedicated receptors on immune cells, so-called pattern recognition receptors (PRRs) and activate immune systems. A well-known family of PRRs is the toll-like receptors (TLRs) in which each member recognizes a specific set of PAMPs. However, not only exogenous pathogens but also several endogenous molecules released from necrotic cells (damaged self) also activate immune systems. These endogenous adjuvants are called damage-associated molecular patterns (DAMPs). It has been reported that high-mobility group box 1 protein (HMGB1), uric acid, heat shock proteins (HSPs) and nucleotides act as endogenous adjuvants. DAMPs are recognized by specific receptors (danger receptors) expressed mainly on antigen-presenting cells such as dendritic cells and macrophages and induce cell maturation and the production of inflammatory cytokines by activating the NF-κB pathway. In this chapter, we will review danger signals released from necrotic cells and its recognition receptors.

INTRODUCTION

In multicellular organisms, many unnecessary or harmful cells, such as those that are generated during development and normal tissue turn over, are eliminated by apoptosis.[1,2] After undergoing apoptosis, cells are divided into small debris, called apoptotic bodies, with the plasma membrane remaining intact and then apoptotic cells are recognized and phagocytosed by 'professional' phagocytes, such as macrophages and dendritic cells (Fig. 1).[3-5] This process is extremely efficient and it is difficult to

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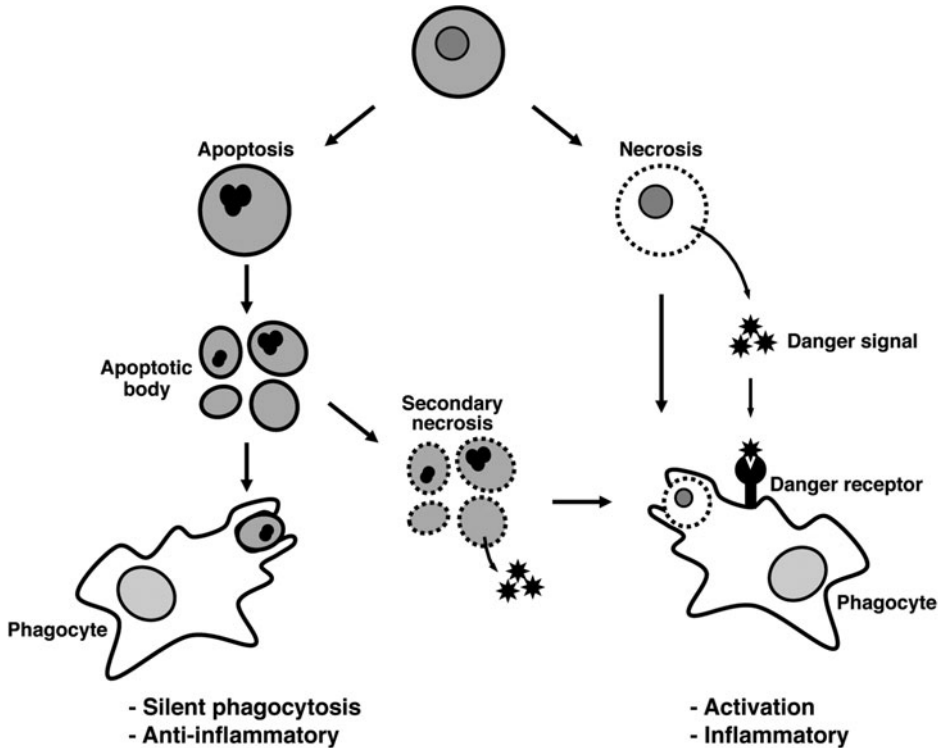

Figure 1. Immune responses of phagocytes after engulfment of apoptotic and necrotic cells. Under physiological conditions, cells die by apoptosis. During apoptosis, cells are divided into small pieces (apoptotic bodies) with the plasma membrane integrity remaining intact. Then apoptotic cells are engulfed by phagocytes. In this process, phagocytes do not activate the immune system and maintain the anti-inflammatory condition (silent phagocytosis). On the other hand, when cells are exposed to excessive physical or chemical stresses, cells die by necrosis. In addition, defect of clearance of apoptotic cells leads to induction of secondary necrosis. Necrotic cells are also phagocytosed or endocytosed by phagocytes. However, the plasma membrane integrity of necrotic cells is lost and cellular components are leaked into the extracellular space. Among the released cellular components, immunostimulatory molecules are involved and these molecules are called danger signals. Danger signals activate phagocytes via danger receptors and induce the production of inflammatory cytokines.

detect apoptotic cells that are not associated with phagocytes under physiological conditions. Rapid removal of apoptotic cells by phagocytes prevents the release of cellular components from dying cells (silent phagocytosis). Moreover, phagocytosis of apoptotic cells induces an anti-inflammatory reaction by producing anti-inflammatory cytokines (e.g., transforming growth factor β and IL-10).[6,7] On the other hand, when cells are exposed to excessive physical or chemical stress (i.e., high or low temperature, strong acid or base, irregular osmotic pressure, mechanical damage and high concentration of detergent), cells die by necrosis.[8] In addition, deficiency in the clearance of apoptotic cells owing to extensive cell death induced by tissue injury/damage by bacterial or viral infection,[9-12] ischemia,[13,14] or acute myocardial infarction[15,16] results in induction of secondary necrosis of dying cells.[17] In necrotic cells, plasma membrane integrity is lost and cellular components are released into the extracellular space. Some of the

released components are potentially toxic or immunogenic and act as endogenous adjuvants that transmit danger signals from necrotic cells.[18] Immune cells recognize danger signals via specific receptors, the so-called 'danger receptors' and induce the production of inflammatory cytokines to initiate inflammation.

DANGER SIGNALS FROM NECROTIC CELLS

High-Mobility Group Box 1 Protein (HMGB1)

HMGB1 is originally described as a DNA-binding protein that stabilizes nucleosomes and facilitates transcription in the nucleus.[19] HMGB1 is normally bound loosely to chromatin, so when a cell becomes necrotic or leaky, HMGB1 is then passively released from the nucleus and promotes inflammatory responses.[20] Released HMGB1 binds to the receptor for advanced glycated endproducts (RAGE), TLR2 and TLR4 and induces the secretion of proinflammatory cytokines and chemokines by immune cells.[21-24] Necrotic cells lacking HMGB1 do not elicit such inflammatory responses. In apoptotic cells, HMGB1 is bound firmly to chromatin because of generalized underacetylation of histone and is not released, which may help prevent unnecessary inflammation.[20] HMGB1 is also secreted actively by macrophages to act as a late mediator of endotoxaemia and sepsis.[25] The production and secretion of HMGB1 is much later compared with those of tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1). Neutralization of HMGB1 by administration of specific antibodies strongly protects against the lethal effects of endotoxin, suggesting that HMGB1 is a critical mediator in endotoxin shock.[26] Although HMGB1 can promote directly the secretion of proinflammatory cytokines and chemokines by immune cells, highly purified recombinant HMGB1 has very weak proinflammatory activity.[27] One possible explanation for this discrepancy is that HMGB1 forms complexes with DNA, lipids and proinflamatory cytokines and these highly inflammatory complexes strongly stimulate cytokine production via TLRs or IL-1R.[28-30]

Uric Acid (UA)

To identify endogenous adjuvants, Shi et al fractionated cytosol from ultraviolet-irradiated BALB/c 3T3 cells by high-performance liquid chromatography (HPLC) on a sizing column and identified UA as a principal endogenous adjuvant released from injured cells.[31] UA is a natural product of the purine metabolic pathway and is soluble inside cells but once released from injured cells, uric acid readily forms into monosodium urate (MSU) microcrystals in the extracellular space. Crystalline uric acid stimulates dendritic cell maturation and, when coinjected with an antigen in vivo, significantly enhances the generation of responses from $CD8^+$ T cells. Eliminating uric acid in vivo inhibits immune responses to antigens associated with transplanted syngeneic cells and the proliferation of autoreactive T cells in an experimental diabetes model.[32] In contrast, uric acid depletion does not attenuate the stimulation of T cells by mature, activated antigen-presenting cells, suggesting that uric acid affects antigen-presenting cells but not T cells. Extracellular uric acid also has major inflammatory properties, most evident when it accumulates in tissues and causes gout. Interestingly, MSU

crystals induce inflammasomes, resulting in the production of active IL-1β and IL-18. Macrophages from mice deficient in IL-1R or in various components of inflammasomes, such as caspase-1, ASC and NALP3, are defective in MSU-induced cytokine secretion and subsequent inflammation.[33]

Heat Shock Proteins (HSPs)

HSPs are a family of molecular chaperones which support the correct folding and refolding of nascent and misfolded proteins. Although HSPs are normally localized in the cytoplasmic region, once released from necrotic cells, extracellular HSPs have immunostimuratory properties. Basu et al reported that necrotic but not apoptotic cell death leads to the release of chaperones such as HSP70, HSP90, calreticulin and Gp96.[34] Released HSPs stimulate DCs and macrophages to secrete inflammatory cytokines and induce expression of major histocompatibility complex (MHC) and costimulatory molecules (e.g., CD80 and CD86) through activation of NF-κB pathway. Several groups have reported that different HSPs can activate DCs and macrophages.[35-37] To identify proteins that bind to Gp96, Binder et al carried out affinity chromatography using immobilized Gp96 and found that CD91 is the surface receptor of Gp96.[38] Basu et al subsequently reported that CD91 could be the common receptor for immunological HSPs, including HSP60, 70, Gp96 and calreticulin.[39] CD40 is a member of the tumor necrosis receptor family, plays a major role in antigen-presenting cell maturation and also functions as the HSP70 receptor. Mycobacteria-derived HSP70 binds to CD40 and induces the maturation of antigen- presenting cells and the release of CC chemokines.[40] It has also been reported that human HSP70 binds to CD40.[41] Millar et al showed the essential role of CD40 in HSP70 stimulation, which promotes antigen-presenting cell function and converts tolerogenic T cell to immunogenic T cell.[42] HSP70 also binds to scavenger receptors, such as LOX-1, SREC-1 and FEEL-1, expressed on antigen-presenting cells and endothelial cells and each of these receptors can mediate HSP70 interaction.[43-45] In addition, several C-type lectin receptor family members expressed on NK cells (NKG2A, NKG2C and NKD2D) interact with HSP70.[46]

DNA/RNA

RNA released from or associated with necrotic cells forms double-stranded structures that can stimulate TLR3 on dendritic cells, leading to interferon secretion.[47,48] This inflammatory response to necrotic cells can be abolished by pretreating necrotic cells with RNAse. Genomic DNA is released from necrotic cells and stimulates antigen- presenting cells.[49] Double-stranded but not single-stranded genomic DNA triggers antigen-presenting cells to up-regulate expression of MHC class I/II and various costimulatory molecules, enhances antigen-presenting cell function in vitro and improves primary cellular and humoral immune responses in vivo. These effects are dependent on the length and concentration of double-strand DNA but are independent of nucleotide sequence. Intracellular nucleotides such as ATP and UTP, which function in energy metabolism and are normally stored in the cytoplasm, are released from various cell types under conditions of hypoxia, ischemia and mechanical stress.[50] Recently, it has been reported that ATP is released from the mitochondria of necrotic cells.[51] These nucleotides can activate DCs mediated by the triggering of the purinergic receptors, P2Z/P2X7.[52-54]

DANGER RECEPTORS FOR SENSING NECROTIC CELLS

Clec9A

C-type lectin Clec9A (DNGR-1) is selectively expressed at high levels by CD8α+ dendritic cells.[55,56] This subset of dendritic cells potently engulfs dead cells and cross-presents dead-cell-associated antigens to CD8+ T cells.[57,58] Clec9A preferentially senses unidentified danger signal that is exposed on necrotic cells. Clec9A-deficient CD8α+ dendritic cells show a reduced ability of cross-presentation of necrotic-cell-derived antigens, although Clec9A deficiency does not alter the phagocytosis activity of necrotic cells. Therefore, Clec9A is dispensable for the uptake of necrotic cells but necessary for efficient cross-presentation of dead-cell-associated antigens by CD8α+ dendritic cells. The cytoplasmic tail of Clec9A contains a hemITAM motif,[59] which allows binding of SYK kinase.[55] Loss of SYK kinase or substitution of tyrosine residue within the hemITAM of Clec9A blocks cross-presentation of necrotic cells, suggesting that SYK kinase is required for Clec9A signaling. The ligand of Clec9A is not characterized yet, which is predominantly localized in the cytoplasmic region and resistant to glycosidase and nuclease treatment but susceptible to the action of proteases, heat and acid.

Toll-Like Receptors (TLRs)

TLR3 has been shown to respond to double-stranded RNA derived from many viruses. Kariko et al reported that endogenous RNA released from or associated with necrotic cells stimulates TLR3 and induces immune activation of DCs, leading to interferon-alpha secretion, which could be abolished by pretreatment of necrotic cells with RNase.[48] Cavassani et al observed the involvement of TLR3 activation in the development of experimental polymicrobial septic peritonitis and ischemic gut injury in the absence of an exogenous viral stimulus.[60] In TLR3-deficient mice, increased chemokine/cytokine levels and neutrophil recruitment characterized the initial inflammatory responses in both injury models. However, the levels of inflammatory chemokines and tumor necrosis factor quickly returned to the baseline in TLR3 KO mice and these mice were protected from the lethal effects of sustained inflammation. Administration of an anti-TLR3 antibody attenuated the tissue injury associated with gut ischemia and significantly decreased sepsis-induced mortality. These observations suggest that TLR3 is a regulator of the amplification of immune responses and serves as an endogenous sensor of necrosis, independent of viral activation.

Mincle

Many of the C-type lectin receptor genes have been mapped to a cluster on mouse chromosome 6F2 and human chromosome 12p31.[61] Among them is the gene encoding Mincle (also known as Clec4e or Clecsf9), a Type II transmembrane C-type lectin receptor expressed in macrophages. Mincle (macrophage-inducible C-type lectin) was originally identified as an LPS-inducible protein in macrophages, as its expression is markedly induced by several stresses in a C/EBPβ-dependent manner.[62] We first determined that Mincle possesses a positively charged arginine residue within the transmembrane region, suggesting that Mincle is associated with some ITAM-bearing adaptors, such as CD3ζ, FcRγ, DAP12 or DAP10. Indeed, Mincle selectively associates with the FcRγ chain but not with other adaptors. This association was mediated by charge because the elimination

of transmembrane charge by introducing R42I mutation in Mincle resulted in the complete loss of its binding capacity to the FcRγ chain.[63]

To search for the physiological ligand for Mincle, we have established an indicator cell line expressing Mincle and FcRγ together with the NFAT-GFP reporter. Intriguingly, when cells of this cell line were cultured without changing the medium for a few days, a large amount of GFP-positive population appeared. The generation of this GFP-positive population was blocked by incubation with a blocking antibody against Mincle, suggesting that 'something' in this culture condition acts as a Mincle ligand. Under this culture condition, a large number of propidium iodide (PI)-positive dead cells were continuously generated. In many cases, GFP^+ cells seem to be in the vicinity of PI^+ dead cells. We assumed that dead cells may provide a Mincle ligand. Indeed, the Ig-Mincle fusion protein selectively binds to $AnnexinV^+PI^+$ late-apoptotic or necrotic cells. Purification of the Mincle-binding protein from the lysate of necrotic cells identified a nuclear protein, splicing associating protein-130 (SAP-130) (Fig. 2).[63] SAP-130 is a component of small nuclear ribonucleoprotein (snRNP), which is also a major autoantigen in autoimmune diseases such as SLE.[64] One may ask how these nuclear proteins are recognized by Mincle. Interestingly, we found that a large amount of SAP130 is secreted into the supernatant from necrotic cells. Thus, the existence of SAP130 in the extracellular milieu

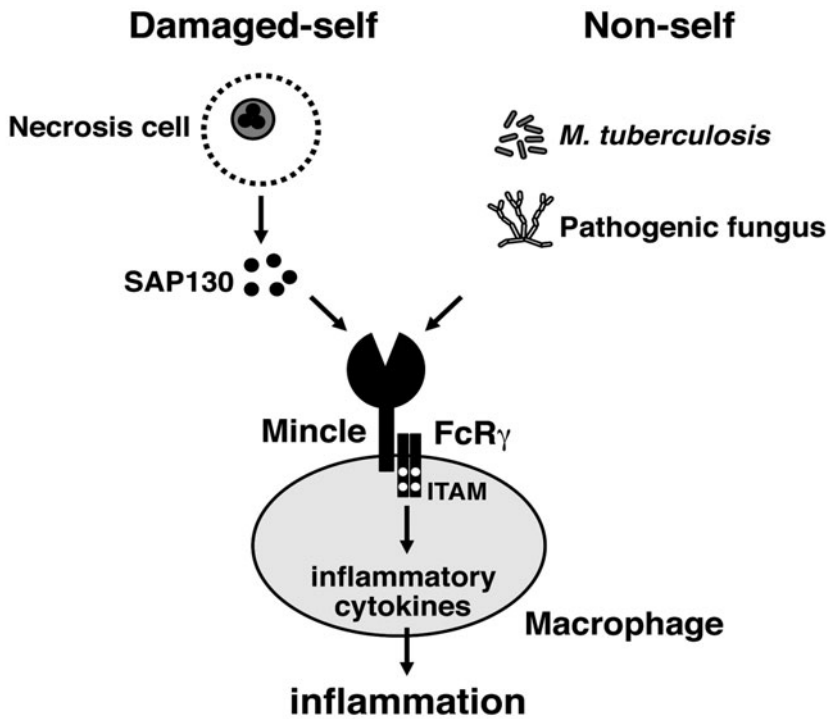

Figure 2. Recognition of danger signals from self and nonself by Mincle receptor. A C-type lectin, Mincle, is expressed in activated macrophages and is constitutively associated with the ITAM-bearing adaptor molecule FcRγ. Mincle binds to SAP130 released from necrotic cells and induces the production of inflammatory cytokines. Mincle also interacts with *M. tuberculosis* and the pathogenic fungus *Malassezia*. Mincle senses both self (necrotic cells from tissue damage) and nonself (invading pathogens) danger signals.

would be a sign for massive cell death and Mincle may sense this sign. Experimentally, irradiation-induced thymocyte death induces transient infiltration of neutrophils into the thymus.[65] However, this neutrophil infiltration induced by dead cells is severely blocked by in vivo administration of anti-Mincle blocking mAb.[63] Thus, Mincle is a critical receptor that senses damaged cells to evoke inflammation.

In addition to damaged-self, we have recently determined that Mincle recognizes nonself pathogens as well, such as pathogenic fungi or *Mycobacterium tuberculosis*.[66,67] One of the ligands for Mincle was found to be a characteristic mycobacterial glycolipid, trehalose dimycolate (TDM).[67] TDM-induced macrophage activation, such as NO production or secretion of inflammatory cytokines, is completely eliminated in Mincle-deficient macrophages. TDM strongly induces lung granuloma in vivo, but it is totally absent in Mincle-deficient mice. More importantly, TDM have been known as a major component of complete Freund's adjuvant (CFA).[68] Therefore, Mincle may be a critical transducer linking innate immunity to acquired immunity.

CONCLUSION AND FUTURE PROSPECTS

In 1989, Janeway proposed that innate immune systems discriminate self and nonself through pathogen-associated molecular patterns (PAMPs).[69] Many PAMPs activate antigen-presenting cells mainly through TLR to evoke acquired immune responses.[70] Some years later, Matzinger expanded Janeway's theory; she proposed the "danger signal theory", which states that the decision to respond or not to respond to a particular antigen depends on whether the antigen is "harmful or not" to our body. In this theory, damage-associated molecular patterns (DAMPs), which are released from damaged cells, are proposed to be determinants that trigger acquired immune responses.[71] However, the precise molecular mechanism underlying this recognition has not yet been clarified. Given that Mincle is markedly induced in the presence of several stresses, recognizes both PAMPs and DAMPs and activates antigen-presenting cells, Mincle might be a late-coming leading player mediating danger signals. The physiological advantages and potential risks of the recognition of DAMPs and PAMPs by Mincle would be intriguing issues to be clarified.

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CHAPTER 10

SENSING ENDOPLASMIC RETICULUM STRESS

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Abstract: This chapter provides an overview of our present understanding of mechanisms of sensing protein folding status and endoplasmic reticulum (ER) stress in eukaryotic cells. The ER folds and matures most secretory and transmembrane proteins. Mis- or unfolded proteins are sensed by specialized ER stress sensors, such as IRE1, PERK and ATF6, which initiate several cellular responses and signaling pathways to restore ER homeostasis. These intracellular signaling events are called the unfolded protein response (UPR). Here we focus on how ER stress and protein folding status in the ER are sensed by the ER stress sensors by summarizing results from recent structural, biochemical and genetic approaches.

INTRODUCTION

The accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER) causes ER stress. The co-ordinated adaptive response to ER stress is called the unfolded protein response (UPR).[1] The UPR is induced to re-establish ER homeostasis by upregulating the protein folding machinery of the ER and protein degradation pathways for ER-associated proteins, inhibiting general protein synthesis and degrading mRNAs encoding proteins targeted to the secretory pathway. Several transmembrane proteins of the ER, including the protein kinase-endoribonuclease IRE1, the protein kinase PERK and Type II transmembrane transcription factors such as ATF6 are activated by ER stress and co-ordinate these physiological responses to ER stress (Fig. 1). Of these ER stress sensors only IRE1 is conserved in all eukaryotes, including fungi, plants and animals. IRE1 possesses a cytosolic effector domain consisting of a serine/threonine protein kinase and endoribonuclease (RNase) domain with homology to RNase L.[2-4] In response to ER

Self and Nonself, edited by Carlos López-Larrea.

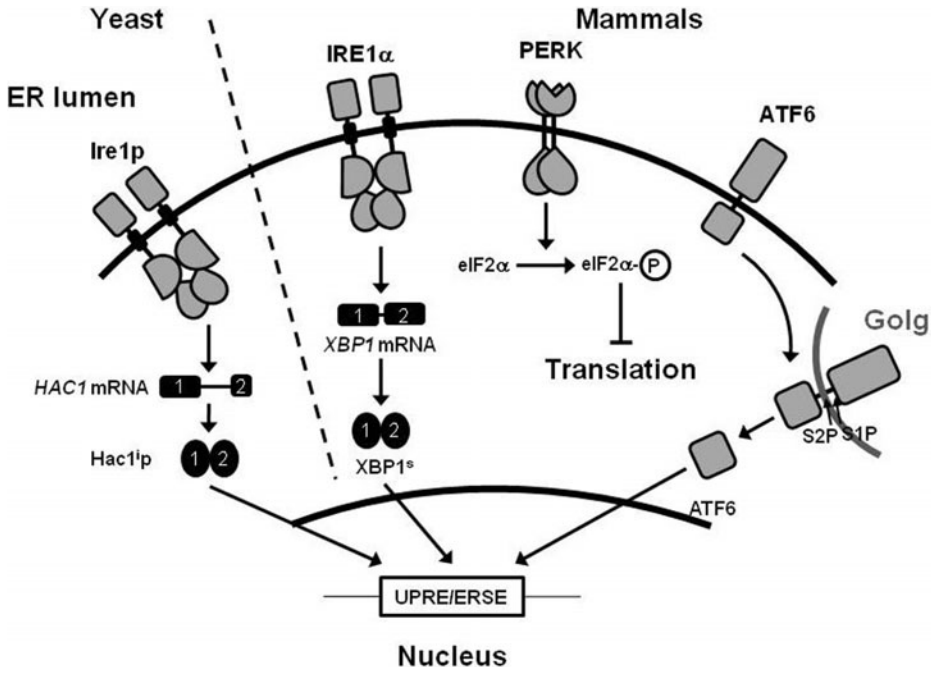


Figure 1. A general overview of the unfolded protein response in yeast and mammals. Upon accumulation of unfolded proteins in the ER, IRE1 is activated and cleaves *HAC1/XBP1* mRNA to remove an intron by a spliceosome-independent mechanism. The protein Hac1^{i}p/XBP1^{s} binds to UPRE or ERSE elements in promoters of target genes to activate transcription of these genes. Activated PERK phosphorylates eIF2α to attenuate cap-dependent translation. ATF6 is activated in response to ER stress and is translocated to the Golgi where it is cleaved by S1P and S2P proteases. Cleaved ATF6 translocates to the nucleus to activate transcription of target genes.

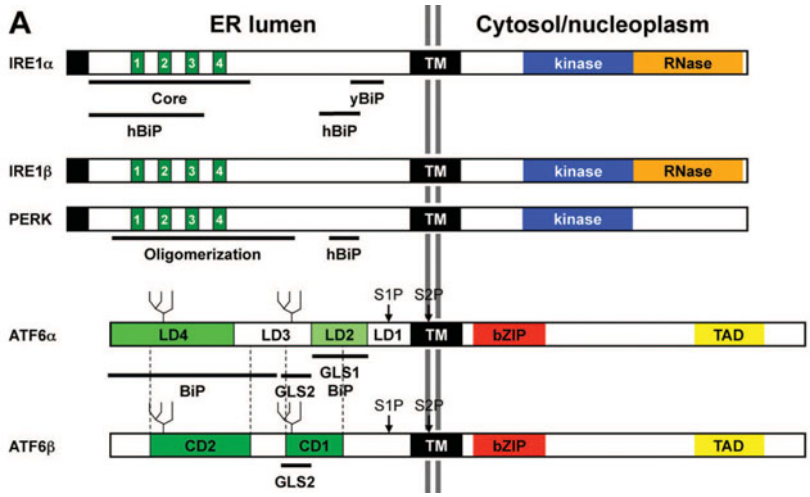
stress IRE1 autophosphorylates and activates its RNase domain.[5] In the budding yeast *Saccharomyces cerevisiae*, the RNase domain of Ire1p cleaves exon-intron junctions in the mRNA encoding the basic leucine zipper (bZIP) transcription factor Hac1^{i}p (Fig. 1).[6] The cleaved exons are ligated by tRNA ligase.[7] Only spliced *HAC1^{i}* mRNA is efficiently translated into Hac1^{i}p, which regulates a wide variety of genes to alleviate ER stress.[8,9] Ire1p is the only known ER stress transducer in yeast. In contrast, mammalian cells possess two orthologs of Ire1p, IRE1α and IRE1β, another ER-localized Type I transmembrane protein kinase called PERK and several Type II transmembrane bZIP transcription factors that respond to ER stress. IRE1α and IRE1β cleave *XBP1* mRNA in an analogous reaction to the *HAC1* splicing reaction. Spliced XBP1 (XBP1^{s}) activates expression of genes encoding ER-resident molecular chaperones, components of the ER-associated protein degradation (ERAD) machinery and phospholipid biosynthetic genes. IRE1 kinase activity-dependent formation of signaling complexes with the ubiquitin ligase TRAF2, the MAP kinase kinase kinase ASK1,[10] and the inflammatory kinase IKK[11] activates inflammatory, innate immune response and apoptotic programs via the MAP kinases p38 and JNK and the transcription factor NF-κB. PERK attenuates general cap-dependent translation initiation by phosphorylating eukaryotic translation initiation factor 2α (eIF2α, Fig. 1).[12-14] eIF2α phosphorylation-mediated translational arrest leads to the preferential translation of mRNAs

containing several short upstream open reading frames (uORFs) in their 5′ untranslated regions (5′ UTRs), resulting in synthesis of the bZIP transcription factor ATF4.[15-17] ATF4 is mainly responsible for inducing the pro-apoptotic bZIP transcription factor CHOP in ER-stressed cells.[18] PERK also phosphorylates the bZIP transcription factor NRF2, leading to disruption of cytosolic complexes between NRF2 and the cytoskeletal anchor protein KEAP1 and activation of an antioxidant response. Type II transmembrane transcription factors, such as ATF6, translocate to the Golgi complex upon ER stress,[19,20] where their cytosolic bZIP transcription factor domains are released from the Golgi membrane by sequential cleavage by the Golgi-resident proteases S1P and S2P (Fig. 1).[21,22] Activated ATF6 induces many ER chaperone genes, but also genes encoding proteins engaging in ERAD.[23,24] The first step in the UPR, nevertheless, is the detection of unfolded proteins in the ER to activate downstream homeostatic, but also apoptotic responses, to ER stress. In this communication we will review mechanisms for how these diverse ER stress sensors sense the accumulation of unfolded proteins in the ER lumen.

SENSING OF ER STRESS BY IRE1

IRE1 was originally identified in *S. cerevisiae* as a gene required for inositol prototrophy.[25] Subsequently, two groups independently isolated mutations in *IRE1* in screens for genes required for *KAR2* activation by ER stress.[2,3] Strains deleted for *IRE1* (*ire1*Δ) show a significant, but not complete reduction in activation of *KAR2*, *PDI1* (a protein disulfide isomerase of the ER),[26] and β-galactosidase reporters under control of the Hac1^{i}p binding site, the UPR element (UPRE).[8,27,28] The *IRE1* gene encodes an ER-resident Type I transmembrane protein with cytosolic serine/threonine protein kinase and RNase domains (Fig. 2A). The *N*-terminal domain resides in the ER, indicating that it may function in sensing ER stress.[4,29-31] IRE1 is conserved in all eukaryotes with each two orthologs in mammals[32,33] and plants.[34-36] The ER luminal domains of these proteins share four regions of sequence conservation. This sequence conservation extends to the luminal domain of PERK, indicating that all IRE1 orthologs and PERK utilize a, common mechanism to survey protein folding status in the ER (Fig. 2B). Indeed, chimeras in which the ER luminal domains of mammalian or *Caenorhabditis elegans* IRE1, or *C. elegans* PERK were grafted onto the cytosolic effector domains of yeast Ire1p were fully functional.[37] Because of this functional conservation between the ER luminal domains of IRE1 and PERK it is widely assumed that studies on the ER luminal domain of IRE1 will also provide insight into the function of the ER luminal domain of PERK. Mammalian IRE1α is expressed ubiquitously, for example in tissues such as placenta, liver and pancreas,[32,38] whereas expression of IRE1β is limited to epithelial cells of the digestive tract.[39] Disruption of the mouse *IRE1α* gene is embryonic lethal in homozygous *ire1*$\alpha^{-/-}$ offspring.[38,40] In contrast, homozygous disruption of *IRE1β* has revealed no major abnormalities, but predisposes mice to colitis.[39] A conceptual understanding for the need of a gut epithelial-specific IRE1 ortholog, however, does not exist.

Several models for ER stress sensing by IRE1 have been proposed. The most popular theory is the ‘competition’ model in which the ER luminal domain of IRE1 and unfolded proteins compete for binding to the molecular chaperone BiP.[41,42] The crystal structure of a large portion of the ER luminal domain of Ire1p has led to the proposal of the major competing model, the direct ligand or unfolded protein binding model.[43] These models will be discussed in the following section.



B Motif 1

PERK	(*H.s.*)	98	LRPRGRSLVIISTLDGRIAALDPENHGKKQWDL--D	131
PERK	(*M.m.*)	94	LRPRGRSLVIISTLDGRIAALDAENDGKKQWDL--D	127
PERK	(*C.e.*)	48	DQNSQVNIIITATLDGVVTALDGET-GEMIWRY-ED	81
IRE1α	(H.s.)	25	TVTLPETLLFVSTLDGSLHAVSKRT-GSIKWTLKED	59
IRE1β	(*H.s.*)	33	HTLRPENLLLVSTLDGSLHALSKQT-GDLKWTLRDD	67
IRE1	(*C.e.*)	37	DEEKTSSTILVSTIDGRLRALDSET-GEIKWTLQEE	72
Ire1p	(*S.c.*)	115	NELSLSDILIAADVEGGLHAVDRRN-GHIIWSIEPE	149

Motif 2

PERK	(*H.s.*)	154	IPSLDGALFQWD-QDRESMETVPFTVESLLESSY-KFG	189
PERK	(*M.m.*)	150	IPSLDGDLFQWD-RDRESMEAVPFTVESLLESSY-KFG	185
PERK	(*C.e.*)	105	MPTLDGRLFSYT-HNTNLIEPLPITTDSLLESSTI-RLG	140
IRE1α	(H.s.)	75	PDPNDGSLYTLGSKNNEGLTKLPFTIPELVQASPCRSS	112
IRE1β	(*H.s.*)	83	SDPADGSLYILGTQKQLGLMKLPFTIPELVHASPCRSS	120
IRE1	(*C.e.*)	88	PNPLDGSLYVL--KN-SSLKKLPFNIPQLVHASPCKGN	123
Ire1p	(*S.c.*)	172	EPFGDGNIYYFNAH--QGLQKLPLSIRQLVSTSPLHLK	208

Motif 3

PERK	(*H.s.*)	190	DDVVLVGGKSLTT-YGLSAYSG--KV	212
PERK	(*M.m.*)	186	DDVVLVGGKSLTT-YGLSAYSG--KL	208
PERK	(*C.e.*)	142	QDAVA-GGKSVTT-KGFDLFTGEQKY	165
IRE1α	(H.s.)	111	SDGILYMGKKQDIWYVIDLLLTGE-KQ	135
IRE1β	(*H.s.*)	120	SDGVFYTGRKQDAWFVVDPESGE-TQ	144
IRE1	(*C.e.*)	122	NDGILYAGSKKDVWFGIDPKTGL-KV	146
Ire1p	(*S.c.*)	220	EDEKVYTGSMRTIMYTINMLNGE-II	244

Motif 4

PERK	(*H.s.*)	231	QEEDILLLQRTQKTVRAVGPRSGNEKWNFSVGH	263
PERK	(*M.m.*)	227	EEEDILLLQRTQKTVRAVGPRSGSEKWNFSVGH	259
PERK	(*C.e.*)	179	PKNPIILIRRTTNSIRAMDTLRGIERWNLSTAE	211
IRE1α	(H.s.)	149	PSTSLLYLGRTEYTITMYDTKTRELRWNATYFD	181
IRE1β	(*H.s.*)	152	PSTPRLYIGRTQYTVTMHDPRAPALRWNTTYRR	184
IRE1	(*C.e.*)	161	NQKQTIFLGRTEYRVSMFDEKNRGKTWNATFND	193
Ire1p	(*S.c.*)	273	ECENMIVIGKTIFELGIHSYD--GASYNVTYST	303

Figure 2. The figure and legend are continued on the following page.

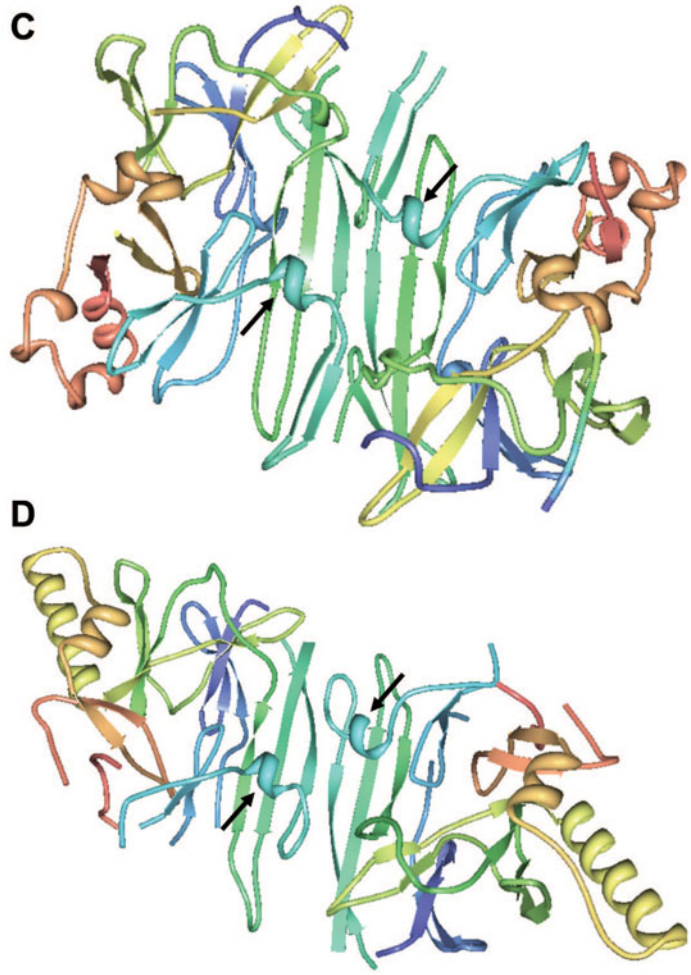


Figure 2, continued. Structure of the ER stress sensors. A) Schematic diagrams of IRE1, PERK and ATF6 (not to scale). Yeast (yBiP) and human (hBiP) BiP binding sites in IRE1 and PERK are indicated by lines. The core region required for signaling in Ire1p and the region in PERK required for oligomerization are also indicated by a line. Abbreviations: bZIP—basic leucine zipper domain, CD—conserved domain, GLS—Golgi localization sequence, LD—luminal domain, S1P—site 1 protease cleavage site, S2P—site 2 protease cleavage site, TAD—transcriptional activation domain, TM—transmembrane domain. Glycosylation sites in ATF6 are indicated by branched trees. B) Sequence alignment of conserved regions in the luminal domains of IRE1 and PERK. Conserved residues are black underlined, similar residues grey underlined. Abbreviations: *C.e.—Caenorhabditis elegans*, *H.s.—Homo sapiens*, *M.m.—Mus musculus* and *S.c.—Sacharomyces cerevisiae.* Gen bank accession numbers: PERK (*H.s.*)—NP_004827, PERK (*M.m.*) NP_034251, PERK (*C.e.*)—AAL30829, IRE1α (*H.s.*)—O75460, IRE1β (*H.s.*)—Q76MJ5, IRE1 (*C.e.*)—NP_495701 and Ire1p (*S.c.*)—CAA77763. C) 3.0 Å crystal structure of the luminal domain of yeast Ire1p (PDB entry 2BE1). D) 3.1 Å crystal structure of the luminal domain of human IRE1α (PDB entry 2HZ6). Arrows in (C) and (D) highlight the α helix that blocks access to the groove in human IRE1α.

THE COMPETITION MODEL

The competition model is based on the law of mass action (Fig. 3A). In this model the ER luminal domain of *IRE1* binds to the ER chaperone Kar2p/heavy chain-Binding Protein (BiP)/glucose-regulated protein of 78 kDa (GRP78) in the absence of ER stress. Upon ER stress an increase in unfolded proteins sequesters BiP away from the luminal domain of *IRE1*, unmasking di- or oligomerization motifs in the luminal

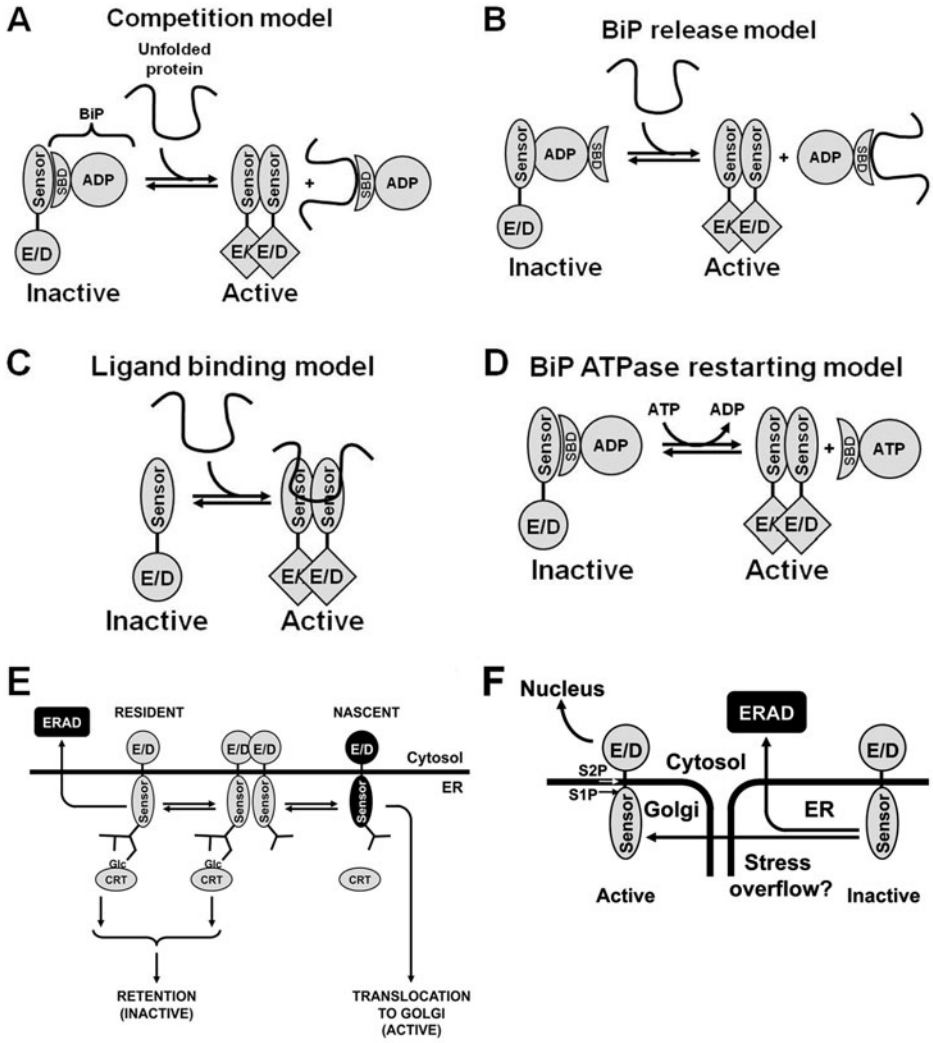


Figure 3. Models for sensing of ER stress. A) The competition model. B) The BiP release model of Gething et al.[65] C) The ligand binding model. D) The BiP ATPase restarting model of Prywes et al.[94] E) Activation of ATF6 by underglycosylation of newly synthesized ATF6. F) Activation of CREB-H by differential partitioning between ERAD and cleavage by S1P and S2P in the Golgi complex. Abbreviation: E/D—effector domain.

domain. Considerable experimental evidence supports the competition model. Elevated expression of secretory proteins induces BiP.[44,45] More slowly folding proteins displaying a prolonged interaction with BiP are stronger inducers of BiP than faster folding proteins.[1,45-48] Overexpression of BiP, but not of the ER chaperone calreticulin or the protein disulfide isomerase PDI, attenuated the UPR,[27,49-51] suggesting that BiP may be a specific negative regulator of IRE1. Decreasing the ER luminal BiP concentration, for example by genetically removing its *C*-terminal HDEL retention sequence in yeast, induces expression of BiP[52] and *KAR2*ΔHDEL *ire1*Δ yeast cells are not viable.[53] Moreover, co-immunoprecipitation of BiP with IRE1 from 'unstressed' cells and a decrease in the amount of BiP co-immunoprecipitating with IRE1 in cells experiencing ER stress has been reported by several groups.[41,42] Despite this large body of evidence in favor of the competition model, alternative explanations for these data weaken the conclusion that unfolded proteins activate IRE1 by sequestering BiP from its ER luminal domain. Overexpression of secretory cargo, especially slowly folding secretory pathway cargo, may induce an UPR and, therefore, provides little mechanistic insight into the mechanism of unfolded protein sensing utilized by the luminal domain of *IRE1*. Further, the function of the UPR is to restore homeostasis by adjusting the ER chaperone concentrations to the concentration of unfolded proteins in the ER. For this reason overexpression of BiP may attenuate the UPR because BiP overexpression decreases the unfolded protein concentration in the ER. Likewise, decreased BiP levels may increase the unfolded protein concentration, leading to activation of the UPR. Finally, interaction of BiP with IRE1 may simply be a client-chaperone interaction that is decreased due to competition of an increased unfolded protein pool in ER stress due to competition for BiP. In conclusion, the current body of evidence does not separate the functions of BiP during ER luminal protein folding, and as a consequence unfolded protein concentration in the ER lumen, from a potential function of BiP as a direct regulator of IRE1.

In an attempt to separate these functions of BiP Kimata and coworkers[54] used several temperature-sensitive mutations of BiP in yeast. BiP belongs to the HSP70 class of molecular chaperones which consist of a *N*-terminal ATPase and *C*-terminal substrate binding domain (SBD).[55] BiP bound to ATP has low affinity for unfolded proteins, whereas BiP bound to ADP has high affinity for unfolded proteins. Substrate binding to the open, low affinity conformation stimulates the ATPase activity of BiP, leading to closure of the SBD and capture of the bound substrate. ADP for ATP exchange, catalyzed by nucleotide exchange factors such as Sil1p and the GRP170 subfamily HSP70 of the ER, Lhs1p,[56,57] terminates the interaction of BiP with substrate. The *KAR2* gene encoding BiP in yeast is an essential gene in yeast.[58] Several conditional, temperature sensitive (*ts*) *KAR2* alleles are available. *ts* mutations in the ATPase domain (*kar2-113*, *kar2-159*, *kar2-191*) trap Kar2p in the low affinity, ATP-bound state, whereas similar mutations in the substrate binding domain (*kar2-1*, *kar2-133*) abolish interaction of Kar2p with unfolded substrates. At the restrictive temperature, *ts* mutations in the ATPase domain blocked activation of the UPR.[54] At the same time these mutants remained associated with Ire1p in ER-stressed cells. In contrast, *ts* mutations in the SBD constitutively activated the UPR and failed to interact with Ire1p.[54] These data suggest that BiP is a negative regulator of Ire1p which interacts with Ire1p via its SBD. However, it again remains unclear to which extent these mutants allow separation of BiP functions in direct regulation of Ire1p and its functions in protein folding and polypeptide chain translocation into the ER. The *ts* SBD mutations should affect all substrate interactions thus leading to elevated unfolded protein levels even in the absence of ER stress. Kar2p *ts* mutations in the ATPase domain are severely defective in protein

translocation into the ER.[59] This translocation defect may account for the severely blunted UPR in these mutants exposed to the ER stressor tunicamycin, which inhibits an early step in *N*-linked glycosylation[60] and therefore requires protein synthesis and polypeptide chain translocation to cause ER stress.

Mapping and subsequent mutagenesis of BiP interaction regions in the luminal domain of Ire1p should address the shortcomings of the data supporting the competition model. To this end Kimata et al[61] divided the luminal region of Ire1p into five subregions based on a series of 10 amino acid deletion scanning mutants from the *N*-terminus of the luminal domain to the transmembrane domain. Subregion V near the transmembrane domain contains the BiP binding site in yeast Ire1p. Deletion of subregion V abolished co-immunoprecipitation of BiP with Ire1p, but also did, surprisingly, have no effect on the UPR. ΔV-Ire1p expressing cells retained full inducibility of the UPR and did not display an elevated basal UPR.[62] These data strongly suggest that BiP release from Ire1p is not the primary event regulating Ire1p activity. Two other mutants, Δ145 and Δ226, were found to be constitutively bound to BiP, but ER stress still led to dimerization of these mutants, but not to activation of an UPRE-*lacZ* reporter.[62] Deletion of the BiP binding sites in subregion V in Δ226 restored tunicamycin resistance to this mutant, but not to the Δ145 mutant. Either the Δ145 and Δ226 mutations lead to formation of an artificial, inactive dimer, or BiP binding to the luminal domain controls another event than di- or oligomerization of Ire1p required for activation of the cytosolic domains. In contrast to subregions V and I, subregions II and IV were indispensable for activation. A 'core' mutant deleted for subregions I and V (also called a ΔI ΔV mutant) showed regulation by ER stress despite of constitutive self association and loss of the ability to bind BiP.[63]

These data suggest that subregions I and V redundantly control di- or oligomerization of Ire1p. Point mutations in S103 (S103P or S103R) rendered the core mutant constitutively active, which may involve conformational changes induced by these point mutations.[63] These data suggest the following activation model for yeast Ire1p. ER stress induces dimerization of Ire1p by inducing conformational changes in subregions I and V. Binding of BiP to subregion V may contribute to inhibition of dimerization of the core domain. After dimerization a second signal induces conformational changes in the core domain, explaining why ΔV-Ire1p and the core mutant are not constitutively active. BiP may inhibit reception of this second signal or conformational changes in the core domain, because the Δ145 and Δ226 mutants which form dimers and are bound to BiP do not signal.[62] This model is further supported by the finding that the S103P mutation alone did not constitutively activate Ire1p.[63] Further, deletion of subregion V in the Δ226 mutant restored the ability to signal to this mutant.[62] In conclusion, the phenotypes of these Ire1p mutants, especially the phenotype of the ΔV mutant, strongly argue against the competition model.

THE BiP RELEASE MODEL

BiP may interact with Ire1p through its ATPase or substrate binding domain. In vitro, ATP dissociates BiP-Ire1p complexes,[41,64] suggesting that the ADP-bound state of BiP interacts with Ire1p, which may not be consistent with the observation of *ts* ATPase mutants constitutively interacting with Ire1p.[54] Gething and coworkers[65] have shown that BiP interacts with Ire1p through lobe IB of its ATPase domain. These authors also showed that BiP mutants locked in the ATP-bound state, but not the ADP-bound state

interacted with Ire1p.[65] These data are consistent with the behavior of the *ts* ATPase mutants reported by Kimata et al.[54] The earlier reported ATP susceptibility of BiP-Ire1p complexes may therefore reflect a classical BiP substrate interaction. Introduction of an *N*-linked glycan into lobe IB of the ATPase domain did not affect the function of BiP in protein folding. However, this mutant (R85T) was not able to attenuate Ire1p signaling.[65] A neighboring Q88E mutant was able to mitigate the UPR, whereas a Q88A mutant was not. The residue corresponding to Q88 displays chemical shift perturbations between the ATP- and ADP-bound states and after substrate binding in *Thermus thermophilus* DnaK suggesting that this residue undergoes conformational changes induced by substrate binding. Thus, movement of Q88 in BiP induced by binding of unfolded proteins to the substrate binding domain of ATP-bound BiP may induce ATP hydrolysis, conversion of BiP to the ADP-bound state and release from Ire1p. These data can be summarized in a 'BiP release model' (Fig. 3B). Whereas mechanistically more elegant than the competition model, the BiP release model has difficulties to explain the behavior of the ΔV Ire1p mutant.[62] In addition, Q88E-Kar2p only rescued viability of *kar2*Δ *ire1*Δ cells, but not of *kar2*Δ cells, suggesting that the chaperone activity of Q88E-Kar2p may be compromised.

THE LIGAND BINDING MODEL

The 3.0 Å crystal structure of the yeast Ire1p core luminal domain revealed the presence of a major histocompatibility complex (MHC)-like peptide binding groove in the Ire1p dimer (Fig. 2C, D).[43] Single point mutations in the dimer interface [interface 1 (IF1), T226W or F247A] partially inactivated Ire1p, whereas the double mutant was nearly completely inactive. Likewise, mutation of amino acids forming the bottom of the groove of this MHC-like peptide binding pocket, M229, F285 and Y301, also interfered with activation of an UPRE-*lacZ* reporter, indicating the importance for the peptide binding pocket in function of Ire1p. Consistent with a peptide binding activity of this MHC-like domain is the finding that the luminal domain of yeast Ire1p prevents aggregation of citrate synthase and firefly luciferase in vitro,[61] indicating that the luminal domain may have chaperone activity. Credle and coworkers[43] suggested that binding of unfolded proteins to Ire1p monomers induces dimerization via formation of the MHC-like peptide binding groove (Fig. 3C). The yeast luminal domain crystal structure also revealed a second crystallographic interface (IF2). Mutation of a residue in IF2 (W426A) severely attenuated activation of an UPRE-*lacZ* reporter,[43] indicating for the first time that a higher order oligomeric cluster or at least linear array is formed by activated Ire1p in the ER membrane. Using immunofluorescence Kimata et al[61] observed a punctuate staining for Ire1p in ER-stressed cells. In unstressed cells, a disperse, ER-localized staining for Ire1p was observed. Mutations leading to constitutive dimerization, i.e., the ΔI ΔV mutant, displayed a punctuate staining even in unstressed cells,[61] whereas mutations in IF1 or IF2 abolished this punctuate staining. These data suggest that Ire1p may form clusters and as a consequence signaling centers in vivo. Cluster formation of Ire1p using mCherry-tagged Ire1p was also reported by Aragón et al.[66] *HAC1* mRNA colocalizes with these clusters.[66] The reduced ability of IF1 and IF2 Ire1p mutants to activate UPRE-*lacZ* reporters suggests that cluster formation is a prerequisite for signaling by Ire1p. Strong co-operativity of RNase domains may provide an explanation for the requirement for clustering in signal transduction by Ire1p in vivo.[67]

These data supporting direct interaction of Ire1p with unfolded proteins and clustering of Ire1p by unfolded proteins to form signaling centers are contradicted by the 3.1 Å crystal structure of the luminal domain of human IRE1α.[68] The crystal structure of IRE1α also revealed an MHC-like groove formed by two monomers, but in contrast to the yeast protein this groove is too narrow to allow for peptide binding. Moreover, access to this groove is blocked by an α helix. Projection of the C termini indicates that the MHC-like grooves in yeast and human IRE1 face the ER membrane and hydrophobic residues lining the bottom of the groove of Ire1p are either buried in the groove of IRE1α or replaced by charged, hydrophilic amino acids, which are unlikely candidates for interaction with unfolded proteins.[68] In solution, the luminal domain of IRE1α formed dimers and did not show any evidence for higher order arrangements in the crystal structure. For all these reasons, it seems unlikely that IRE1α directly interacts with unfolded proteins. Consistent with this conclusion is that the luminal domain of IRE1α does not possess chaperone activity in vitro.[69] One possible explanation for these differences between the yeast and human proteins may be that both proteins use different mechanisms to sense unfolded proteins. However, conservation of function of the luminal domain from yeast to humans[37] suggests otherwise. Another puzzling and difficult to rationalize observation in the field is that the leucine zippers of MafL and JunL could fully substitute for the luminal domain of Ire1p.[37] The ability of heterologous dimerization domains, such as the leucine zipper domains, as a minimum suggests that one critical function of the luminal domain is to provide a good proportion of the affinity required for dimer formation. Provided these leucine zippers fold in the ER as in the cytosol, the ability of these chimeras to successfully splice *HAC1* mRNA as well, if not better than WT Ire1p, also argues that clustering of the ER luminal domain may be dispensable, possibly because the cytosolic domains retain sufficient affinity for each other to induce oligomerization after dimer formation.

SENSING OF ER STRESS BY PERK

Double stranded RNA activated protein kinase (PKR)-like ER kinase (PERK) or pancreatic eIF2α-subunit kinase (PEK) was independently identified by two groups.[12-14] PERK is a Type I transmembrane serine/threonine protein kinase. The protein kinase domain is localized in its cytosolic portion (Fig. 2A). The luminal domain of PERK has limited sequence homology to its counterpart in IRE1 (Fig. 2B). PERK phosphorylates Ser 51 in eIF2α resulting in the attenuation of general protein synthesis.[12,16] ER stress sensitivity of *perk*$^{-/-}$ cells can be partially rescued by the translation inhibitor cycloheximide.[70] Phosphorylation of eIF2α shuts down general translation by reducing the frequency of AUG codon recognition. However, a subset of mRNAs are selectively translated due to the presence of 5′UTR open reading frames in these mRNAs or the presence of internal ribosomal entry sites to promote cap independent translation initiation.[71] Thus, PERK decreases the load of unfolded proteins in the ER by shutting off general protein synthesis. PERK also phosphorylates the bZIP transcription factor NRF2 to release NRF2 from cytoskeletal stores. NRF2 activates an antioxidant response to ER stress[72-75] to deal with reactive oxygen species (ROS) produced in ER-stressed cells.[76,77]

The luminal domain of PERK oligomerizes in the presence of ER stress.[41] The luminal domains of IRE1 and PERK show some homology (Fig. 2B). They are interchangeable in yeast and their function is evolutionary conserved.[64] Compared to IRE1 considerably less work has been done on how PERK senses unfolded proteins, presumably because

lessons learnt from the luminal domain of IRE1 are thought to be applicable to the luminal domain of PERK. Nevertheless, informed by the competition model proposed for IRE1,[41] two groups have reported experimental data in support of such a model in regulation of PERK. Co-immunoprecipitation experiments have revealed that BiP and another molecular chaperone of the ER, the HSP90 family member GRP94, bind to the luminal domain of PERK in unstressed cells.[41,78] In the presence of ER stress these interactions are lost.[41,78] The regions required for dimerization and BiP binding are separate in PERK.[79] BiP may interfere with the oligomerization of the luminal domains by steric hindrance or inhibiting conformational changes in the luminal domain.[80] The *N*-terminal domain lacking the BiP binding region is required for both oligomerization and activation of PERK. BiP may directly control PERK activation because PERK mutants lacking the BiP binding region were constitutively active.[79]

SENSING OF ER STRESS BY ATF6

Activating transcription factor 6 (ATF6) is the prototype for several Type II transmembrane basic leucine zipper (bZIP) transcription factors in the ER membrane that are activated by ER stress. There are two isoforms for ATF6, ATF6α[21] (90 kDa) and ATF6β[81] (110 kDa). Other Type II transmembrane bZIP proteins in the ER membrane are OASIS,[82,83] CREB3/Luman,[84] CREB-H,[85,86] CREB4[87] and BBF2H7.[88] These transcription factors consist of a bZIP and transcriptional activation domain in the cytosolic N-terminal region followed by a transmembrane segment and ER luminal domain (Fig. 2A). The ER luminal domains of these proteins serve as ER stress sensing domains. There is little to no sequence conservation between these luminal domains. Mechanistic studies on how these membrane-bound transcription factors sense ER stress have only been reported for a subset of these proteins.

Activation of ATF6 has been explained on the basis of the competition model (Fig. 3A). ATF6 is bound to BiP under nonstressed conditions, but upon ER stress it is released from BiP.[20,89] BiP release unmasks two Golgi localization sequences, GLS1 and GLS2. GLS1 binds to BiP, while GLS2 stays inactive. On dissociation of BiP, GLS2 translocates ATF6 to the Golgi. Thus BiP retains ATF6 in the ER by suppressing the activity of GLSs. BiP binding by itself is not responsible for retention of ATF6 in the ER because loss of BiP binding in the absence of GLS motifs was not sufficient to translocate ATF6 to the Golgi.[20] These regions are conserved between ATF6α and ATF6β, suggesting that at least these two transcription factors share a common mechanism of activation. ATF6 is translocated to the Golgi via COPII vesicles[89,90] and cleaved sequentially by the Golgi-resident proteases S1P and S2P. S1P cleaves in the luminal domain and S2P in the transmembrane domain,[22] releasing the cytosolic transcription factor domain from the Golgi membrane. The activated, cytosolic fragment of ATF6 migrates to the nucleus and induces transcription of genes containing ATF/cAMP response elements (CRE) or ER stress elements (ERSE) I and II.[91,92]

Compared to its interaction with unfolded proteins the interaction of BiP with ATF6 is stable,[93] suggesting that BiP binds to ATF6 in its ADP-bound form. A point mutation in the SBD of human BiP (P495L), analogous to the *kar2-1* mutation in yeast, inhibited binding of BiP to ATF6.[93] A mutation in the BiP ATPase domain (T37G) prevented dissociation of BiP from ATF6 by ATP when these complexes were purified in the presence of detergent. These data indicate that BiP recognizes ATF6 as an unfolded

client protein. However, ATP did not dissociate the BiP-ATF6 complex in vitro when this complex was isolated in the absence of detergent. Induction of ER stress with the reducing agent DTT dissociated BiP-ATF6 complexes more efficiently than complexes of BiP with other unfolded substrates.[93] Based on these data it has been suggested that ER stress restarts the ATP hydrolysis cycle of BiP bound to ATF6. Sequences in ATF6 required for restarting the ATP hydrolysis cycle have been identified. More research is required to understand why the ATP hydrolysis cycle of BiP bound to ATF6 is stalled.

An alternative mechanism for activation of ATF6 by ER stress has been proposed by Lee and coworkers.[94,95] Lee et al reported that ATF6 is retained in the ER by the lectin chaperone calreticulin. ATF6 synthesized in ER-stressed cells is underglycosylated, which inhibits its interaction and retention by calreticulin in the ER. At the same time fully glycosylated ATF6 is an efficient ERAD substrate. This degradation of fully glycosylated ATF6 prevents retention of underglycosylated ATF6 in the ER through dimerization via the bZIP domains (Fig. 3E). For CREB-H, differential partitioning between ERAD and activating proteolytic release of its cytosolic transcription factor domain in unstressed and stressed cells has been proposed (Fig. 3F).[86] Competitive inhibition of CREB-H targeting to the proteasome by elevated levels of unfolded proteins, which themselves are ERAD substrates, in stressed cells is one explanation for partitioning of CREB-H toward S1P and S2P cleavage in the Golgi of ER-stressed cells, provided that ERAD activity is limiting in ER-stressed cells. Alternatively, differential partitioning of CREB-H may be mediated by, yet to be identified, specific CREB-H targeting factors which may selectively target CREB-H toward ERAD or activation in the Golgi by S1P and S2P. This model may also explain activation of other Type II transmembrane transcription factors, including ATF6α and ATF6β.

Another aspect of activation of these transcription factors is how they are distinguished from other unfolded proteins in the stressed ER to allow their sorting into COPII vesicles. The luminal domain of ATF6 has been shown to form inter- and intramolecular disulfide bridges between two conserved cysteine residues. In the absence of ER stress, ATF6 is found as a monomer, dimer and oligomer in the ER.[89,96,97] Under ER stress, reduction of disulfide bridges in ATF6 leads to deoligomerization and translocation to the Golgi.[89,96,97] This behavior is in contrast to other sensors of ER stress in that ATF6 depolymerizes on sensing ER stress, while IRE1 and PERK oligomerize. The authors suggested that reduction of ATF6 was carried out by an enzyme in the oxidizing environment of the ER in the presence of the reducing agent DTT and the nonreducing ER stressor tunicamycin. These data explain the longstanding observation that DTT is the most effective inducer of ATF6 cleavage. The identification of the enzyme responsible for ATF6 depolymerization, probably one or several of the 17 protein disulfide isomerases of the ER,[98] will provide further insights into the mechanism of ATF6 activation.

CONCLUSION

Three principal ER stress sensors exist in mammalian cells, IRE1, PERK and ATF6. Unfolded protein sensing for all three ER stress sensors has initially been explained on the basis of the competition model. For IRE1, and because of sequence and also functional conservation of luminal domain function between IRE1 and PERK, also PERK, more recent work has shown that the competition model cannot explain how these proteins sense ER stress. As an alternative explanation direct interaction with unfolded proteins of

the luminal domains of IRE1 and PERK was proposed. Experimental data supports this model for yeast Ire1p, but not human IRE1α. These discrepancies need to be resolved in future work. A conceptual problem with crosslinking of Ire1p by unfolded proteins is that rapid reversibility of the UPR, or in other words, inactivation of Ire1p by other means than degradation of the crosslinked Ire1p clusters, is difficult to reconcile with cluster formation, if not specific 'declustering' activities exist. Recent work on ATF6 activation also suggests that ER stress sensing by ATF6 is more complex than predicted by the competition model. Here, experimental work needs to be extended to other Type II transmembrane transcription factors. In conclusion, despite considerable efforts to understand unfolded protein sensing by IRE1, PERK and ATF6 much more remains to be learnt.

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CHAPTER 11

AUTOPHAGY AND SELF-DEFENSE

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Abstract: Autophagy is a highly conserved mechanism which is essential for the maintenance of cellular homeostasis in response to cellular stress. Autophagy has been conserved from yeast to humans as a quality control process that is involved in the recognition and turnover of damaged proteins and organelles. It is also a response mechanism to nutrient starvation. In mammals, autophagy is involved in antigen presentation, tolerance, inflammation and protection against neurodegenerative diseases. The decrease of autophagy during aging reduces the removal of damaged organelles and increases the accumulation of waste products in the cells. In this chapter, we review these aspects of autophagy along with their role in self-nonself distinction, their implication in innate and adaptive immune response, and its dysregulation in the pathology of certain inflammatory and autoimmune diseases.

INTRODUCTION

Autophagy is a lysosomal-mediated process in which intracellular components are degraded. This adaptive mechanism occurs in response to stress and promotes the survival of the cell under these conditions. The stress conditions include nutrient starvation, invading microbes, or tumor formation. Autophagy is also involved in the clearing of defective proteins and damaged organelles. Therefore, it has an important physiological role in development, innate and adaptive immunity, cancer, neurodegenerative diseases, aging, and cell death. The importance of autophagy is illustrated by its conservation from yeast to human. Many of the genes first identified in the study of autophagy in yeast have orthologues in other eukaryotes, including human homologues. There are three forms of autophagy, designated as chaperone-mediated autophagy, microautophagy and macroautophage: (1) Chaperone-mediated autophagy

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consists of the selective degradation of soluble proteins that are translocated into the lysosome by the chaperon hsc70 and the transmembrane protein LAMP-2A. (2) Microautophagy involves a direct capture of cytosolic components by the lysosome by invagination of its membrane. (3) Macroautophagy is a tightly regulated process in which a newly formed, double-membrane vesicle captures a proportion of cytoplasm that can include damaged organelles and eventually fuses with a lysosome.

Macroautophagy in yeast starts in the phagophore assembly site (PAS), which is a nucleation point for recruitment of the proteins involved in the first steps of macrophagy. Here a new vesicle, the phagophore, appears, expands, and surrounds the portion of cytoplasm to be degraded. The vesicle enlargement and their fusion generates a double-membrane organelle, the autophagosome. Subsequently, the autophagosome fuses with a lysosome to form an autolysosome, and the autophagosome cargo, together with its inner membrane, is degraded by lysosomal enzymes. Eventually, the molecules generated in this process are released into the cytosol to be reused by the cell (Fig. 1A). Although a distinct PAS has not been found in humans and other higher eukaryotes, the phagophore, which is a group of multiple membranes throughout the cytosol, appears and eventually fuses to form a unique membrane around the material to be degraded.

Macrophagy (which we will call simply autophagy in this chapter) evolved in unicellular eukaryotes, in which the supply of nutrients depends on their availability in the environment. Unicellular eukaryotes must be able to react to unfavorable conditions in which precursors for proteins or other necessary components are not available. Autophagy is one mechanism that allows cell survival in these stressful situations. Autophagy recycles existing cytoplasmic components to generate the molecules that are required to sustain the most vital cellular functions. Surprisingly, autophagy is a highly conserved system that has been maintained in metazoans, in which the concentration of extracellular nutrients is normally controlled. Autophagy is essential during times of inadequate nutrients because cells need adequate precursors or building blocks to support basic functions to maintain their viability.[1] For example, cells could need amino acids and ATP that can be obtained by the degradation of cytoplasmic components. These generated metabolites can be recycled to synthesize new proteins or to obtain ATP. One situation in which the reuse of cellular components is crucial for survival is during neonatal starvation. Soon after birth, mammals face a severe period of starvation until milk provides the nutrient supply. Autophagy remains at a low level throughout the embryonic period but is extensively induced in various tissues, such as heart muscle, diaphragm and alveolar cells, after birth. Several studies have shown that mice deficient in proteins related with autophagy such as Atg5 and Atg7 die the first day after birth.[2,3]

In addition to its crucial role during starvation, autophagy also contributes to cell viability through the removal of damaged organelles, in particular mitochondria, and the disposal of aggregated proteins during hypoxia. Age-related accumulation of damaged organelles is related to a failure of autophagy which removes damaged organelles by a poorly understood mechanism.[4] Suppression of autophagy can trigger the accumulation of aggregated proteins, which play an important role in age-related diseases, including neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and Huntington's disease.[5] Hypoxia is also a stressful situation in which upregulation of autophagy appears to be protective. Its protective function against hypoxia has been studied especially in ischemic injury during heart failure.[6]

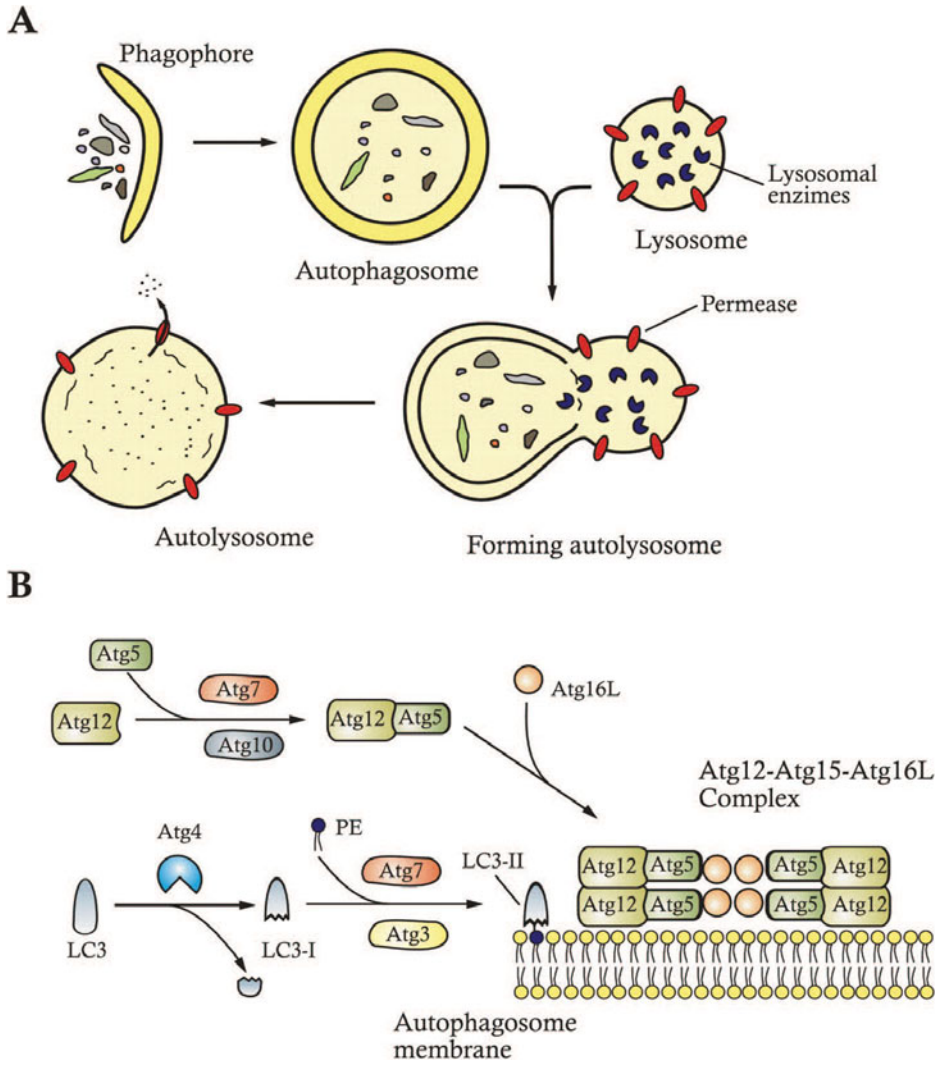

Figure 1. Cellular and molecular events in autophagy. A) Creation and expansion of the phagophore, formation of the autophagosome and fusion of the outer membrane of the autophagosome with a lysosome. The autolysosome content is degraded and the resulting molecules are released into the cytosol. B) Molecular events involved in the expansion of the phagophore membrane.

For simplicity we will consider the proteins and protein complexes described in the human autophagy pathways. We will review the protein complexes involved in autophagy, the signals that regulate this process, and the signaling pathways implicated. We will finally analyze the role of autophagy in aging and in apoptotic cell death as well as its role in autoimmune disorders, inflammatory diseases, and the immune response against intracellular pathogens.

AUTOPHAGY MACHINERY

The understanding of the molecular machinery implicated in autophagy began with the discovery of the autophagy-related (ATG) genes in yeast. Currently, more than 30 ATG genes have been identified. Orthologues have been identified to many of these genes in other eukaryotes. In humans, the pathways involve genes that are analogous to those found in yeast as well as other genes that do not have a known counterpart (Table 1). Proteins encoded by these genes generally form multi-protein complexes that comprise the 'core' autophagy machinery and are essential for autophagosome formation.[7] Other proteins have a role in its regulation. The core machinery in mammals is composed of four complexes: The unc51-like kinase (ULK) complex, the phosphatidyl inositol 3-kinase (PI3K) complex, the ubiquitin-like proteins (Atg12 and LC3), and the membrane protein mATG9 and related proteins.

The ULK complex in humans contains the ULK1/2, mAtg13 and FIP200 proteins. ULK1 and ULK2 proteins are homologues of yeast Atg1; mAtg13 and FIP200 are homologues of yeast Atg13 and Atg17, respectively.[8,9] mTORC1 regulates the activity of ULK complex by selective binding that is dependent on the nutrient conditions, as we will discuss later. The ULK complex is essential for the autophagy induction.

Table 1. Molecular machinery of autophagy. Mammalian genes and their orthologues in yeast

Function	Mammals	Yeast
Induction of autophagy		
	ULK1	Atg1
	ULK2	
	FIP200	Atg17
	Atg13	Atg13
	Atg9	Atg9
	WIPI	Atg18
Vesicle nucleation		
	Class II PI3P	
	Beclin 1	Atg6
	Barkor or Atg14L	Atg14
	URAG	Vsp38
	p150	
Maduration		
	Atg12	Atg12
	Atg5	Atg5
	Atg7	Atg7
	Atg10	Atg10
	Atg16L	Atg16
	Atg4	Atg4
	Atg3	Atg3
	LC3	Atg8
	Atg9	Atg9

The mammalian class III PI3PK complex (PI3KC3) is composed of Vps34 (essential for the kinase activity), p150, Beclin 1 and Atg14-like protein (also known as Barkor). PI3KC3 phosphorylates phosphatidyl inositol (PI), generating phosphatidyl inositol 3-phosphate (PI3P). PI3P is required for autophagosome formation since it mediates vesicle nucleation. There are two additional PI3PC3 complexes: The UVRAG complex includes UVRAG and the rubicon complex in place of Atg14L. These three complexes act differently: Atg14L complex has a role in the formation of autophagosomes, the UVRAG complex acts in autophagosome maturation, and the Rubicon complex inhibits autophagosome maturation.[10]

The conjugations of ubiquitin-like proteins, Atg12 and LC3, are mediated by several proteins (Atg7, Atg10 and Atg5 for Atg12; Atg7 and Atg3 for LC3) and have been proposed to occur during the expansion of the phagophore membrane.[11,12] Atg7 and Atg10 mediate the conjugation of Atg12 and Atg5. The noncovalent association of multiple Atg12-Atg5 conjugates with Atg16L proteins generates the Atg16 complex (Fig. 1B). Atg4 cleaves LC3, which generates a protein fragment (LC3-I). LC3-1 binds covalently with a phosphatidyl ethanolamine (PE) molecule by the action of Atg7 and Atg3, and becomes a nonsoluble conjugate called LC3-II which is associated with the autophagosome's outer membrane.

Atg9 is another core protein that is highly conserved across species. This transmembrane protein is also required for mammalian autophagy. It colocates with LC3 positive autophagosomes, and is also found in several vesicles such as late endosomes and the trans-Golgi membranes. The function of mAtg9 protein is still unknown, although it might deliver lipids or other components to the forming autophagosomes.[13-15]

REGULATION OF AUTOPHAGY

Autophagy is a basal process that helps maintain cellular homeostasis. The elimination of defective proteins, aggregates of proteins and damaged organelles is part of the normal cellular turnover which involves autophagy. The importance of basal autophagy was revealed in animal models: The suppression of autophagy triggered neurodegenerative diseases.[16,17] Autophagy is also required when critical changes occur in the environment and the organism cannot provide an appropriate physiological response, as in starvation conditions.[1] These processes are regulated by several key components: mTOR and the kinases c-JUN N-terminal kinase 1 (JNK1) and the death-associated kinase (DAPK) (Fig. 2).[18,19] mTOR (mammalian target of rapamycin) is a large protein kinase that forms two distinct multi-protein complexes, mTORC1 and mTORC2. The mTORC1 complex is involved in autophagy regulation whereas the role of mTORC2 in autophagy remains less definitive.[20] mTORC1 consists of the mTOR catalytic subunit and the four associated proteins, raptor, GβL/mLst8, PRAS40, and DEPTOR. mTORC1 a key inhibitor of autophagy, integrates multiple upstream signals associated with the regulation of autophagy (see below) and inhibits the ULK complex, an inducer of autophagy. mTORC1 interacts with the ULK complex and induces phosphorylation of ULK1/2 and Atg13, which inhibits ULK complex activity. Conversely, conditions that trigger autophagy such as nutrient starvation causes mTORC1 to dissociate from the ULK complex. The ULK complex undergoes conformational changes and becomes active.[20-22]

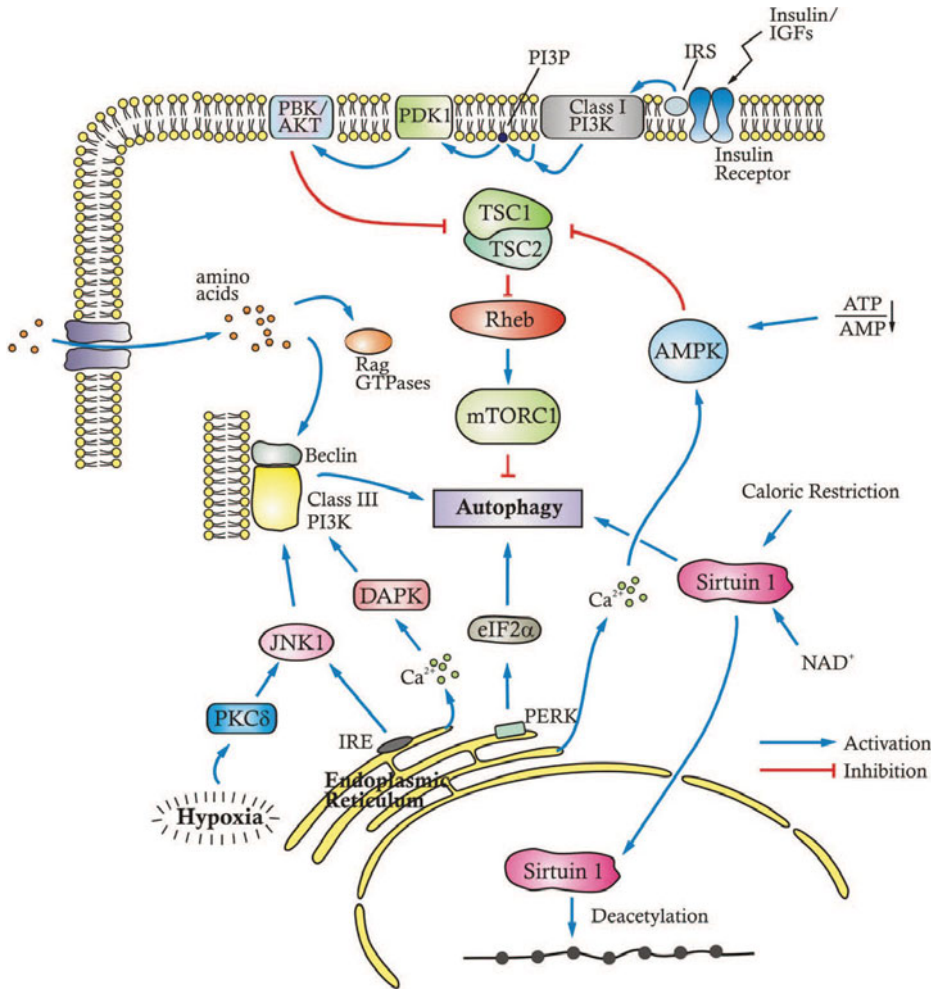

Figure 2. Regulation of autophagy in mammalian cells. Autophagy is regulated by nutrients, growth factors and stress. In this process mTORC1 has an essential role, although there are also some mTORC1-independent types of regulation, such as the regulation of the PI3KC3 by JNK-1 and DAPK.

JNK1 and DAPK are involved in the regulation of the PI3KC3 complex. During this process, the anti-apoptotic protein B-cell lymphoma/leukemia (Bcl)-2 binds Beclin 1 and inhibits the PI3KC3 complex. PI3KC3, which is essential for the nucleation and assembly of the phagophore, is only active when Beclin 1 dissociates from Bcl-2. This indicates that the anti-apoptotic activity of Bcl-2 is distinct from it anti-autophagic activity.[10] JNK1 and DAPK induce autophagy by phosphorylating Beclin 1, which promotes dissociation of the Beclin-1/Bcl-2 complex. The JNK1 and DAPK kinases are involved in autophagy regulation in response to stress.[10] Bcl-2 can also be dissociated from Beclin 1 by mechanisms independent of phosphorylation. For example, MyD88 and TRIF, two Toll-like receptor adaptor proteins, interact with Beclin 1, reduce Beclin 1 binding to Bcl-2 and lead to autophagy. In this way, Toll-like receptors can trigger autophagy.[23]

SIGNALING REGULATION OF AUTOPHAGY

Autophagy participates in the regulation of growth and metabolism in response to environmental changes. During starvation, a decline in the intracellular concentration of amino acids induces autophagy. Extracellular amino acids use specific transporter proteins to enter cells. Their intracellular concentration is detected by the mTORC1 complex. The increase of amino acid concentration leads to mTOC1 activation, which downregulates autophagy. mTORC1 may detect the different amino acids via diverse mechanisms. It has been proposed that Rag GTPases mediate amino acid activation of mTORC1 in a process mediated by the GTP-loaded Rheb (Ras homolog enriched in brain). The GTP-loaded Rheb interacts directly and activates mTORC1.[24,25] PI3KC3 (hVps34) may also be involved in the regulation of mTORC1 by amino acids.[26] Other unknown pathways may also be involved or amino acids concentrations may be sensed by mTORC1 directly.

mTORC1 is also involved in the regulation of autophagy by insulin or insulin-like growth factors (IGFs). These factors activate mTORC1 via the class I phosphatidyl inositol 3-kinase (PI3KC1) complex that inhibits autophagy, in contrast to PI3KC3 (reviewed in ref. 7). The binding of insulin or IGFs to their receptor lead to its autophosporylation, which results in the recruitment and phosphorylation of the insulin receptor substrate (IRS) that binds to PI3KC1. Then, PI3KC1 catalyzes the generation of PI3P at the cell membrane, subsequently recruits PBK/Akt and PDK1 to the membrane, and activates PBK/Akt via PDK1. Activated PBK/Akt phosphorylates the heterodimer of tuberous sclerosis protein (TSC)-1 (hamartin) and TSC-2 (tuberin) which disrupts and inactivates the TSC-1/TSC-2 heterodimer. Functional TSC-1/TSC-2 is a GTPase-activating protein for Rheb, which binds to and activates mTORC1 in its GTP-bound form.

The level of cellular energy also regulates mTORC1. A reduction in the level of cellular energy (low ATP/AMP ratio) triggers its inactivation. The AMP-activated protein kinase (AMPK) in conjunction with LKB1 kinase acts as a sensor of the cellular energy. A low ATP/AMP ratio activates AMPK, which results in the subsequent phosphorylation and disruption of the TSC1/TSC2 heterodimer and ultimately the inhibition of mTORC1.[27] Activated AMPK also stimulates pathways that generate ATP as fatty acid oxidation.

There are other stress signals that induce autophagy such as hypoxia and endoplasmic reticulum (ER) stress. Hypoxia induces the hypoxia-induced factor (HIF)-1, a transcription factor that promotes the transcription of hundreds of genes in response to low levels of oxygen. One of these genes encoded the regulated in development and DNA damage (REDD)-1 protein,[28] which downregulates mTORC1 via the TSC1-TSC2 complex.[29] The response to hypoxia also can be HIF-1 independent via the protein kinase Cδ (PKCδ)-JNK1 pathway.[30] ER-activated autophagy is triggered by the accumulation of unfolded proteins (reviewed in ref. 31). Many misfolded proteins show a tendency to polymerize and form insoluble aggregates that cannot be reached by enzymes normally involved in their degradation. This situation activates autophagy by multiple signals, such as PERK and IRE1 pathways and calcium-mediated signalling. The PERK pathway involves eIF2α, which is necessary for the conversion of LC3-I to LC3-II and increases the transcription of *Atg12*. JNK1 participate in the IRE1 pathway and induces autophagy by interfering in the interaction between Bcl-2 and Beclin 1. JNK1 triggers the liberation of Beclin 1 and induces autophagy.[32,33] The release of Ca^{2+} from the RE to the cytosol also induces autophagy. The increase in the cytosolic calcium activates the calmodulin-dependent kinase kinase-β (CaMKKβ). CaMKKβ activates AMPK which induces autophagy as seen previously.[34]

AUTOPHAGY AND CELL DEATH

Cell death is a normal event that occurs in healthy organisms. Thus, just as cells destroy and renew their organelles or other molecular components, cells die and are renewed in a given organism. Several forms of cell death include apoptosis, autophagic cell death, and necrosis. Apoptosis, also known as Type I cell death or programmed cell death, results in the condensation of nuclear chromatin and DNA fragmentation. Autophagy is denoted as Type 2 cell death. Necrosis, also called Type 3 cell death, has been generally considered as an uncontrolled form of death, although it probably undergoes a type of regulation. Autophagy generally preserves cells when they are subjected to stress, protecting them from cell death under these conditions. However, in some cases autophagy acts as a distinct form of cell death and depends on the type and degree of environmental stimuli.[35] Autophagy and apoptosis are different processes that generally use inverse regulatory signals. For example, autophagy induction in conditions of stress generally protects cells against apoptosis; conversely, inhibition of autophagy leads in many cases to a higher susceptibility to apoptosis.[36] The molecular pathways involved in autophagy and apoptosis as far as their regulatory mechanisms are different. However, mammals (but not in unicellular organisms) have proteins that are involved in the regulation of both activities, such as the Bcl-2 protein family.[10]

Bcl-2 family of proteins contains at least one Bcl-2 homology (BH) region. In humans and other mammals, this family comprises (1) The anti-apoptotic multidomain proteins (such as Bcl-2 and Bcl-X_L), which contain four BH domains (BH1, BH2, BH3 and BH4), (2) The pro-apoptotic multidomain proteins (such as Bax and Bak), which contain three BH domains (BH1, BH2 and BH3), and (3) The pro-apoptotic BH3-only protein family (such as Bad and Noxa). Bax and Bak are necessary for the mitochondrial outer membrane permeabilization (MOMP). They form channels in this membrane, which release apoptosis-inducing proteins that are normally present in the space between the outer and inner membranes and induce apoptosis in this manner. On the contrary, the anti-apoptotic Bcl-2 and Bcl-X_L reside in mitochondria, stabilize their membranes by an incompletely elucidated mechanism and prevent apoptosis.[37] The pro-apoptotic protein BH3 domain can bind to a binding groove in Bcl-2 and Bcl-X_L and inhibit the function of Bcl-2 and Bcl-X_L. One component of the PI3KC3, the protein Beclin 1, also contains a BH3 domain that can interact with Bcl-2 and Bcl-X_L. This interaction inhibits autophagy.

Two mechanisms disrupt the interaction between Bcl-2 proteins and Beclin 1, and therefore, induce autophagy. First, as aforementioned, cell stress activates JNK1 and DAPK proteins which phosphorylate Beclin 1. Second, proteins with BH3 domains that bind to the BH3-binding groove of Bcl-2 and Bcl-X_L block their binding to Beclin 1. Although autophagy and apoptosis are interconnected by common regulatory proteins, we still need to elucidate the environmental or intracellular conditions that trigger cell death by apoptosis or by autophagy and the converse conditions that lead to the use of autophagy as a stress adaptation to maintain cell viability and therefore suppress apoptosis.

AUTOPHAGY AND AGING

aging involves an accumulation of intracellular waste consisting of defective and damaged cellular components that interfere with normal cellular activity. Accumulated waste is due to the decline of autophagic degradation since one of its functions is

to carry out a quality control surveillance and maintenance of the cell components. Therefore, autophagy has been suggested as a mechanism involved in aging and longevity (reviewed in refs. 38,39). There are several animal models that have been used in these studies. First, the nematode *Caenorhabditis elegans* model identified several factors that modified its lifespan and were related with autophagy. When *C elegans* undergoes caloric restriction, a treatment that increases autophagy, its lifespan is increased. Conversely, the inhibition of genes required for autophagy using RNAi, such as Atg-7 or Atg-12, shortened it lifespan. Likewise, the knockout of bec-1 (ortholog of mammalian Beclin-1) also shortened the lifespan of *C elegans*.[40]

Several known longevity factors, such as SIRT1 and p53 have been implicated in autophagy. The Sir2 protein, which is a SIRT1 homolog in yeast, can extend the life span of yeast. SIRT1, a class III protein deacetylase ubiquitously expressed in different organs, regulates cellular metabolism, and has been associated with aging. Interestingly, SIRT1 has a role in the regulation of autophagy.[41] Chronic caloric restriction increases the level of SIRT1 and mice that are defective in SIRT1 do not completely activate autophagy. In these animals, the introduction of SIRT1 restores autophagy. SIRT1 deacetylates cytoplasmic proteins involved in autophagy, such as Atg5, Atg7 and LC3 although it also could act by deacetylating histones. This result indicates that the previously known effects of SIRT1 in longevity could be mediated by autophagy and, on the other hand, SIRT1 may be an important regulator of autophagy. With regard to p53, the knockout of its orthologue in *C elegans*, CEP-1, induces an extension of lifespan.[42] CEP-1 is an inhibitor of autophagy. Thus, depletion or inhibition of p53 induces autophagy whereas cytoplasmic p53 represses it.[43]

Autophagy can also be related to the maintenance of balance. One study described that mice deficient in autophagin-1 protease (Atg4b), which is a protein necessary for autophagosome maturation, showed a balance disorder related with an inner ear developmental defect.[44] These mice showed defects in the development of the calcium carbonate crystals (otoconia) essential for the sense of balance. This defect could be related to age-related degeneration of vestibular otoconia and therefore related with the decline of autophagy with age.

AUTOPHAGY IN INNATE AND ADAPTIVE IMMUNOLOGY

Autophagy Regulation by Immune Signals

Autophagy has important functions in many aspects of the immune response, both innate and adaptive. It is involved in processes such as Toll-like receptor (TLR) response to pathogens, antigen presentation, pathogen degradation, and regulation of inflammation. Cytokines can induce autophagy in some cases or conversely, suppress it in other cases (reviewed in refs. 45-47). Likewise, autophagy is involved in the pathology of certain inflammatory diseases such as Crohn's disease (CD).

Recently, the signaling pathways leading to activation of autophagy during the immune response have been described. Some of the immune signals that induce autophagy include IFN-γ, TNF, CD40-CD40L interactions and TLRs. In contrast, autophagy is negatively regulated by T helper Type 2 cytokines, interleukin (IL)-4 and IL-13. TLRs are a type of pattern recognition receptors (PRRs), which detect molecular structures shared by pathogens. They are membrane receptors located at the cell surface, like TLR2, or

in endosomes, like TLR7. When TLRs recognize their specific ligand, for example lipopolysaccharide (LPS) derived from cell walls or viral single strand RNA (ssRNA), they induce the maturation of phagosomes.[48] The stimulation of TLR2 with zymosan (a cell wall component of fungi that is recognized by TLR2) triggers the recruitment of the autophagosome marker LC3 to the phagosome. The adaptor molecule MyD88 is not required, although possibly involved, in this process. These data indicate that the pathway involved in the TLR2 induction of autophagy is unknown.[49] The induction of autophagy by LPS involves TLR4. The signaling pathways include the adaptor TRIF, the receptor-interacting protein (RIP)-1 and the p38 mitogen-activated protein kinase, but not MyD88.

TLR induction of autophagy is important in the destruction of mycobacterias.[50] TLR7 signalling also contributes to the induction of autophagy in macrophages and dendritic cells in response to pathogen invasion. TLR7 is expressed in endosomes where it detects viral ssRNA. TLR7 stimulation activates autophagy through MyD88 and also stimulates production of Type I IFNs, an important promoter of innate immune response. Therefore, viral recognition via TLR7 can induce IFN production during the innate immune response, and TLR7 stimulation reduces viability of mycobacteria in infected macrophages.[51] The importance of autophagy during immune responses is also supported by the existence of evasive strategies developed by some pathogens. For example, the herpes simplex virus Type I (HSV-1) encodes a neurovirulence protein ICP34.5, which is expressed early, binds to Beclin 1, and inhibits autophagy function.[52] Escape mechanisms have also been described in bacteria such as *Brucella*, *Shigella*, *Listeria*, and others.[53,54] *Shigella* evades autophagy by secreting IcsB that inhibits autophagy by competing with the protein VirG for the binding to the autophagic protein Atg5. *Shigella* needs VirG for intracellular actin-based motility. However, VirG binds to Atg5 and triggers autophagy, which can clear the infection.[53]

Type I IFN production is also induced by TLR9 in plamacytoid dendritic cells (pDCs) by the same signaling pathways induced by TLR7.[55,56] These two receptors share the pathways involved in autophagy. TLR9 recognizes viral double-stranded DNA, such as herpes simplex virus, and is involved in antigen capture and presentation by pDCs. B cells constitutively express TLRs, such as TLR7 and TLR9, as well as their characteristic B-cell receptors (BCRs). Although both types of receptors initiate downstream independent signaling pathways, TLR9 and BCRs can act synergistically. For this reason, DNA-containing antigens can induce hyperactivation of B cells, which could produce a strong response to common antigens in these cells, as occurs in systemic lupus erythematosus (SLE).[57] SLE is a chronic inflammatory disease that can affect multiple organs such as skin, joints, and kidneys. SLE pathogenesis involves IFNα and autoantibodies that recognize self cellular components such as complexes of proteins and nucleic acids. The autoantibodies can form immune complexes that accumulate in the renal glomerulus, joints, and other tissues. Genetic studies indicate that TLR9 as well as some downstream TLR-signaling molecules could be involved in SLE.[58] Chaturvedi et al have proposed a mechanism that integrates TLR9 signaling from endosomes with BCR signaling initiated at the plasma membrane and that could predispose genetically susceptible individuals to autoimmunity by connecting the innate and adaptive immune systems.[59] In their model, synergism begins between BCR and TLR9 when the BCR is stimulated by a DNA-containing antigen. This binding triggers the internalization of the BCR-antigen complexes and their subsequent fusion with endosomes containing TLR9 into autophagosomes. The internalized DNA is recognized by TLR9 and induces the TLR9

downstream signaling, together with the BCR signaling pathway, result in a synergistic activation through MAPK. This dual activation is useful for responding to pathogens, but autoimmunity can arise from incorrectly regulated dual activation.

Autophagy and Inflammation

Nucleotide-binding oligomerization-domain (NOD)-like receptors are intracellular, cytoplasmic receptors that detect specific bacterial molecules.[60,61] NOD-like receptors, TLRs, and retinoid acid-inducible gene (RIG)-1, are the main classes of known PRRs. The minimal structure recognized by NOD1 is the bacterial dipeptide D-γ-glutyamyl-meso-diaminolimelic acid (iE-DAP) whereas NOD2 recognizes the muramil dipeptide (MDP) moieties found in peptidoglycan. The activation of NOD1 and DOD2 by these bacterial components induces the production of cytokines, antimicrobial peptides, adhesion molecules, and other inflammatory mediators. As happen with TLRs, inadequate regulation of the NOD1 and NOD2 response can lead to inflammatory diseases, such as CD. Genetic studies have revealed the association of the NOD2 gene with CD and subsequent functional studies have confirmed its role in the disease.[62,63] NOD1 has also been involved in the susceptibility to CD.[64] A recent study has revealed that NOD1 and NOD2 have an important role in the autophagic response to invasive bacteria, and have linked bacterial sensing through peptidoglycan detection to the initiation of autophagy.[65] Autophagy activation by NOD1 and NOD2 requires the recruitment of ATG16L1 to the plasma membrane, and it is independent of the adaptor protein RIP2 or activation of NF-κB. Interestingly, the NOD2 polymorphism L1007InsC is the most prevalent polymorphism associated with CD, impairs recruitment of ATG16L1, and significantly decreases autophagy in response to bacteria. These results concur with the previously described association of a single nucleotide polymorphism in the ATG16L1 with CD and provide a mechanistic link between two of the most important genes associated with CD.[66,67] Therefore, NOD1 and NOD2 trigger an unknown pathway in which the adaptor protein RIP2 and the transcription factor NF-κB are not involved. Perhaps these receptors are only related to the formation of bacteria autophagosomes since NOD1 and NOD2 are not necessary in autophagy induced by starvation or by rapamycin. Together, these findings connect the deregulation of autophagy with the excessive inflammatory response found in some diseases, and may spur new approaches in their treatment.

Autophagy and Antigen Presentation

The implication of autophagy in antigen presentation could be crucial during the immune response to intracellular pathogens. Autophagy has also been implicated in cancer and tolerance.[68] Most cells are able to display peptide antigens on their MHC class I or class II molecules. The peptide-MHC complex is recognized by a specific T-cell receptor, which is the critical step for T-cell activation.[69] Peptides presented by MHC class I and class II are different. Class I molecules join intracellular peptides originated from viral, bacterial, or cellular transformed proteins, and present it to CD8 T cells, whereas class II molecules present peptides from extracellular origin to CD4 T cells. Strong evidence indicates that autophagy is involved in class II presentation. It may also participate in class I presentation although this still needs to be confirmed. Autophagy may also play a role in cross-presentation, which is the presentation of extracellular antigens by MHC class I molecules. This very important immune mechanism allows, for example,

antigen-presenting cells (APC) to present viral antigens that are not able to infect them. Autophagy also may be involved in cross-presentation of extracellular tumor antigens.[70] Tumor cells would include tumor antigens in the autophagosomes, where they would be degraded. These autophagosomes may be released into the extracellular milieu and be internalized by dendritic cells. However, this research does not explain how tumor antigens could reach class I cells.

Autophagy may also participate in the CD8 T-cell response against certain intracellular microorganisms. For example, *Toxoplama gondii*, survives in macrophages by residing in vacuoles that do not fuse with lysosomes. It is eliminated when autophagy triggers the fusion of vacuoles with lysosomes.[71] On the other hand, CD8 T cells are required for protective immunity against *T gondii.*[72] Therefore, cross-presentation is required to eliminate this pathogen. However, it is not known how peptides derived from it gain access to the class I molecules.

Similar to class I cross-presentation, some studies have described the presentation of endogenous antigens by the class II molecules. This cannot be explained solely by the classical route of presentation. The presentation of cytosolic antigens is possible by the continuous fusion of autophagosomes with the compartments originating from the ER and loaded with class II molecules.[73] Class II presentation of endogenous antigens is necessary to generate a robust CD8 T-cell response, and thus, class I and class II molecules must present the same antigen.[74] Indeed, the mechanisms of cross-presentation could have implications in self-tolerance. For example, autophagy was disrupted in thymic epithelial cells of mice which eventually developed severe colitis and inflammation in multiple organs.[75] TECs exhibit a high constitutive level of autophagy and the alteration to autophagy interferes with their endogenous class II-peptide repertoire, which reduces the number of tissue-specific self antigens presented. This reduced autophagy alters T-cell selection and increases the possibility of autoimmunity.

Autophagy in Immunity Against Intracellular Pathogens

Autophagy contributes to immune response against intracellular bacteria, parasites, and viruses. Phagophores have the capacity to include large intracellular structures such as bacteria and the capability to destroying them when the phagophore fuses with the lysosome. This mechanism can act when other systems fail. For example, when group A *Streptococcus* infects nonphagocytic cells, it can escape from endosomes into the cytoplasm. However, the bacteria can be sequestered in an autophagosome and can then be destroyed with the fusion of this bacteria-containing compartment with a lysosome.[76] The clearing of cytoplasmic pathogen by autophagy have been demonstrated in other bacteria such as *Mycobacterium tuberculosis* and *Toxoplasma gondii*, *Salmonella*, and others. *M tuberculosis* can survive within phagosomes unless interferon (IFN)-γ activates autophagy pathways. The INFγ-induced autophagy overrides the mycobacterial block of phagolysosome formation, perhaps induces the fusion of bacteria-containing phagosomes with autophagosomes, and the subsequent fusion with a lysosome.[77] Similarly, *T gondii* survive within vacuoles after being ingested by macrophages. In this case, the induction of autophagy and the subsequent destruction of the pathogen are induced by CD40 stimulation.[71]

There is less evidence that autophagy participates in the degradation of virus during the immune response. However, herpes simplex virus (HVS)-I virions can be captured by autophagosomes and destroyed in an in vitro model.[78] Hepatitis C virus (HCV) infection could also induce an autophagic response in hepatocytes. Infection of immortalized human

hepatocytes by HCV induces the formation of autophagic vacuoles.[79] Autophagy is required in some cases for the recognition of virus, like the vesicular stomatitis virus (VSV). This ssRNA virus is recognized by TLR7, which is expressed on endolysosomes. Immune recognition requires the transport of cytosolic viral replication intermediates into the lysosome by autophagy.[55] Autophagy also interacts with human immunodeficiency virus (VIH) biogenesis. During HIV infection, autophagy appears to have an ambivalent role. Early stages of autophagy promote HIV infection in macrophages, and thus, induction of autophagy either with rapamycin or by starvation increase HIV production.[80] Activation of TLR7 and TLR8 signaling induces autophagy during HIV infection.[51] However, HIV during the maturation stages is not degraded by autophagy due to the protective effect of Nef, which inhibits terminal, degradative stages of autophagy. Nef colocalizes with Beclin 1, and increases the distribution of hVPS34 on the membranes. The protective effect of Nef during autophagy became evident by using HIV defective in Nef; these HIV mutants are degraded by autophagy. In other circumstances, HIV can inhibit autophagy. In DCs, HIV replication produces envelope proteins that activate mTOR, which in turn inhibits autophagy. This inhibition evades early immune control by DCs.[81] Therefore, autophagy mediates a direct destruction of intracellular pathogens that has to be circumvented by the microorganisms to produce an effective infection. The importance of this process in whole immune responses to invading microorganisms still needs to be determined.

CONCLUSION AND FUTURE PROSPECTS

Autophagy has an important role in physiological and in pathological circumstances. The elimination of altered proteins and organelles by autophagy occurs during normal physiological conditions. However, it is also involved in stress situations in which it promotes the maintenance of cell integrity. A rapid advance in the knowledge of the molecular mechanisms implicated in this process has occurred during this last decade, and includes the discovery of their molecular pathways and insights into their regulation. However, our understanding of some aspects of autophagy is still limited. Many aspects of the autophagosome formation still have to be resolved. Autophagy is clearly involved in numerous pathological processes including infection, inflammation, neurodegenerative diseases, and aging. The knowledge of its regulatory mechanisms, the multiple inhibitory and stimulatory signals and their interactions remain incomplete. A better understanding of how autophagy is regulated would allow us to manipulate these signals, and in this way, develop strategies to modulate, prevent and treat the multiple diseases in which this mechanism is implicated. Autophagy is a highly conserved process involving proteins conserved from yeast to humans. However, in pluricellular organisms, autophagy has acquired new functions. The lack of these additional functions in simple organisms has made it challenging to elucidate some aspect of this process, especially those related with apoptosis and cell death. Probably the resolution of this and other aspects of autophagy will allow us to obtain valuable information to understand the pathological aspects of the diseases in which this process is involved.

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CHAPTER 12

VIRUSES AND HOST EVOLUTION: Virus-Mediated Self Identity

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Abstract: Virus evolution has become a topic that involves population based selection. Both quasispecies based populations and reticulated mosaic exchange of populations of genetic elements are now well established. This has led us to the understanding that a cooperative consortia can be a crucial aspect of virus driven evolution. Thus viruses exist in groups that can cooperate. However, consortial based evolution (group selection) has long been dismissed by evolutionary biologist. Recently, biocommunication theory has concluded that the evolution and editing of any code or language requires a consortial based process in order to adhere to pragmatic (context) requirements for meaning (in conflict with survival of the fittest concepts). This has led to the idea that viruses are the natural editors of biological codes or language. In this chapter, I present the view that the persistence of virus information in their host provides a natural process of host code editing that is inherently consortial. Since persistence requires mechanisms to attain stability and preclude competition, it also provided mechanisms that promote group identity. Accordingly, I review the viral origins of addiction modules and how these affect both persistence and group identity. The concepts emerging from addiction module based group identity are then generalized and applied to social identity systems as well. I then examine the prokaryotes and the involvement of viral elements in the emergence of their group identity systems (biofilms). Here, integrating dsDNA agents prevailed. In the eukaryotes, however, a large shift in virus-host evolution occurred in which the role of dsDNA agents was diminished but the role of retroviruses and retroposons was greatly enhanced. These agents provided greatly expanded and network based regulatory complexity that was controlled by sensory inputs. From this perspective, the role of virus in the origin of the adaptive immune system is then outlined. I then consider human evolution from the perspective of the great HERV colonization. The origin of a large social brain able to support the learning of language is presented from this viral perspective. The role of addiction modules in the origin of extended social bonding of humans is outlined and applied to the emergence of language as a system of group identity.

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INTRODUCTION: IDENTITY—LESSONS FROM THE BOTTOM

Self recognition can be defined as an aspect of an identity systems in which self is distinguished from nonself. However, prior to this volume, identity as a specific field of study has not previously been considered as a separate or coherent body of knowledge. And yet, as a highly social species, humans are fascinated by the topic of identity and are often interested to know what biology may be able to tell us about the social and culture nature of human identity.[1] This interest is not specific to human identity. It doesn't require too much contemplation to realize that the concept of identity is a fundamental biological issue. Identity, therefore, must have been an ancient feature and all organisms will need such systems. Evan with the viruses (and sub-viral defectives), composed as they are of only genomes and capsids when outside of cells, they too must be able to identify themselves and to differentiate and replicate their genomes apart from those of host and other competing genetic parasites. Therefore, all organisms, including viruses, would fundamentally need to identify self from nonself and a whole array of molecular and other systems can be considered from this perspective. These systems would include all those involved in the innate and adaptive immune response as immunity is clearly identity related. Such systems also seem to be involved in cell fate and tissue identity for multicellular organisms. In the case of viruses with their basal simplicity, we may thus see the most elemental characteristics of identity systems involving groups or populations. Viruses might have a lot more to tell us about identity systems then had previously been realized. Recently, I have developed this idea into an extended evaluation of how the viruses were likely involved in the origin of many early identity systems, but also how the viruses help us understand some of the basic principles of group identity.[2] We have only recently come to realize that many viruses (mostly RNA, retro, but also some DNA) exist and evolve in quasispecies based populations that are essentially consortial in character.[3] This informs us that the population, not the average or master fittest type as we have long thought, can provide a clear (and sometimes essential) selective advantage to the group.[4] But in such a consortia, the population must still maintain various ways by which membership is identified. Identity still matters to a population. Indeed, recent studies of the evolutionary dynamics of prokaryotic DNA has led some to propose that the DNA viruses of prokaryotes also operate as a 'cloud' or consortia of viral derived elements that will dynamically colonize host genomes.[5] From the perspective of virus, it is the regulatory (essentially informational) relationships between population members that become crucial for group identity. Indeed, when any organisms exists in colonies or groups that maintain self identity, they too must maintain some mechanism to identify members from nonmembers. Members would need to be supported (maintained and/or replicated) whereas nonmembers would be precluded and/or destroyed if not consumed as prey. Such group based identity would thus include associative or altruistic features as well as preclusive or aggressive features. Previously, the existence of any such potentially altruistic systems had been rationalized as emerging from selection of the fittest type (essentially selfish genes) that in accordance to the various assumptions of cost-benefit analysis and game theory can result in the selection for individuals that support other (genetically related) individuals within a group. Numerous mathematical models have thus sought to account for the emergence of group related phenotype that did not violate the central tenant of neo Darwinian theory, namely that variation and natural selection of the fittest type drives such adaptations, see[4] for references. In some cases, it indeed appeared that such theories could account for

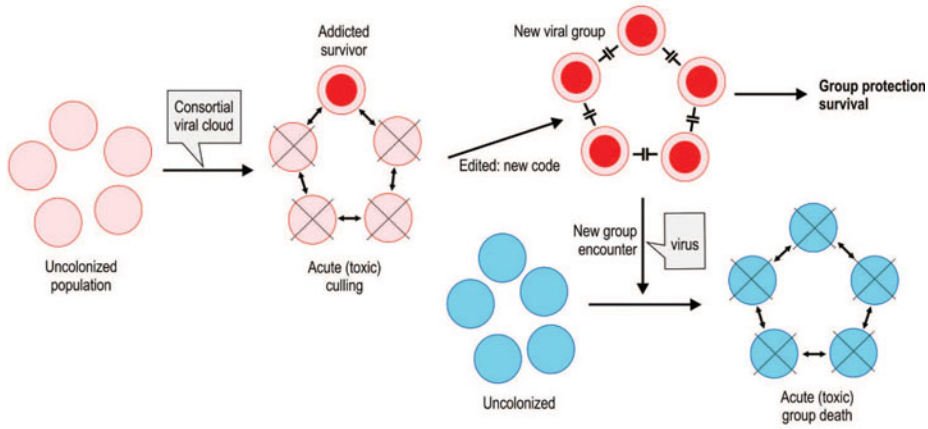

Figure 1. Schematic of virus affects on population based host survival. The five diffuse red circles represent a host population free of the infectious virus in question. When exposed, many members will succumb to the toxic (acute), affects of virus infection (crossed lines). Some, however, may be stably colonized (shown with dark red center). This host population has acquired a new virus derived instruction set that also provided immunity to the same (and often other) viruses (shown by broken lines between cells). If this population retains some capacity to produce infectious virus, or if the virus remains prevalent, when it encounters another naive population (blue circles), the uncolonized population will crash due to virus toxicity. The virus colonized population will be favored.

some forms of group behavior. But the simple virus informs us differently. Quasispecies based group selection does not require any of these cost-benefit like assumptions or even depend on a fittest (master) type, although an average type always prevails.[6] A consortia inherently results from what has been called 'high error' and recombination rates but these 'errors' also provides a much enhanced capacity for a genetic system to explore sequence space (which is astonishingly vast even for a small 10 kb RNA virus). This enhanced exploratory capacity is essential in order to attain solutions (which are sometimes dynamic and/or counteracting) by a genetic system whose genetic capacity is ironically also limited by high 'error' rates (via error threshold). Thus the high error rate both enhances and limits the genetic capacity of simple viral systems, but the enhancement is fundamentally consortial.[3,7] In this realization, we may see an underlying general principle by which a group or colony of organisms can attain enhanced survival.[4] A diverse population must be generated that can also provide a diversity of solutions (including counteracting solutions) to any particular issue. But this population must retain some degree of stability and still have someway to identify or include individual members of the population. In the context of humans and their inherently social group identities, it might seem implausible to propose the involvement of some type of genetic plasticity in group identity. But if we consider that behavior (including social bonding) is a central element of human group identity, then we only need to understand how behavioral diversity (plasticity) is generated or maintained in order to apply this concept to humans and to explain tendencies towards associative or altruistic behaviors.[8] Thus, it is most ironic, that in the viruses, considered the most selfish of genetic elements, we may define the fundamental principles by which groups can form cooperative consortia that work together. This chapter thus explores this idea and relates it to others presented in this volume.

THE CONSORTIA STORY FROM VIRUS

The existence of consortial genetic action is not a broadly accepted concept. Such a state appears equivalent to that of group selection, an idea that has been soundly rejected by most evolutionary biologist as having no sound theoretical foundation. In spite of this strong consensus, the consortial genetic action by viruses has been experimentally established[9] (see ref. 3). Most evolutionary biologist, however, would likely question the applicability of this virus genetic biology to their host organisms. Yet we now know viruses to be the most abundant and diverse genetic entities on Earth and that all life has survived the unending onslaught of these agents. So, if we can suspend our skepticism and accept the idea that consortial genetic action is selectively favored and broadly essential for group identity, we would still need to address how and why such group identity is established (mechanistically) and how its stability is maintained. Returning to the fundamental simplicity of viruses, here too we can find some answers. By considering the relationship of bacteria and their viruses to bacterial colony (group) behavior and survival, we can observe that some viruses colonize their host in stable relationships (persistent) but in so doing, alter the identity of their host. In bacteria, many persisting viruses are DNA viruses that integrate their DNA into their host to promote genetic stability. This addition also brings to the host capacity to resist similar viruses (self immunity). However, some persisting viruses of bacteria do not integrate their DNA yet also attain a highly stable extra chromosomal persistent infection (e.g., P1). It is with these viruses that we can define a fundamental strategy that promotes group identity.

CODE EDITORS MUST BE CONSORTIAL

A topic that has not previously received much attention concerns the need to originate and edit code in biological systems. It has long been assumed that the process of natural selection from individual variation (fittest type) can explain the origin and development of code. In the context of human evolution and their social groups, this issue is of uniquely special interest as it is crucial to account for the biological origin of language.[1] The application of and mathematical modeling of natural selection to explain the origin of biological information systems was first presented by M. Eigen (see refs. 10-11). The aim was to understand how error prone replication systems (such as a precellular RNA world) might be able to conserve information content and syntax in order to get evolution by natural selection started. This line of reasoning was to lead to the quasispecies equations which described the population based behavior and error/information limits of such replication systems. Since then, the quasispecies theory has been much evaluated and developed in the context of RNA virus studies. However, early on it also became evident that the focus on only syntax and point changes presented basic problems for understanding code.[12] Essentially, it was appreciated that context, in addition to syntax, was as important for determining ‘meaning’ of code. This context dependence established that ‘pragmatics’ not simply syntax, was key to understand codes. However, after an extended period of evaluation, it was concluded that the pragmatic nature of code or language also required the participation of populations, not individuals for it was the living population of agents that would assign meaning to the context dependent code.[12] A direct deduction from this analysis was the individual fittest type (master template of quasispecies theory) as used by neodarwinian natural selection, could not have led to the origin and editing of code.

Code must have originated from a population based process and a similar population based process must also have contributed to the origin of human language. The problem with this conclusion, however, was that there was no accepted mechanism by which populations could operate as selective units to promote such code. Group selection, for example, had been dismissed as unsupported and lacking theory, see.[4] However, the possibility that virus populations might provide the underlying 'group-based' mechanisms had not yet been developed or considered when group selection was dismissed. This was due to several reasons: one, early on these virus populations were assumed to also operate via fittest type as described in early quasispecies theory. And two, the frequent and large scale occurrence of viral derived genetic information in the genomes of all life forms, had been dismissed as being due to 'selfish DNA', which had no fitness or code functions.[13] However, during the 1990's, an extended set of experimental studies established that viral populations showed much interaction (see ref. 3). And by 2005, compelling evidence established that viral fitness could be cooperative and consortial in which a fittest type was not essential.[9] In addition, it was realized that the selfish virus derived DNA could frequently affect the relationship of a host population with other viruses and that these elements also had to potential to superimpose a type of coordinated regulation onto the genetic loci they colonized. These two observations together led some to propose that the viruses provided the poplulations that were competent to edit and create code.[12,6] Such a conclusion would place viruses in a central role as code (and genome) editors. What was then needed, was to mechanistically explain how a population of genetic colonizers could alter or create a population based group identity.

THE CONCEPT OF ADDICTION MODULES AND STABLE GROUP IDENTITY

It has long been realized that in the prokaryotes, colonization by prophage (genomic viruses) constitutes a major process by which prokaryotic DNA is changing. With the sequencing of nearly 1000 prokaryotic genomes, we are now confident that viruses and their defective elements account for the majority of DNA variation between genomes.[14,15] It has also become very clear that the horizontal transfer of DNA is a major and ongoing process by which prokaryotes adapt (discussed below).[16,17] Accordingly, it has been thought that by integrating into host DNA these viruses attain a stable relationship with their host. It had also long been recognized that such prophage integration normally involved immunity genes that control virus replication and provide immunity against related and sometimes unrelated (lytic) viruses. Thus, a prophage colonized host has altered its susceptibility to such viruses. In this relationship, we start to see how colonizing viruses can promote group identity. A bacterial colony that host a particular prophage, will be immune from (and likely also produce) that same prophage. However and identical colony that does not harbor the prophage (or its defectives) will be susceptible to lysis from that (and other) virus. Given that phage are the most abundant and diverse entities in most all habitats,[18,19] viruses provide an inescapable selective pressure that due to their transmission, operates on groups of host. If an individual bacteria were to lose its resident prophage, it will also become susceptible to group specific virus mediated killing. Thus we see a form of group selection where on group (colonized) can threaten another group (uncolonized) via a transmissible agent. We might think that the colonized bacteria is addicted to its virus, since if it loses the virus, host death by lysis is likely. However, this

concept of addiction can be expanded to provide an even more fundamental way to think about group identity and its stability. In the 1990s, the idea of an 'addiction module' was proposed by Yamolinsky in order to explain the ability of a non-integrating phage to attain stable persistence in its host (see refs. 20,21). The virus was P1 phage, which does not integrate but can stably persist as an episome in its host for highly extended periods. P1 thus establishes a noncovalent form of genetic stability and it does so by using various gene pairs that counteract one another (PhD/DOC) as well as other mechanisms. One gene in the pair is toxic to the host (such as an endonuclease) but its toxicity is inhibited by a second gene that specifically binds to it. However, the toxic gene is stable while the inhibitory gene is unstable. This then requires that the inhibitory gene be continuously expressed to prevent a toxic reaction. Thus, if the P1 DNA is lost, its transcription is also lost resulting in death to the cell. The cell is thus addicted in that it must maintain the P1 genome to avoid programmed cell death and continue living and the responsible gene pair is called an addiction module.[22]

GENERALITY OF FEATURES

Although such modules can promote stability of the virus and are thus crucial for persisting viruses, they can also respond to the infection of the colonized host by other agents. Both acute viruses (T4) and temperate viruses (lambda) will disrupt addiction modules when they infect a P1 colonized host (for example by affecting ongoing transcription or protein stability). The result is that before such an infection can produce new virus, the disrupted addiction module results in lethal toxicity to the newly infected host (programmed cell death). In this way, a population of P1 infected cells will be immune to these second transmissible agents by terminating the production and transmission of virus in individually infected cells and preventing transmission to neighbors, see.[23] This viral addiction module has thus promoted three distinct states. (1) it compels the host to stably maintain P1 to avoid death, (2) by maintaining P1, established a state of immunity to lytic P1 infection that it can also produce and (3) by expressing the P1 addiction modules it has provided a form of immunity (via self destruction) against various other agents.[2] It is also important to note that defective versions of P1 may be able to fulfill most of these states. These three states can be considered to act together to from a new state of group identity (see Fig. 1). To envision this group identity situation, let us consider two adjacent populations of otherwise identical *E. coli* colonies, one colonized by P1 and the other not. Since all the P1 colonized *E. coli* have the potential to also produce lytic P1, only P1 colonized *E. coli* will make the needed immune functions to be able to grow within the colony. This virus has thus imposed a distinct state of group identity (P1 immunity) to the colony. And the P1 addiction module has also imposed a new state of more generalized immunity to other viruses (e.g., T4 and Lambda). Given that such types of tailed prokaryotic DNA virus are the most numerous and diverse genetic entities in all aqueous habitats, a colony that has acquired a generalized resistance to other viruses will be highly favored. But in addition to favoring the survival of the colony, the persisting P1 can also disfavor the survival of any nearby colony that is not also P1 colonized. For example consider an adjacent *E. coli* colony that is not in direct contact with the P1 colonized colony, but is near enough for some of the low level P1 virions made by the P1 colony to diffuse to them. Clearly, such an unprotected colony will be susceptible to lytic P1 infection. Its fitness will be conditional, not determined on the situation of the

habitat, but what its neighbors might be producing virus wise. Such conditional fitness states will not be susceptible to mathematical models that don't also include specific viral states of colonization. Since such states are completely stochastic, they will not likely be readily modeled. In this example, we therefore see the clear operation of group based selection in which the group identity has been determined by the colonizing viral agent. Thus we can now state the essential and generalized features of this version of group identity to evaluate if or how it might apply generally to group based identities in other living systems. These general features are the following:

1. In order for a parasitic genetic agent (e.g virus or defective) to successfully and persistently colonize its host, it requires a strategy that promotes the maintenance (stability) of this element. The colonization can be either genomic or epigenetic.
2. This strategy can be considered as an addiction module which compels the host to retain the agent in order to avoid harm that the agent or its associates will otherwise impose. That harm is generally a form of self destruction which is also prevented by the genetic parasite.
3. In molecular terms, an addiction module is composed of a gene pair encoded by the parasite in which a stable but toxic gene (or function) is counteracted by an unstable protecting gene that inactivates the toxic function. An addiction module can also be mediated by virus or 'defective' genetic parasites that colonize their host, but protect the host against destructive (lytic) consequences of prevalent acute nondefective viruses in the habitat.
4. Colonization by such genetic parasites superimposes a new form of group identity in that the harmful component of the addiction module can be transmitted to and harm those populations that are not similarly colonized.
5. The establishment of group identity in new populations or descendents is a time dependent process (i.e.,via a developmental window) and requires that the protective element of the addiction module be transmitted or activated before the destructive element or agent is active. Group identity is thus an acquired state that is not necessarily or directly determined by genetic composition of the population.

These five features can now be considered in generalized terms to see if they are inherent to other systems of group identity. One overarching generality that emerges is that group identity states are beneficial to or protective of group members and a disadvantage for or harmful to nonmembers. The nonmembers are often very similar to if not identical to the members. Thus the harm that results can often appear to be a from self destruction and can involve toxic genes made by the organisms itself (such as those of apoptosis). These toxic functions are sometimes actively transmitted or exported to harm populations that differ in identity (especially in prokaryotes and lower Eukaryotes). At its origin, viral derived lysis functions or toxins appear to have originated many identity systems in the prokaryotes (see below). Indeed, prokaryotic viruses still provide the most useful way to identify specific bacterial populations via the well established process of phage typing. In addition, the prevalent occurrence of toxin/antitoxin gene pairs (T/As) in prokaryotes can also be best understood from the perspective of group identity (see below). Thus in prokaryotes, the viral origins of many if not most group identity systems seems clear. However, in eukaryotes the origins of group identity are less apparent. This is because diffusible or transmissible feature of the group identity in Eukaryotes became mediated initially by sensory mechanisms involving signal transduction such as surface molecule

detection, then sensory odor detection (i.e., peptides and pheromones) but also evolved to be mediated by visual and audio sensory systems (requiring the evolution of learning and the associated neuronal CNS functions). Yet even in these more derived systems of Eukaryotic group identity, we can still see the essential features of group identity as outlined above. Thus the acquisition of group identity in Eukaryotes often has an inherently sensory dependent (epigenetic) character to it which seems unrelated to the more biochemically determined systems in prokaryotes. And in the more complex Eukaryotes, such as humans, group identity has become predominantly a learned state involving little apparent toxic biochemistry. However, even in the most complex of Eukaryotes we can still identity an essential role for viruses and genetic parasites in promoting the origin of new systems of group identity. A striking example of such an underlying viral role is found in the adaptive immune system of vertebrates. The acquisition of this most complex system of group identity was directly mediated by the action of endogenous viruses. Yet the adaptive immune system still adheres to all general features of group identity as outlined above (see below).

SOCIAL IDENTITY AND LANGUAGE ADHERE TO THESE GENERALITIES

What about the culturally acquired and highly social character of human based group identity? Can the general features of group identity outlined above still apply to such a highly social and learned states? On the surface of this question, it would not seem that some of the above features are applicable. For example, the toxic and anti toxic gene pairs involved in addiction module mediated identity would not seem relevant to defining human social based group identities. I know of no observations that support such an idea. However, the more generalized state in which harmful and protective features work together to set a state of addiction and promote stable group identity clearly does appear applicable to human group identity, (ref. 8 see below). It has long been asserted by some social scientist that various features of Darwinian selection do not apply well to explaining the origin of the highly associative nature of human group interactions. Survival of the fittest, competition and natural selection did not appear well suited to explain extended human social structures. Historically, evolutionary biologist have come to explain the existence of the highly associative nature of human group identity as a product of some indirect consequence of selfish gene based evolution. These are thought to operate via mechanisms such as kin selection. Basically, the idea is that survival of the fittest can be made to promote kin survival (inclusive fitness) if the application of various form of cost-benefit analysis or game theory are incorporated (see ref. 4). Although kin linked human social structures might be explainable by such methods, broader social groups lacking any kin or genetic relationship that are characteristic of many human social structures are not explained by such models. However, possibly the most troubling of all human social capacities to explain is the evolution of language. Indeed, this was a troubling issue for Darwin himself. Language is the essential human trait that most distinguishes humans from the other great apes and required the evolution of a large social brain to learn, produce and use it. In evolutionary terms, the evolution of language is a very recent and essentially unique evolutionary phenomena. But with the evolution of language, we encounter a most fundamental theoretical problem that was not initially appreciated. Language, like any natural code, must convey meaning and meaning must

be learned. For some years, it was thought that meaning resided in the syntax (sign sequence) of the code, thus models of information theory that were focused on syntax were applied to understand the evolution of biological codes.[24] This approach was first formally proposed in the 1970s . However, by the 1990s it had become apparent that meaning must also be context dependent and not simply syntax determined. This led to the dominance of pragmatics (context dependent meaning) as a way to understand the meaning of language (and the emergence of biocommunication theory). However, with the realization of the dominance of pragmatics, another direct conclusion was reached; that pragmatics demanded that a population (not an individual fittest type) be the source of new and collective meaning.[12] This indicated that for language to have evolved, it must have involved a selective process that was population based. Thus, all of the prior theories of evolutionary biology that involved a fittest type (inclusive fitness, cost-benefit, game theory) became problematic as their selective basis still stem from the fittest individual type. However, if we consider that language itself is a form of group identity, that adheres to the features outlined above, we can more easily understand why language must have evolved in a group based process.

Let us now consider how language and group identity relate to the general features as outlined above. Language can also be considered as an information parasite that has colonized a group of large social human brains, affected their development and group identity. In so doing, it provides this group of brains with a common social identity via a shared ability to communicate social traditions and membership (culture). Language also provides a large selective advantage to the group by providing a mechanism to socially communicate the memory of experience that can have big consequences on group survival (i.e., culture -relating to knowledge of food, shelter, survival etc.). Thus the maintenance of language is made essential for group survival. However, once colonized, the human host population is distinguished from other populations that have become colonized by other languages since social and other forms of communication are not supported. This difference can lead to group antagonism and conflicts. Given that the human (but not chimpanzee) brain goes through a period of early post birth brain development in which the learning of language is facilitated, but later becomes much more difficult for adults to learn new languages, we can also see that language acquisition has both an underlying biological basis in brain development, but additionally has the needed temporal (open window) features of a system of group identity. That is, language acquisition requires a developmental window during which group identity can be transferred to new members but after which it becomes resistant to further transfers of language (as required for group identity). Thus the biologically based and addictive nature of language can be recognized. Its acquisition is associated with clear and crucial advantages to those groups that have learned it. And, it also provides a strong disadvantage to any individual that does not learn language. Language based identity also likely promotes conflict between groups with distinct social identity and communication.

So then, how does a language mediated from of group identity relate to forms of group identity that exist in other mammals. Are they biologically similar? Consider, for example, an encounter between human groups compared to that of other mammals. Humans will likely attempt conversations, that seek to learn language, cultural, tribal or religious based group identity. All these aspects of identity are learned socially. However, some of our close social companions of another species, such as the domestic dog (and most mammals), will not be as fully dependent on learned social states of group identity. They will also seek to evaluate identity by more biochemical means involving intense

sensing of odors (pheromones). Thus the marking urinary odors of a competing male or a competing pack of dogs or from a menstruating female will be of primary social interest to evaluate and this applies to essentially all terrestrial tetrapods. Thus at least some of these canine group based responses, although possibly learned, are not the products of culture but have a more basic biological basis to them. As we consider even less complex but ancient species, say like a gold fish, we might further conclude that odor based group identity is prevalent in evolution and almost mechanical in its application to many vertebrate species.[25,26] Yet this ancient biochemical sensory system used for social and group identity was essentially lost in the African primates, especially the great apes.[27,28] Indeed, the associated vomeronasal organ (VNO) involved in such social detection has been incapacitated in African primates (see ref. 2). This loss was not due to a single genetic locus, but involved numerous retroposon genetic alterations of all the relevant receptors. I have argued that such a loss was principally mediated by a great HERV colonization that occurred in the African primates. In addition, as presented below, we can also see evidence that the emergence of our large social brain was also promoted by the further action of such genomic viruses. Thus a great genetic upheaval in human evolution was involved in the transition of human group identity from an ancient process dependent on order detection to a social process dependent on language and social learning. Although what resulted was a socially learned form of group identity, it was still promoted by the most ancient of creative processes, the colonization of external genetic parasites we collectively know as virus. Let us now note one final commonality between us and our dogs. In both cases identity is being communicated by invisible and transmittable agents (odor or vision compared language, thought and belief). But this media regardless of its physical characteristic must act via sensory channels and be able to elicit an appropriate biological response (such as behavior). That behavior will have a general character to it, being either be associative (supportive, friendly, emotionally positive) or antagonistic (aggressive, emotionally negative) towards groups. In this essential features, human and dogs (or bacteria) don't seem so different. And it also appears that the action of externally derived genetic parasites was key in the origin of these group identity systems.

THE NATURE OF PROKARYOTES

The prokaryotes constitute the most ancient, numerous, diverse and dynamic genomes of all cellular life forms. We now appreciate that they can rapidly adapt via complex genetic alterations to a great number of selective shifts in their habitat. They are the ultimate inventors of genetic solutions. Indeed, it has been proposed that essentially all gene functions can be traced to prokaryotic origins. Yet with the completion of about 1000 prokaryotic genomes, comparative genomics informs us that this adaptability is most often mediated by viruses (external sources). The term horizontal gene transfer (HGT) has often been used to characterize how prokaryotic genomes are most frequently observed to change and adapt. Since it was noted long ago that many of the transferred genetic elements and gene sets involved were defective in their ability to function as an infectious agents (virus), it was assumed that nonviral mechanisms were prevalent in mobilizing such DNA. However, more recent reevaluations of this notion indicates that infectious agents (viruses) are indeed the main sources of HGT (see references above). And we have also come to realize that viruses themselves are the most numerous, diverse and ancient genetic entities in all aqueous and soil habitats

on Earth. Both in clinical, culture and also natural habitats, viruses have been reported to mediate the HGT associated with host genetic adaptability of prokaryotes. A more recent characterization of the dynamics of prokaryotic genomes suggest that an external 'cloud' of mosaic viral elements is mediating host genetic adaptability.[5] Indeed, it has been observed that in oceanic cyanobacteria, for example, phage islands characterize the genetic changes associated with habitat specific populations of this crucial photosynthetic host and furthermore, that viruses are manipulating both the regulation and the core genes of photosynthesis.[29-31] Similar observations have been reported for photosynthetic eukaryotic algae.[32] The most sequenced of all prokaryotic genomes are those of *E. coli*, associated with clinical isolates. These have clearly been shown to vary by gene sets that can alter about 20% of the genome. And as these gene sets are mostly adjacent to tRNA integration sites and also generally have identifiable proviral elements, they clearly appear to have been acquired via the action of viruses. However, as noted above by Banfield and Young, these viral derived elements do not appear to act individually, but most often in populations or clouds that work in concert to affect host fitness. This has recently been most apparent from studies of the most intensely sequenced of all *E. coli* genomes, those of enteropathogenic and toxigenic strains of *E. coli* (serotypes 0157:H7 0127:H6) whose genomes have been sequenced and compared to commensal *E. coli* strain (0152:H28).[33-35] And in these studies, it has been shown that various and distinct external genetic elements that are phage/plasmid derived were found to stably colonize these strains, although most elements were individually defective. It was most interesting that these elements were shown to work in coordination with each other to mobilize and transmit infectious (albeit defective) DNA.[36] And that this transmissive process also incorporated the use of phage mobilized insertion sequences to generate diversity in the host DNA.[37,38] Furthermore, comparing the two distinct genomes of the sequenced pathogenic and commensal *E. coli*, established that although distinct viruses had been involved, all these genomes essentially followed parallel patterns of evolution.[35] In addition, it was shown that the transcription of these regulated 'horizontal' oathogenic genes was orchestrated by the captured regulatory genes and regulatory elements.[39] In this observation, we see direct evidence of how to edit and coordinate a complex code (the genome of *E. coli*). It involved stable colonization by a mixed and cooperative population of viral derived elements. In general, these observation directly support the views of Villarreal[2] and those of Banfield and Young noted above[5] regarding the mosaic mixture or 'cloud' like nature of virus mediated prokaryotic evolution. Thus the viral-host situation in prokaryotes is often more complex then envisioned by classical Darwinian thinking in which individual variation in virus and host combine to affect fitness. However, what is seldom considered even in such recent studies as those above, is the likelihood that these persisting viral genetic elements have attained stability because that have imposed a viral derived addiction module onto their host. Yet this conclusion seems almost inevitable since at the very least these agents would likely provide immunity against similar nondefective lytic viruses. But if we accept this as a likely outcome of such mixed colonization, we also can infer that the group identity of such bacteria will also likely have been altered and attained. However, we are not accustomed to thinking about prokaryotes in terms of groups or populations.

For many years, bacteria were thought of in terms of clonal haploid organisms in which group interactions were not significant. Since a colony was the product of a single cell, group interactions or group identity systems seemed unnecessary to account for bacterial survival. As noted above, however, proviral and defective viral elements

(missing genes needed to make independent virus) can none the less provide most of the features of addiction modules, thus promote a from of prokaryotic group identity. In addition, there are frequent interactions with other colonizing viruses or their defectives, such as P2/P4, very much like that characterized for toxigenic *E. coli* noted above, that can function as a complementing network of agents that promotes host resistance to other agents, as well as adaptability to changing circumstances by promoting the mobilization of these viral derived but coordinated gene sets. Indeed, in my recent book I examine both the consequence of phage production/resistance and the origin of bacterial toxins to consider their likely role in prokaryotic group identity.[2] One conclusion is that the bacteriocins (bacterial toxins active against other bacteria) can clearly mediate group based competition, but an examination of the likely origins of these toxins indicates that they have mainly originated from defective viral sources.[40-44] Thus these transmissible toxins so common in may prokaryotes can be considered as components of identity systems that are harmful to similar bacterial populations. Along these lines, the frequent occurrence of toxin/antitoxin (TA) gene sets in most bacterial genomes can also be thought of as elements of addiction modules[45,46] that appear to have no direct fitness consequence.[47] However, these modules can promote the stability of group identity by providing counter measures to infection and colonization by viruses.[48] Indeed, the emerging study of TA gene sets in biofilms, as presented below, strongly support this idea. In the context of cyanobacteria, it is especially noteworthy that these organisms are well known for being able to produce a large diversity of diffusible toxic molecules.[49] Indeed, the synthesis of these cyanotoxins provides an effective means of group or strain identification for the cyanobacteria.[50] The involvement of these molecules in group identity however, has not been explored.

The historic view of bacteria as essentially clonal organisms thus has changed considerable in the recent years. With the discovery of quorum sensing systems in bacteria and their role in the formation of bacterial communities such as in biofilms (that also involve other organism), we have witnessed a big shift in how we think of bacterial survival (for review see ref. 51). Indeed, clinically it appears that the most states of bacteria resistant to drug therapies are to be found in biofilm communities. And here too, studies with toxigenic *E. coli* have been crucial in understanding how multidrug resistance can relate to biofilm formation. Related studies have recently made it clear that TA addiction modules are involved. It was noted by Bigger many years ago[52] that a small population of bacteria in a community were able to resist various drugs (but with no known genetic alteration). These bacteria were called persisters (see refs. 53, 54). More recently, it has become clear that TA addiction modules are involved in these persisting (dormant) states of resistance,[55-57] including the Phd-Doc TA system,[58] described above in P1 addiction. As mentioned, when first discovered, TA modules were thought to have no fitness consequences as deletion analysis suggested, but more recent evaluation based on biofilm survival establishes their important role in community formation.[59,60]

The existence of the altruistic-like self destruction associated with TA addiction modules and viral-stress exposure in a unicellular organism had long puzzled evolutionary biologist. As these gene sets appeared to offer no observable fitness advantage to growing individual cells,[47] their ubiquitous presence was hard to account for. Although the usual Darwinian based cost-benefit modeling for kin selection have been applied to explain TA modules, however, since the majority of known TA modules are phage or plasmid encoded, this would indicate that any such 'kin' selection would be defined by the persisting parasitic agent,[61] not the *E. coli* genome itself. An like the P1 viral episome

addiction maintenance outlined abovem,[62] other low level plasmid maintenance can also be supported by the plasmid encoded TA modules,[63] (see ref. 46). Thus a general proposal that sought to explain the existence of five TA modules in *E. coli* K-12 suggested that these TA modules were important for cellular resistance phage. In keeping with this, the highly studied mazEF TA module will cause individual cells to die upon P1 phage growth, an altruistic act of this 'self destruction' that is beneficial for the bacterial population by excluding P1 propagation.[64] The term abortive infection (Abi) systems has also been used to describe how TA modules would contribute to more generalized forms of phage exclusion or resistance.[48] Similar TA mediated anti-phage altruistic suicide has also been reported to be encoded on parasitic plasmids in Erwinia bacteria.[65] These observations allow us to present an alternative explanation for the origin and evolution of prokaryotic TA addiction modules. They are the products of persisting genetic colonizers (viruses and plasmids) that are essential and used to compel parasite persistence and stability, but are also active against and preclude other viral colonization by inducing self destruction. Thus, the vast, ever present diversity of viruses that all prokaryotes must always contend with, compels all surviving cells to have these viral derived systems in order to exclude both self and other competing viruses. Accordingly, any fitness measurement done in the absence of this virus habitat (or stress) will be misleading. In this way we can also understand another prokaryotic immune process: the ever present and highly adaptable clustered regular interspaced short palindromic repeats (CRIPSRs), are also essential in regulating lysogeny.[66,67] In biofilms, these elements also appears to undergo a population based response to virus, but have themselves been acquired from and are the products of rapid evolution by lateral DNA transfer.[68] Since the addiction modules outlined above can all stem from and also oppose transmissible/infectious agents, such infections provide the very mechanisms that define bacterial group identity. Indeed, such reasoning would suggest that persisting phage are a principle driving force in the creation of prokaryotic communities and biofilms. Thus a biofilm colony would need to be compatible with its own viruses and TA modules (group identity) to continue its group living and oppose (by self destruction) the loss of this identity by common viruses in their habitat. Thus it is most interesting that the very exposed exocellular polysachharides that defines biofilms, are also important for some phage resistance.[69,70] Yet other specific phage can be induced during TA induction and biofilm formation[71] and in some cases, this phage can provide the very killer genes of the TA modules.[72] It has also been observed that in pathogenic proteus species, persisting exrtachromosomal parasites can provide biofilm associated TA gene pairs.[73] It is interesting and has long been known that most phage have a significant density dependence regarding their ability to infect their host.[74] Thus biofilms with their very high cell densities, provide an ideal habitat for phage maintenance and transmission. But biofilms are known to limit the diffusion distance of resident phages and thus appear to act as 'active' phage reservoirs in that amplified viral particles can be held and protected from harsh environments by the biofilm.[75] Phage are also the most efficient agents able to paraitize biolifIms as some viruses can penetrate, depolymerize and invade biofilms.[76] Viruses can also be specifically constructed to efficiently degraded the exopolysachharide and lyse most of the cells in the biofilm colony.[77] No other know agents appear to have this power to affect biofilms. A biofilm community can thus present opportunity for virus inclusion or virus preclusion. Thus we can think of biofilm as a highly favored viral/host habitat. An ideal circumstance to define a population based viral mediated identity. Accordingly, it seems likely that biofilms are communities that share common group identity via their viruses, other genetic parasites and addiction modules.

There is more to the idea that viruses and transmissible plasmids can be responsible for prokaryotic based group identity then can be covered in this chapter. For example, the well known ability of phage and plasmids to exclude each other has long been a basis of the classification for these agents, as well as the importance of phage typing or lysogenic trnsformation for bacterial strain classification. This issue in the context of group identity is presented in greater detail in my book and will not be further developed at this chapter.[2] However, here too, strong arguments can also be made that a viral or infectious agents likely underlie the origin of these systems.

As outlined above, clear examples of group identity and group survival in bacteria has been established. Although such communities are of major clinical importance, we have only recently come to appreciate the general significance of bacterial groups. In addition, that viruses occupy such a central position in all habitats on Earth and provide such a basic role in the evolution of prokaryotes is also a realization that has only recently become clear. These realizations both come well after the development of most of the theory behind evolutionary biology. Thus historically, the evolutionary mechanisms that promoted the origination of group identity states did not invoke any HGT mediated mechanisms. Past theories were and remain exclusive of the possible role of viral populations in host group based adaptation. In no review that I am aware of on possible mechanisms behind group biology, has the perspective of viral involvement been evaluated or even presented. The only concept that appears relevant in the extant literature is that of natural selection of parental variation acting through survival of the fittest type (individual). Infectious (lateral), origin and spread of new information (and group identity) not originating from parental types is not considered. Colonization and preclusion of information, although now undeniable, was also considered only from an individual survival based perspective. This limited view is clearly wrong and inconsistent with current comparative genomics. Similarly, studies on bacteria that are known to undergo forms of cellular differentiation (e.g., dictyostelium) are also not evaluated from the perspective of possible viral involvement.[78,79] However, dictyostelium are also known to have many specific and distinct relationships with various genetic parasites.[80-82] Strikingly, this includs the earliest emergence of significant numbers of retroposon based genetic parasites.[83,84] Interestingly, in their stress response, we also see the example of G-coupled proteins receptors being used for signal transduction.[85] Historically, the question of a possible viral role was simply not considered relevant to the issues of social biology. Yet in our postgenomic era, it has become clear that the dynamics of prokaryotic genomes are mostly viral mediated. And as presented below, the dynamics of eukaryotic genomes are also mostly viral mediated (albeit by distinct type of viruses).

Prior to the emergence of eukaryotes, followed by meazoans, there was a great global transformation that created the O_2 atmosphere which became able to sustain metazoan organisms using O_2 based oxidative metabolism, was a major development within the prokaryotes. However, this massive release of photosynthetic O_2 was the product of community cyanobacteria living in stromatolites. We can expect that viruses were also crucial for this community development. This global transformation of Earth's atmosphere from a reducing to oxidizing state was though to have occurred about 2.4 to 2.7 billion years ago[86] and correlated with massive stramatolites found in the geological record, the product of large surface communities of cyanobacteria. And extant stramatolites that host cyanobacteria as well as other organisms are still to be found in some habitats, such as Sharks Bay Australia.[87] These modern mats are composed of mixed species of bacteria, but the filamentous form of cyanobacteria that make large quantities of self absorbent

extracellular matrix are though to provide that matrix from which the laminar community is built.[88] The similarity of this process to biofilm formation presented above is clearly obvious. What is not understood, however, is how these films and communities establish systems of group identity and what role viruses have in these cyanobacteria. Given the above discussion, we might that such group living involves the TA addiction modules. Indeed, in modern stomatolites, phage are present in highly numerous and diverse types.[89] However, their role in community structures remains unknown. In contrast, phage-host relationships of the free living cyanobacteria are much better understood, since their genomes are known and how they adapt has been examined. Cyanobacteria are susceptible to large population crashes that can be viral mediated. The free living cyanobacteria are also well known for making a inordinate diversity of toxins, an observation that seems relevant to group biology. And it is known that some cyanobacteria use cyanotoxin like molecules to establish host colonization.[90] As also noted previously, it has been established by genomic sequencing that habitat specific variation in cyanobacterial genomes is mostly mediated by virus colonization (phage islands) and involves viruses that can provide viral versions of core photosynthetic enzymes, as well as much light dependent gene regulation.[91] Thus, we would fully expect that viruses and TA modules would also have been involved in the origin of stomatolite communities and their systems of group identity. But we await direct evidence for this view. However, with the photysynthetic oceanic bacteria, we also see the emergence of major new sensory systems, that of light detection.[92] Coincidentally, light can also affect cyanophage production.[93] As alluded to above, sensory systems are of special relevance to group identity in the eukaryotes which have evolved to depend heavily on such processes. But regarding the photosynthetic oceanic bacteria, with the related Vibrio fisheri we can see the presence of the LuxS system with its ability to both produce and detect light and to have this detection affect their group behavior.[94] Such as a systems of quorum sensing and/or directional swimming, is most interesting to us from the perspective of group behavior.

THE NATURE OF EUKARYOTES

If we consider the emergence of eukaryotes from the perspective of viral involvement in creating identity systems, we can see that the eukaryotes generally represent a major transition in the host-virus relationship and in the dynamics of their genomes with respect to the nature of a viral involvement. Indeed we might call this the second big bang in virology (the first being the origin of DNA[95]) that signals a fundamental transition in virus-host relationships.[96,2] No longer would most host genomes evolve predominantly via the multi-gene acquisition of colonizing dsDNA proviruses and plasmids, as had been conserved in all prokaryotes. Instead, what emerged was the dominant role for retroviruses and retroposons in the evolution of multicellular host genomes, elements which were to numerically dominate the eukaryotes. We also see a major shift in the types of viruses that can infect or persist in their host, especially RNA viruses, including the emergence of negative strand RNA viruses, which were absent from the prokaryotes. Thus the ubiquitous integrating dsDNA viruses were mostly lost from Eukaryotes. This transition also correlates with a general shift away from large scale gene acquisition seen in Prokaryotes to the emergence of more complex and network-like regulatory control seen in Eukaryotes. In this light, we should recall the discussion above in which the problem of how to edit code was presented. In order to edit a complex regulatory

code, agents with the competence to read the code and assign meaning would be needed. Since this will require a pragmatic circumstance to assign meaning, the agents will need to function as a population or consortia. From this perspective we start to see a distinct and essential role for the retroviruses and retroposons as code editors as they are genetic agents that fundamentally operate and adapt via quasispecies based evolution, in which fitness can be determined to a large degree by cooperative or consortial interactions. The involvement of retroviruses as agents of dynamic change in eukaryotes, however, also implies that virus driven evolution acquired a new enhanced capacity with respect to expanded direction and consortial nature of information flow. The population of viral agents that successfully colonized and persisted in host genomes were now also able to re-emerge (via reverse transcriptase) as elements of infectious populations and evolve very rapidly by quasispecies (QS) based adaptations then, possibly, recolonize as a QS population the genomes of other host. Thus both highly dynamic and highly stable information resulted. We now call this process endogenization. This is also a form of lateral information transfer, but one that is much less gene centric and much more associated with the superimposition of new sets of QS based regulatory networks. Thus, this retrovirus based colinization is much more prone to create and manipulate regulatory networks then the 'gene-set' (islands) evolution previously seen in prokaryotes. These new networks stem from the very identity (regulatory) regions that are used by the new colonizers for group identification. It is from this perspective, they we might better understand why all the lineages of eukaryotes have their own peculiar version of endogenous retroviruses as well as their specific but various and numerous retrotransposons. These are not simply the defective residue of 'selfish DNA' that is of no consequence to host survival, as currently thought. Rather they were the successful and persisting competent editorial consortia that created the new regulatory networks that henceforth underlie the identity of that specific lineage of host organism. Thus, in contrast to the prokaryotic portion of the tree of life, the eukaryotic portion of the tree of life can be defined and traced by patterns resident ERVs and retroposon populations. All mammals, for example, fit this characterization, including mice, chimpanzees and humans. Thus, with the emergence of the eukaryote, a transition from DNA virus driven to retrovirus driven host genome evolution was established. This transition set the stage for more complex, network and regulatory based evolution. Thus a more complex form of identity control using the same or similar gene set became possible. This complex identity was to promote the evolution of multicellular organisms from a common DNA via the action of TA addiction systems that can induce programmed death (such as apoptosis, oxidative burst, etc.). Such self identity systems could also now be mediated via small toxic RNA molecules, originally derived from retroviral regulatory elements (LTRs). This complex regulation in linkage to the more capable sensory systems, allowed the differentiation and emergence of neurons, whose purpose was initially to learn group identity via early olfactory based systems that could detect small identity peptides (pheromones) and affect neuron based learning (sex behavior, as seen in C. elegans). Social (and sexual) behavioral learning was thus at the origin of neuron based learning. This scenario thus outlines the steps that were to set the stage for evolution group (social) identity in vertebrates and ultimately in humans. With humans, group identity became a mostly learned state via the emergence of language and was no longer very dependent on biochemical cues (such as olfaction). Much more detail regarding this scenario can be found in my book.[2]

However, before the above scenario might get going in eukaryotic evolution, we needed to invent the first eukaryotes. That is, we need cells with a nucleus which can

support larger genomes and allow more complex and epigenetic regulation that will be essential for the identity of multicellular organisms. The origin of the nucleus is enigmatic and has long occupied the interest of evolutionary biologist. Various scenarios involving symbiotic joining of different types of prokaryote cells and loss of a cell wall has been proposed to account for the nucleus and the a cytoplasm.[97] Although still problematic, these scenarios are believed to provide the most likely explanation to the majority of current evolutionary biologist. More recently however, here too the enormous potential for viruses to manipulate host has been proposed to provide crucial solutions to complex problems. Indeed, one idea is that the nucleus itself is the product of successful and persistent colonization of a prokaryotic host by a large membrane bound dsDNA virus.[98-101,95,96] Thus, the very distinct multi chromosome, the chromatin bound DNA and highly coordinated multi origin DNA replication (cell cycle), the separation of transcription from translation and the post transcriptional modification, processing and transport of mRNA can all be proposed to have originated from viral (not prokaryotic) systems. According to the principles of group identity as outlined above, however, such successful virus would also have needed to superimpose a major new form of self identification onto its host to attain the necessary stability. Indeed, we might seek to understand the strong selective pressure imposed onto this new symbiotic virus-cell organism from the perspective of virus. Thus we might evaluate if this new eukaryote would be able to resist the highly abundant dsDNA viruses that would have been so prevalent in this ancestral prokaryotic world. It does indeed appear to be the case that this resulting eukaryotic was now highly able to oppose the colonization and chromosomal integration by large DNA viruses. Integrating DNA viruses are rare in the eukaryotes. Possibly, the nucleus itself, with its internal membrane, pores that restrict access and distinct chromatin based replication process, could provide the essential features to oppose DNA virus colonization so common to prokaryotes. For example, restriction/modification systems, a major prokaryotic systems to oppose virus infection and integration, were not maintained in the eukaryotes. Nor was the CRIPSRs repeat system mentioned above maintained in Eukaryotes. These two highly prevalent anti-sviral ystems (which themselves most likely originate from virus) are no longer found in the eukaryotic decedents. Along with this, prophage like entities (remnants) , seen in all prokaryotes, are also not found in genomes of eukaryotes. Most DNA viruses (like the herpesviruses) would henceforth persist as episomal, extrachromosomal elements and require noncovalent strategies to maintain persistence, much like the episomal P1 phage. However, the resulting new systems of eukaryotic identity would allow the colonization by populations of retroviruses and retroposons (as seen in dictyostelium), which would mediate the establishment of complex regulatory networks. Retroviruses and retroposons, with their inherent consortial QS based capacity to adapt, would superimpose new regulatory networks and group identity onto their host. This process would establish a positive feedback state, that would further promote ever more complex and network based identity which is predominately regulatory in nature.

THE EXEMPLAR OF ADAPTIVE IMMUNITY: COMPLEX SELF IDENTITY FROM COMPLEX VIRUS COLONIZATION

It is clear in contrast to prokaryotes that eukaryotes have evolved a distinct set of systems that can provide nonspecific self and group identity. For example, with the emergence of the mitochondria and with the corresponding emergence of mitochondrial

pore and anti-pore proteins, we also see the emergence of the apoptosis system that will destroy self cells by oxidative damage when the dynamics of these two gene functions are perturbed (by virus infection, stress or differentiation, for example, see ref. 2). We also see other systems (called innate immunity) that also typically have toxic and anti-toxic elements working together to destroy self when signaled to do this. The complement system clearly fits the characteristics of an addiction module as it is composed of a large pore forming attack complex along with binding proteins that prevent the toxic activity of this pore. It is also clear that individual cells, such as macrophages, can recognize nonself by various means. For example, when a macrophage is observed under a microscope to chase and engulf individual motile bacteria it must recognize nonself. These mechanisms are likely to involve small molecule detection which directs cellular behavior (motility), similar to that of quorum sensing in many bacteria or olfaction invertebrate eukaryotes. These same innate systems are also often used to destroy virus infected self cells (such as natural killer cells), thus they must be able to recognize a change in status from self to nonself, as could result from interferon induction, for example. However, the most remarkable and complex of all these identity systems is that of adaptive immunity as found in jawed vertebrates. This system has the capacity for 'anticipatory immunity' in that it has evolved the mechanisms for the generation of diverse and novel recognition capacity in 'anticipation' of responding to specific pathogens that it has not yet encountered. It also retains a memory of this response which is the basis of immune memory. Yet, the origin of this system has also been enigmatic. Early vertebrates, such as tunicates, lack essentially all of the components of the adaptive immune systems. Yet they do have rather complex systems of self recognition (used for colony formation) that also depend on the generation of diversity.[102] And it is clear that both these early vertebrates as well as the enormous numbers of oceanic invertebrate animal species all have lived successfully in a highly virus infested habitat of the ocean. Clearly all these species that lack an adaptive immune system have done well. Yet their immune systems lack any evolutionary homology to those of the adaptive immune system. Nor do invertebrates all live short lives as some have proposed since some species can live over 200 years. So then, what was the selective pressure to originate the complex adaptive immune system? The ability invertebrates to survive in the presence of infectious agents does not suggest that they are less successful with respect to virus. How and why did this complex system of self identity emerge? Adaptive immunity first evolved in bony fish in the ocean, a habitat that is intensely virus infested. As we know that eukaryotic genomes are most susceptible to colonization by ERVs and retroposons, we can examine the possible origin of adaptive immunity from the perspective of these ERVs as an acquisition of complex group identity mediated (see ref. 103). Although adaptive immunity currently involves diverse genetic elements and cell types, it is understood to be broadly composed of CTL mediated cellular immunity and B-cell mediated humoral immunity. Since the latter differentiates from components of the cellular immunity, it has long been thought that the T-cell receptor and the induction of CTL immunity must be the basal function that evolved first and lead to the humoral arm of immunity. Thus it is striking to realize that the bony fish did indeed undergo a major transition in gypsy-like chromovirus ERV colonization relative to the tunicates and other invertebrate sea animals. A large and diverse set of such ERVs is found in the genomes of all bony fish, including those that have otherwise compact genomes (such as the puffer fish). There was also a significant expansion of other retroposon elements (LINE) in jawed vertebrates.[104,105] Along with this ERV colonization, the genomes of jawed vertebrates became significantly larger then those of the ancestral tunicate, which

at about 65-75 MBP and only about 15,000 genes are the smallest of all animal genomes. Tunicated did have some ERVs in their genomes, but only from 6 clades, compared to the much greater diversity (over 30 ERV families) in jawed vertebrates. The result of this colonization in the jawed fish was a genome that had about 20 fold decreased gene density, but a much enhanced capacity for coordinated regulation. Thus it is clear that a great ERV colonization did indeed occur during the transition to the jawed vertebrates.

The core functions we seek to explain is the origin of the T-cell receptor (TCR) which is subjected to genetic rearrangement and surface expression in the context of MHC presentation and a peptide portion of the antigen. This results in a genetically modified white blood cell that must then undergo a form of selection that eliminates those cells that are self reactive via a process of apoptosis (through immunological education). The resulting white blood cells is one that can recognize and kill virus infected cells, that is a self cells that now differs only in the specific pattern of virus gene expression. Basically, a white cell that kills virus infected cells can be considered as the original core function of adaptive immunity. We can now consider the origin of this antiviral state from the perspective of retrovirus: an ERV originated addiction module mediated system of identity that resulted from a complex (QS based) event of stable virus colonization. Since the T-cell receptor (TCR) can be considered as a basal element, we must consider the origin of this gene and how it might have become linked to the RAG1/2 mediated rearrangement system. Phylogenetically, TCR is a member of a large family of Ig-like proteins that is a membrane spanning, surface protein. It is composed of two chains from distinct loci; heavy and light. The light chain has multiple V, J and C domains that undergo gene (site) specific and RAG mediated recombination to generate a VJC light chain. The heavy chain is similar but has an additional D domain between the V and J domain resulting in a VDJC heavy chain. The most basal (simplified) version of this gene appears to be the heavy chain and phylogenetic analysis indicates that the JAM/CTX/PVR (aka CTX-like) family of proteins are the most basal versions of these genes. Ciona does have a homologue of this protein, but it does not appear to have a role in immunity. Also, the VLR system of lampreys does not resemble the T-cell receptor nor does it use the RAG1/2 mechanism to generate VLR diversity. However, what is clear is that this CTX family of proteins are viral receptors of various types (e.g., poliovirus, reovirus). As previously presented, the origin of viral receptors can often be traced to the viruses themselves.[2] Clearly, in bacteria, phage conversion in which viral encoded surface receptors are expressed and function as virus receptors is well established. And it is also known that these bacterial viral receptors can be linked with coordinated systems that generate genetic diversity of the viral receptors. These two observations allow us to lay out a scenario for how the TCR and its associated RAG based recombination system came to colonize the genomes of jawed vertebrates together via the action of viruses. A particularly enlightening example from bacterial viruses regarding this idea is found in the T7 related phage of Bordetella.[106-109] This phage encodes a reverse transcriptase that introduces variation in the amino acid sequence within a specific domain of the viral surface receptor. This receptor (Mtd) has a C-type lectin fold, which like the Ig fold, is the only other protein domain known to be able to tolerate tremendous sequence diversity while still retaining its general structure.[110] Lysogenic Bordetella harboring this virus were significantly more able to resist infection by other viruses.[111] Thus, we can propose that a similar logic applied to the origin of the TCR, which along with the RAG1/2 gene specific rearrangement system, were originally found in some ancestral virus population and that together these systems colonized the genome of bony fish by some ERV mediated

process, resulting in motile blood cells that were able to kill subsequent competing virus. Furthermore, both retroviruses and DNA viruses of acquatic animals (and avians) are well known for their ability to induce clonal expansion of virus infected hematopoetic stem cells. Thus these agents also bring with them the ability to reprogram host cells to both proliferate and express viral receptors. Such virus infected blood cells are also know to be susceptible to large scale induction of apoptosis.

From the above perspective, we can now evaluate the Rag1/2 integrase to see if a viral origin or addiction strategies appears likely. These integrases are members of the Mu family of integrases that operate via cut and paste mechanisms.[112] With Mu, however, the integrase is essential for virus replication and consequently we find the largest diversity of these types of integrases in related viruses, not host. Since the cut and paste process of integration is not like that seen in most retroviruses, however, some have suggested this argues against possible retrovirual origin of Rag1/2. Yet RAG 1 and 2 both conserve the DDE metal binding motif and have striking structural similarity to the RNAse H fold of retroviral integrase. Given how common virus mixtures (consortia) are involved in host evolution, these observations are still entirely consistent with the likely viral origin of RAG1/2. Also consistent with this view is that RAG 1 or 2 alone is toxic since it will cleave chromosomal DNA. Normally they are found associated to one another to hold them in inactive states. Only during T-cell differentiation is RAG active. Thus they clearly have features of a TA gene set. Finally, the gene specific nature of RAG1/2 mediated integration has always posed a problem to explain by traditional (Darwinian) evolution. The integration site involves an RSS repeat with clear similarity to LTR mediated integration. But we know of numerous aquatic viruses (such as the phycodnaviruses), that conserve a large array of homing endonucleases that are often gene specific (such as for the viral DNA polymerase[113,114]). These are thought to function to preclude viruses with similar DNA polymerase genes. Other DNA viruses of invertebrates are also known to encode Tc1-like transposase, (granulovirus cydia pomonella; TCP3.2) which closely resembles the V(D)J recombination system and also result in the preclusion of similar viruses.[115-117] Such observations are entirely consistent with virus mediated addiction as the basis of the adaptive immune system whose initial objective was to preclude infection by other viruses.

There is another entirely distinct line of evidence that also supports the idea that viruses (ERVs) are especially involved in the evolution of the adaptive immune system.[118-121] The MHC locus contains the TCR as well as many other rearrangeable Ig superfamily genes involved in antigen presentation. These are the Class I and II genes. These genes are also mostly absent from tunicate genomes. This loci is very gene dense. But it is especially interesting that the MHC locus is also the one region of the chromosome that is most dense with respect to ERV and LTR composition (outside of the Y chromosome). Such an observation suggest that viral mediated coordination of regulation is most responsible in driving the evolution of this region. Based on phylogenetic analysis, the MHC I locus appears to be ancertral to the MHC II locus. The MHC locus appears to have evolved by a process of gene dupliocation and divergence, a process that involved identifiable blocks of genes that have been called a duplicon. Thus it is revealing that the most basic duplicon unit of MHC evolution also contains one ERV and an ERV element that appeared essential to mobilize and duplicate this region. Nor has this ERV mediated process of MHC evolution abated during primate evolution. Indeed the significant differences between human and chimpanzee MHC locus are also observed to be the result of ERV action. Thus the role of ERVs in the dynamic adaptations of the immune system have continued into more recent evolutionary periods.

The above outline of the adaptive immune system thus justifies several general ideas. One, it is best thought of as a highly elaborate system of identity that originated the capacity of white blood cells to kill virus infected 'self' cells. It did not originate because of a distinct advantage regarding pathogens or selective sweeps by pahtogens. Two, it was the product of a complex colonization by viruses (especially ERVs) and other genetic parasites that superimposed complex and coordinated addiction modules that created the new system of identity. Ironically, this very system of cells also made the host susceptible to various viruses (retroviruses and DNA viruses) that specifically infect the immune system.

If we can indeed accept the idea that the immune system is really a complex identity system, then we can also start to understand some additional but otherwise curious associations. For one, it has been known for some time that the MHC locus also contains many genes that are olfactory receptors (OR genes).[122-125] Their presence and conservation within the MHC locus has always been difficult to rationalize. However, if they are constituents of a gene set associated with identity, then their presence in this locus makes sense. Along these lines, it is of particular interest that the specialized receptors genes for the vomeronasal organ (VNO) are also concentrated in the MHC locus. These genes are responsible for the detection of peptide pheromones that directly affect social interactions, including offspring recognition by mothers, mate identity and social identity. In Voles, for example, the VNO is essential for mate bonding. Indeed, in mice, there is also the known phenomena of the 'Bruce effect' that is mediated by both MHC peptide composition and olfactory detection.[124] In this case, a pregnant mouse dam will reject (abort) her fetus if she becomes associated with a second male that was not the sire of the fetal pup. This is mediated by olfaction of different MHC peptides of the second male by the dam. Here we can clearly see the interface of olfaction, social bonding, MHC immunity and fetal immunity all in the context of the locus of the adaptive immune system. There is reason to think that this association of olfaction to MHC composition is ancient and seen in early jawed vertebrates since it is clearly present from goldfish to all terrestrial tetrapods. Thus it is striking to realize that in the African primates, but especially in the great apes, this system underwent a significant genetic alteration and degradation.[28] Indeed, it appears that during the great HERV colonization of the African primate genomes (described below), there was also a great interruption of essentially all of the VNO receptors and many OR receptors in the African, but not New world primates. This event was especially true for the human MHC locus in which all the 7 VNO receptor families became psudogenes via the action of retroposons. In addition, of the 34 OR genes in the human MHC I locus, 60% became psudogenes, whereas chimpanzees retained more of the OR open reading frames. This contrast with the mouse MHC I genome which has retained and even coduplicated the corresponding OR genes. Thus, African primates do not appear to have retained olfaction based social identification to any great degree. There is no 'Bruce' effect in the primates. Nor is there the scent marking by urine so common to other mammals. Instead, the African primates have become much more dependent on visual communication and also on audio communication for social purposes. Thus, from a group identity perspective, we can note that the great HERV primate colonization was associated with a major transition in this ancient olfactory based systems used for group identity which were degraded via the action of retroviruses and retroposons, setting the stage for the emergence of a larger social brain that would depend more initially on vision then language (learning) for group identification. From this perspective, we can assert that group identity based selection, was crucial for the evolution of human social attributes.

HUMAN SPECIFIC EVOLUTION: THE GREAT HERV COLONIZATION

This brings us to the doorstep of human evolution. And regardless of the likely negative visceral reactions by most evolutionary biologist that surely viruses are not relevant to human evolution or even members of the Tree of Life,[126] we have laid out a clear path and rationale that retains this fundamental concept of viral mediated persistence via addiction that sets new systems of group identity. Viruses are central to the Tree of Life.[127-129,15,6] Humans are no exception to this general process by which viruses affects all life.[130] Viruses inform us that groups, not individuals (quasispecies or mosaic networks) can provide distinct selective advantages and can vastly accelerate adaptation. They provide edited, functional and coordinated text elements, not simply point errors, that can work together in networks. They also inform us of some of the strategies by which new information will persist, via addiction modules. Such modules inherently promote group identity. The result is an unending tendency to add coordinated code sets to their host which furthermore explains the general tendency for more complex organisms to have more complex identity systems along with more complex viral remnants in the DNA. Indeed, such complexity will not likely be attained without virus; they are essential participants.[6] What we can now clearly see is the power of the diverse and many, made into one group by their common addiction states. This is population based survival which is not dependent on any cost-benefit feature as derived from an individual or its direct kin. Individual cheaters, if they evolve, will simply lose their protective addiction module and become negatively selected. From these principles, the emergence of the highly social human brain does not seem so impossible. Human evolution is also the product of virus population based colonization and (social) addiction. There have long been doubts by those that study human and primate social interactions[131,132] regarding the applicability of selfish gene based concepts.[133,134] Since the great ape social structures are much more associative then competitive, it seemed much more likely that group associated positive aspects must underlie such structures. Primates simply like each other and like being together. They seem inherently social and not compelled by competition and spite.[135] However, these doubts continue to be dismissed by evolutionary biologist since there appeared to be no underlying accepted theory (Darwinian based) that would directly promote social based or group based selection. Humans are even more social, so the discomfort of some social biologist regarding human evolution was even more troubling. However, according to the reasoning above, we must first evaluate the underlying viral populations based changes associated with human evolution, before we might be able to understand resulting human group identity and addiction mechanisms. And when we do this, we are not disappointed for we see evidence of massive human genomic editing mediated by viruses. Like the evolution of eukaryotes, the evolution of the bony fish, the evolution of primates and the evolution of the great African apes and humans, in all these genomes we see clear evidence of a transition mediated by major colonization event involving various types of retroviruses and retroposons. The African primates in particular underwent a great HERV-K colonization (which also incapacitated the importance of olfaction for group identity). Curiously, HERV-K represents a virus family that exists as an infectious endogenous viruses in numerous rodents, but not primates. This endogenization process has continued into human evolution, whose HERV-K, LTRs and Y chromosomal makeup is distinct from that of chimpanzees. This is the biggest genetic difference between us and our closest relative.

VIRUS DRIVEN HUMAN EVOLUTION

There are numerous issues that relate to the idea that viruses were involved in driving human evolution and I recommend that the reader refer to my recent review on this topic for those references.[2] The above presentation has been much more focused on genomic viruses (ERVs) and retroposon. However, it is likely that extra-chromosomal persisting viruses were also very important for human evolution. But a commonality for all persisting viruses is the need to establish a mechanism the compels stable persistence. Thus all these human-viral relationships should involve some form of an addiction module. And in this, the original phage P1 examplar still informs us regarding the link between the essential elements of addiction and group identity. Primates have many specific relationships with the viruses that infect them, especially those that establish persistent infections. These include primate foamy viruses (PFV), SIV, large DNA viruses (herpes family), adenoviruses, papillomaviruses and others. Indeed, most African monkey species are known to harbor their own peculiar versions of PFV and SIV, often together. With persistent viruses, however, sexual and group behaviors are often crucial for their maintenance. For example, SIV appears to generally be transmitted from mother to young in nursing monkeys. Also, human HIV-1 infection is clearly dependent on risky sexual and other group behaviors. Thus there is a direct link between social behavior and virus maintenance and transmission. With this realization, we start to see how more elaborate and learned social behaviors would also be highly affected by viruses. For example, the human behavioral response to the HIV pandemic is quite distinct from the essentially absent behavioral response of koala's to the leukemia virus currently infecting them. Clearly learned group behaviors matter for virus. Group behavior in the chimpanzees are distinct from monkey species in that the males (after breaking maternal bonds) learn group hunting of monkeys from older males. But the consumption and sharing of monkeys as food by such hunting bands would clearly and efficiently expose these groups to those persisting viruses harbored by their monkey prey. Thus this learned group hunting by chimpanzees would clearly alter the viral ecology, hence evolution of chimps. Yet, in fact, most wild chimp populations are not SIV infected. And it does indeed appear to be the case that the chimpanzee MHC locus shows evidence of recent selective sweeps, probably mediated by retroviruses (given their altered APOBEC3C makeup). And it is also known that gorilla and chimp social structures makes them susceptible to ebola virus as well as to various human viral infections.[136] But the learning of adolescent chimpanzee males to group hunt is also used directly as a form a group identity. Groups of chimpanzee from the same troupe will hunt and attack chimpanzees from other groups (if the numbers are sufficiently one sided). Thus learned attack behavior is clearly the harmful element of group identity. Conversely, shared support (common defense, communal feeding etc), is clearly a beneficial feature of group identity. But in the case of Chimpanzees, these group identities are mostly learned during social development of the young (not by HMC peptide imprinting as in mice). Thus, in the great apes, group identity is established by social learning.

ADDICTION REVISITED: SOCIAL BONDS (LOVE) AND COGNITION

What does it mean to establish a social bond? This question is of special relevance to human social evolution since it seems clear that humans have much more extended

social systems then the other great apes.[137] In addition, our large social brain must be more adapted to such states. We would like to understand how selective pressures, possibly involving virus might affect this process and how language might be involved. The most fundamental social bond seems to be that of a mother and its offspring. In placental mammals, the need (obsession) to nurse the young is mediated by maternal bonds that initiate from the placenta producing various pheromones that emotionally affect the mother and the fetus, but continues after birth via lactation and milk.[138] Clearly, molecules (peptide pheromones) such as oxytocin, vasopressin and others along with their cognate receptors are crucial for this process.[139] Since all mammals must preserve this fundamental bond system, these bonding systems are not subjected to the type of evolutionary variation that could readily indicate the underlying genetic mechanisms involved. However, mate bonding is much more variable (uncommon) within the mammals and it is known that two otherwise very similar species can differ considerably in their mate bonding systems. This has been most evaluated in the voles in which similar species can be either monogamous or polygamous. Here it was reported that the role of vasopressin and its receptor that is expressed in the central nervous system are crucial for the bonding that occurs.[140-142] Indeed, the regions expressing elevated levels of this receptor corresponded to brain regions that are also known to be involved in drug addiction.[143] That the receptor was responsible was definitively shown by the use of a virus, in this case a recombinant DNA virus, that would over-express the receptor when injected into the specific brain region.[144,145] Indeed, this virus was able to also induce a degree of mate bonding into other rodent species (mice) that do not normally mate bond. The presence of a bonded mate is clearly comforting (and can be measured by reduced stress hormones). Interestingly, in bonded voles, the incorrect MHC olfaction of a strange mate will quickly induce negative response (angry attacks).[146] Olfaction is clearly involved in these pair bonds.[146-148] Evaluation of the genetic difference between these two vole species established that variations in regulatory DNA associated with the respective receptors was the notable difference.[149] The usually simple repeat elements involved are called satellite DNA and are often thought to vary in length due to errors (strand slippage) during DNA replication, even in humans.[150] However, the length (and sequence) variation is often specific to particular genetic loci (such as seen between human, chimpanzee and bonobo VP promoter region[151]) which indicates that site specific variation must somehow be occurring.[149,152] Because of this, I have asserted that this variation is more likely due to the action of retroposons resulting from unequal insertion and excision as originally proposed by Smith.[153,2] The direct implication, is not only can a constructed virus directly manipulate social bonding, but it appears that viral action may have generated the underlying natural genetic regulatory variation involved in different social bonding states.

Given this information, let us now consider the situation with respect to human evolution and social bonding. In contrast to the chimpanzees and in addition to the maternal infant bond, humans also from strong extended social bonds. The two most apparent of these are paternal infant bond and mate bonding. But there are clearly more extended bonds as those that include family, tribal, cultural and religious bonds. All of these are forms of extended (not necessarily kin based) group identity. I have also suggested that they all have similar addiction elements as well. The presence of the bonds brings social pleasure and contentment. However, when broken, they rapidly induce a reproducible series of pathological emotional and physiological reactions known collectively as grief. Whether it be the death of an offspring, mate or tribal,

political or religious leader, similar responses will occur. These clearly have the features of an addiction module. Indeed, one of these social bonds has been studied in greater detail by using various brain imaging methods that can measure brain activity within various regions; that is romantic love. The pleasurable feelings of romantic love can be induced visually by showing the subjects pictures of their loved ones. Thus it is very interesting that the brain regions thus stimulated were similar to those of the vole study and also associated with the addiction centers of the brain. Indeed, this led some researchers to conclude that romantic love is a from of addiction.[154,155] But these extended social bonds in humans also demonstrate a distinction between humans and the great apes. Clearly, chimpanzee mothers will experience a grief response when their offspring die. This usually requires the mother to experience the presence of her dead infant. However, in humans, these extended grief reactions can be induced with language. Being informed verbally of the death of a loved one will just as effectively induce a grief response. This informs us that humans have adapted language to be a potent media of social bonding. What types of genetic events might have allowed such extended social bonding to evolve so that emotional attachment centers and cognitive language centers are linked? We can assume that the underlying bonding system was that of the mother-infant. Somehow, this system which historically depended on placental and lactation pheromones to establish the bond, must have become activated by less biological (e.g., placental) but more cognitive processes. However, it would still likely involve the underlying addiction systems of the central nervous system would need to link to the needed basal emotional systems to generally (instinctively) affect behavior. What must have changed for this linkage to occur was the sensory and neuronal media by which the CNS addiction systems become engaged. Clearly vision can be involved (hence the ability of pictures to induce brain activity during love states). But if extended social bond maintenance became able to use language as a media, as well, then this requires the prefrontal cortex become part of the transmission or establishment system of group identity. This can only mean that learned states (belief states) must be also involved in bond maintenance. In this way, when language informs us that a love bond has been broken, we disrupt a belief state that maintains the social bond and induce the pathological and emotional grief response. Thus we can assert that language, belief and social bonding all must have become linked for extended human group identity.

The basic role of the placenta in maternal bonding and the assertion that this likely underlies other more extended social bonding systems is of particular interest from the perspective of ERVs. For the placenta has long been know to be the one tissue most active for the expression of old and recent ERVs (expressed mostly as RNA[156-158]). It is also curious how variable all the mammals are with respect to their specific placental (reproductive) biology which can require ERV expression,[159] including distinctions between human and chimpanzee placentas. No other organ shows such variation. In addition it is also interesting that human placenta has an expanded set of pheromone genes that it can express. However, none of these observations has been linked to human social bonding. Such an evaluation now seems warranted. What we should seek to understand how the selective pressures associated with group identity led to an expanded neocortex which essentially doubled the size of the human brain,[160] but greatly expanded human social and group identity.

A LARGE SOCIAL BRAIN AS A PRODUCT OF GROUP IDENTITY

The scenario I have outlined above suggests that the African primates underwent a major viral mediate genomic colonization which led to the incapacitation of their olfaction based self and group identity systems (e.g., VNO social pheromone or MHC peptide detection). The selective pressure for this would be related to that associated with how viruses have always affected group identity. Primates shifted the prevalent mode of their group identity to become much more visual, involving the brain visual systems more so then most other mammals (hence visual cortex, color vision, eyes forward, reduced snout from ancestral primates). This must have also been the basis for the origin of mirror neurons which we now see visually connecting the mind of the African primates into groups. In the evolution of the Great apes, this trend continued such that the brain based learning became the predominant mode by which group identity was established. In humans, however, there was, in addition, another wave of HERV colonization which further incapacitated the remaining olfactory based identity as well as incapacitating some brain based systems of group identity that had been more 'hard wired". By this I mean that the various behavioral instincts that most mammals express immediately after birth were also mostly lost from humans. What resulted was a species that was much more dependent on learning from others via input sensory during early brain development, then was previously found in the other primates. Thus the hominid species was in many ways very incapacitated and depended more heavily on extended social bonds for the long-term maintenance and development of social capacity. These extended social bonds were mechanistically derived from the fundamental maternal bond, but also became based on social learning and brain development (via the neocortex). The mechanisms involved were related to those used by prolactin, oxytocin and vassopresin in that addiction regions of the CNS were linked to visual and language based sensory learning. From this, a positive feedback loop was established for the evolution of ever more brain based social learning that would result in an ever expanding group identity. But with this expanded learning capacity, there also occurred expanded capacity for humans to learn from their environment and socially communicate this to other group members, via a general intelligence which further developed a positive feedback loop for brain based social learning. It was under such social based selective pressure that we see the emergence of a large social brain whose underlying selective pressure was to be able to learn and use language as a major mode of group identity. With the emergence of language, however, a different type of nongenetic information system comes into existence. One that is no longer strictly tied to the genetic code for its continued (viral mediated) evolution. Thus, possibly for the first time in the evolution of life on Earth, genetic viruses are no longer essential for the creation of new information and group identity. New group identity can now occur as a product of social learning. Thus human attained a sort of liberation from their biological origins regarding information they have acquired as well as how they establish their identity. All this was made possible by a large social brain that became dependent on learning language to establish social identity, but was initially made possible by the incapacitation following viral colonization. Thus, language (and other learned social/cultural and written information) became the equivalent of virus for the origins of new identity. And like virus, was dependent on

consortia (populations, not individuals) to assign meaning. Language retained some virus-like features. Language can be transmitted to and colonize groups of brains from older group members and imprint these brains with a social identity during an open developmental window. This colonization, like a virus, will also modify their development of their host brains such that these brain-host would become resistant to learning other identities (second language). In this we can see why early brain growth is biologically linked to language acquisition.

In the scenario I have outlined, HERVs (and likely other viruses) did the dirty work, they were the systemic editors of the underling human genome. They took out numerous regulatory systems and other functions. And in what seems like a distributed scramble of defective viral elements along with their 'hyperparasites (Alu's. LINES), created new networks of control whose main selective pressure was to generate new brain based (social/learned) systems of group identity. In this they succeeded. The resulting large brain was accomplished by viral promoted transformed basal layer of the neocortex. Although this was initially made less functional, the resulting layer was more proliferative, invasive and controlling of other brain structures that it could now use to control group learning. This was mainly accomplished via a viral mediated regulatory regime, not by the acquisition of many new genes. Thus we can possibly explain why HERV-K LTRs are often used as promoters. The capacity for neuron based control was also viral mediated by enhanced systems of neuron apoptotic development, a product of learning. Thus here too we can see examples of ERVs active in many brains transcription programs that control apoptotic genes and also in genes crucial for human brain development. Thus it seems possible that viral agents could have promoted the evolution of the enlarged and controlling prefrontal cortex, especially if this was essential for language meaning to be developed.

But how might a virus affect host behavior? For it is really in the resulting social behavior (and social bonds) that came to differ so much between humans and their ancestors. Viruses are known to have rather specific effects on brains and behavior, including affecting various memory systems. They are also known to affect emotional behaviors, such as being able to induce rage or obsessive behavior. Their transmission can also be closely associated with sexual and risky behavior and even be strongly affected by political behavior (such as in South Africa and HIV). However, it is not at all clear how such general viral effects might have affected the evolution of human specific behaviors. And it is certainly not clear how learning of language might relate directly to virus selective pressures. Yet even with this issue we see some evidence of a relationship. Consider for example the languages currently spoken in the New World. Although hundreds of native languages were originally spoken by these populations, none of them have been maintained by large modern populations. Only European languages (English, Spanish, Portuguese) have prevailed following the European colonization. This outcome was strongly affected by the population based viruses (e.g., smallpox, measles) and the population crash and genetic sweep that followed the introduction of these European adapted viruses to the Americas.[161] Thus the consequences of the viruses brought over by the European colonizers were population specific and in this they did help select for a shift in population based language and identity.

CONCLUSION

Although the study of viruses and disease has long been pursued, the deeper significance of virus effects on host evolution has not been historically recognized. Most theories of evolutionary biology have not incorporated the transmissive and horizontal capacity of viruses to colonize host populations and their genomes or how this affects host-virus relationships. The concept that viruses might also affect host identity systems has not previously been explored.

In the last 20 years, several lines of investigation have provided new perspectives on the nature and general importance of virus for all life. Comparative genomic sequencing and analysis has informed us that virus derived information represents the most dynamic information found in all domains of cellular life. This information has clear consequence to virus-host relationships and affects host population survival and identity. Metagenomic (shotgun) sequencing of various habitats has informed us that viruses are the most abundant and diverse genetic entities in these habitats. Comparative viral genomics has further informed us that the majority of genes found in various viral clades are virus derived, not derived from host.

It has also been experimentally observed that virus populations can often have mixed and cooperative behaviors and fitness. Thus, modern quasispecies theory indicates that a cooperative populations of virus (not simply the fittest type) can affect fitness. The mosaic nature of DNA virus evolution, especially in prokaryotes, also indicates the importance of virus mixtures.

The persistence of viral derived information in its host has been explained here by the use of viral derived 'addiction' modules (linked toxin/antitoxin). Although these modules promote persistence, they also promote the establishment of new host population identities and affect the competition and survival of host populations. The existence of bacterial populations that employ these mechanisms is now being explored. The concept of an addiction module, however, can also be broadly generalized to explain the nature and origin of most systems of host identity, including the adaptive immune system of vertebrates. Such identity systems can also promote group identity.

These observations along with other analysis have led to various theories that viruses are providing new and complex information that fundamentally contributes to host evolution. However, there has emerged an entirely new line of theory based on essential requirements of any natural language and the premise that DNA is a natural genetic language that adheres to these same parameters. Thus, issues of syntax and context (pragmatics) apply to DNA as well as other languages. To understand the biological basis for the origin of DNA language and the ability to edit this language during evolution, the term 'biocommunication' has been introduced.[12] However, one conclusion of this theory is that an individual (fittest type) cannot create or edit language due to the required context dependence (pragmatics). Instead, this requires the participation of a "population of agents" that are competent to understand, use and edit DNA code.[12]

Since viral fitness can use population based processes and can affect otherwise identical host populations, viruses provide the population of agents that are competent to edit and create new code. And since stable virus colonization employs addiction modules, viruses also provide new population identity along with edited code. Using this concept, we can trace primate to human evolution in the context of endogenous retroviral (ERV) colonization. In so doing, we see much evidence for viral colonization directly associated with primate and human evolution. We also see the emergence of learned systems of

group identity that are linked to brain development, but still use CNS based addiction circuits to establish group (social) bonds. These primate social identities still adhere to the basic attributes of addiction modules, albeit resulting from learned, not new genetic information. It is from this perspective that we can propose that human language is a form of learned group identity which still adheres to the basic parameters of addiction modules.

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CHAPTER 13

THE EVOLUTION OF ADAPTIVE IMMUNITY

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Abstract: The concept of adaptive immunity suggests de novo generation in each individual of extremely large repertoires of diversified receptors and selective expansion of receptors that match the antigen/pathogen. Accordingly, adaptive immune system is also called "anticipatory". It allows each individual to have a unique repertoire of immune receptors corresponding to its life history. The memory of an antigen gets encoded in the clonal composition of the organism's immune cells instead of being encoded in the genome. Consequently, the immune response to repeated encounter with the same antigen becomes stronger, a phenomenon called immunological memory. Elements of adaptive immunity are found at all taxonomical levels, whereas in vertebrates, adaptive mechanisms have become the cornerstone of the immune system. In jaw vertebrates, adaptive immune receptors of T and B lymphoid cells belong to immunoglobulin superfamily and are created by rearrangement of gene segments. In jawless vertebrates lamprey and hagfish, recombination of leucine-rich repeat modules is used to form variable lymphocyte receptors. Striking functional similarity of the cellular and humoral branches of these systems suggests similar driving forces underlying their development.

INTRODUCTION

The main challenge for the immune system is to create in an economic way a repertoire of receptors able to discriminate between "self" molecules and cells and the vast arrays of pathogens. Immune system also needs to detect defective, damaged and transformed "self". Depending on the type of receptors, immune mechanisms are traditionally subdivided into innate and adaptive. The most ancient and universal innate immune mechanisms are based on germ-line encoded receptors that evolved to recognize molecular patterns common

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for large groups of pathogens or for defective "self".[1,2] Pathogens, however, constantly develop mechanisms for escaping immune surveillance and new pathogens arise. One of the ways pathogens escape immune system is by extensive diversification of their surface molecules. For example, the human malaria parasite *Plasmodium falciparum* generates diversity within the multi-copy variant antigen gene families by gene recombination.[3]

To meet the challenge, the host organisms need to maximize the scope of immune recognition. Extensive populational polymorphism is one strategy. In case of Drosophila melanogaster, variability in immune competence is associated with nucleotide polymorphism in at least 16 immunity genes.[4] Duplication of receptors is another strategy widely used by invertebrates.[5] Combinatorial use of receptors is employed to increase the range of recognition using the same number of receptors.[6] Somatic diversification of receptors such as gene recombination, conversion, or alternative splicing is used to increase the number of receptors produced from a limited number of genes. In insects and crustacean, large repertoire of receptors is generated through alternative splicing of the Downs syndrome adhesion molecule.[7,8] 185/333-gene family in the purple sea urchin is diversified by recombination and point mutations.[9] Highly adaptive nucleic acids based immune mechanisms evolved in all phyla, including bacteria.[10,11]

Somatic diversification of receptors became a dominant strategy in the adaptive immune system of jaw vertebrates (gnathostomes). From sharks to humans, antigen receptors of T and B lymphoid cells (TCR and BCR) are encoded as gene segments,[12] and functional genes are produced by rearrangement of these segments by recombination activated genes (RAGs).[13] Each lymphoid cell expresses unique receptors and can clonally expand in response to a matching antigen.[14] Rearranged BCRs can be further diversified by gene conversion, class switch recombination and somatic hypermutation, mechanisms dependent on activation-induced cytidine deaminase (AID).[15] Despite the similarity between TCR and BCR, T and B cells differ functionally and in the way they recognize antigens. B cells recognize antigens directly, in contrast, T cells recognize antigens in association with the major histocompatibility complex (MHC) proteins and related molecules serving as markers of "self".[16] T cells therefore combine recognition of self and nonself. T cells are responsible for the cellular immunity and regulatory functions, while B cells are involved in humoral immunity by producing antibodies.

Till recently, adaptive immune system was thought to be unique to jaw vertebrates.

However, recently a strikingly functionally similar, albeit based on a completely different type of receptors, adaptive immune system has been discovered in jawless vertebrates (cyclostomes) lamprey and hagfish.[17] In this system, leucine-rich repeat (LRR) modules are incorporated into noncomplete variable lymphocyte receptor (VLR) gene to form a functional gene. Two types of receptors, VLRA and VLRB are expressed in different cell populations that seem to divide functions, as do T and B cells of jaw vertebrates and to mediate, correspondently, the cellular and humoral immunity.[18] VLRB receptors can be secreted as antibodies. In contrast, VLRA resemble TCR in that they are found only in membrane-bound form and do not bind antigens directly. Moreover, VLRA positive lymphoid cells express genes typical for T cells of jaw vertebrates, while VLRB-bearing cells express B-cell types of genes. Remarkably, assembly of VLRs may depend on the agnatha's homologue of AID.[19]

This discovery changes the view of the evolution of adaptive immune system. If both jaw and jawless vertebrates have B- and T-like cells and AID is involved in diversification of receptors in both systems, then their common ancestor might have already had subdivision of lymphoid cells into two branches. It is also possible that AID

was already involved in diversification of some immune receptors. It is now clear that somatic diversification of antigen receptors takes place in many invertebrate species.[20] Therefore the ancestors of vertebrates already had a sophisticated immune system. Two events likely contributed the most to the divergent evolution of the immune systems in jaw and jawless vertebrates; whole genome duplication (WGD)[21] and the development of the Rag-mediated receptor diversification in jaw vertebrates.[22] The knowledge of two different adaptive immune systems opens new opportunities for understanding the principles of the underlying design and evolution of the immune mechanisms.

THE MAJOR FEATURES OF THE ADAPTIVE IMMUNE SYSTEM OF JAW VERTEBRATES

The adaptive immune system of jaw vertebrates combines strong conservation of its general features with fast evolution of immune genes. The structure of immune receptors of T and B cells, mechanisms of receptor diversification and signaling mechanisms are conserved from sharks to humans while sequences can have only residual similarity. The adaptive immune system is built around three major molecules, TCR, BCR and MHC. TCR and BCR are dimers.[16] BCR contains immunoglobulin (Ig) heavy (H) and light (L) chains; TCR is composed of alpha and beta chains in αβT cells or gamma and delta chains in γδT cells. Antigen-binding site is formed by hypervariable complimentarity determining regions (CDRs) from both chains. Two CDRs are encoded by variable segments while CDR3 is created at V(D)J junctions. The signaling from TCR and BCR depends on the immunoreceptor tyrosine-based activation motif (ITAM) in accessory molecules, which is CD3 chain for TCR and immunoglobulin alpha and beta chains for BCR.[23]

The variable regions of TCR and BCR are encoded as segments, variable (V), diversity (D) and joining (J) flanked by recombination signal sequences (RSSs)[12] (Fig. 1). During rearrangement, RAG1 and RAG2 together with other proteins complex on the RSS and nick the DNA at the border between the RSS and the gene segment.[13] This leads to a DNA hairpin at the end of the coding sequence. Then, proteins from the nonhomologous end joining repair pathway open and ligate the hairpins. During this process, nucleotides can be removed or added to the junctions, increasing the variability of receptors. In addition, nontemplated nucleotides can be added by terminal deoxynucleotidyl transferase (TdT).[24] Each lymphoid cell typically expresses only one type of receptor by a mechanism known as allelic exclusion.[25]

BCRs are diversified additionally by somatic hypermutation, gene conversion and class-switch recombination.[15,26,27] These processes start with deamination of cytidine to uridine by AID with subsequent steps performed by DNA repair enzymes. The RGYW motif (where R = purine, Y = pyrimidine and W = A or T) and the reciprocal WRCY motif are the preferred targets for AID.[28,29] Somatic hypermutations coupled with selection by antigen leads to evolution of BCR with high-affinity for antigen (Fig. 2). This process is especially efficient in mammals, while in cold-blooded vertebrates it is much slower. The structure of V regions has evolved to direct somatic hypermutations mostly to CDR1 by concentrating AID target sequences in this region.[30] However, the relative density of such targets in human CDR1 is much higher than in the corresponding zebrafish sequences.[31] It may be one of the reasons why maturation of immune response is slower in fish.

The process of receptor diversification brings the danger of autoimmunity since some of the newly generated receptors can be self-reactive. These receptors are modified by

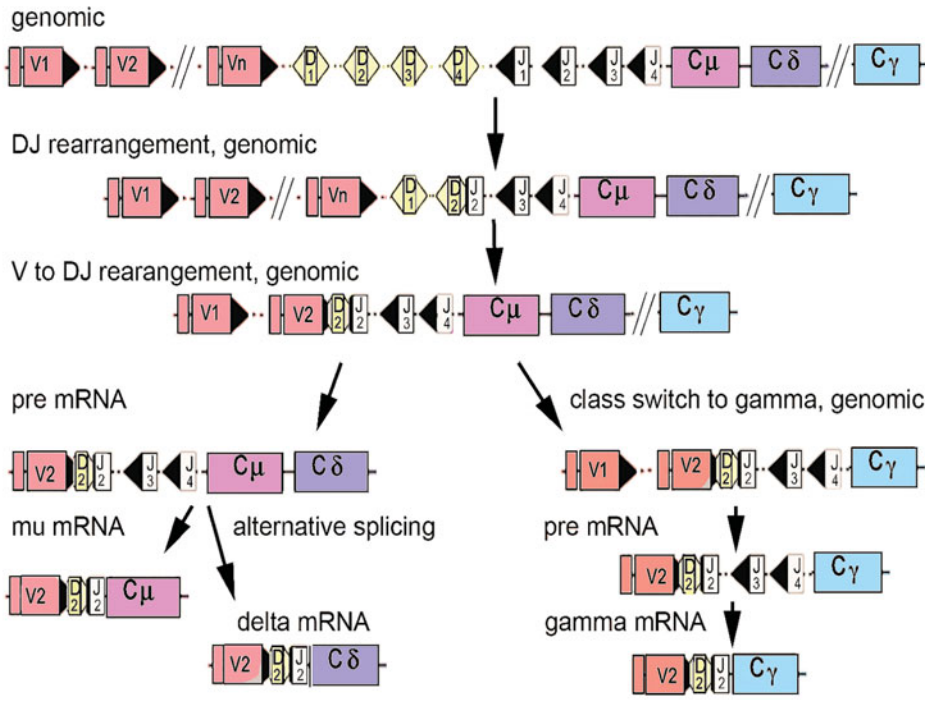

Figure 1. Diversification of immune receptors in jaw vertebrates. VDJ rearrangement of a hypothetical immunoglobulin heavy chain is shown.

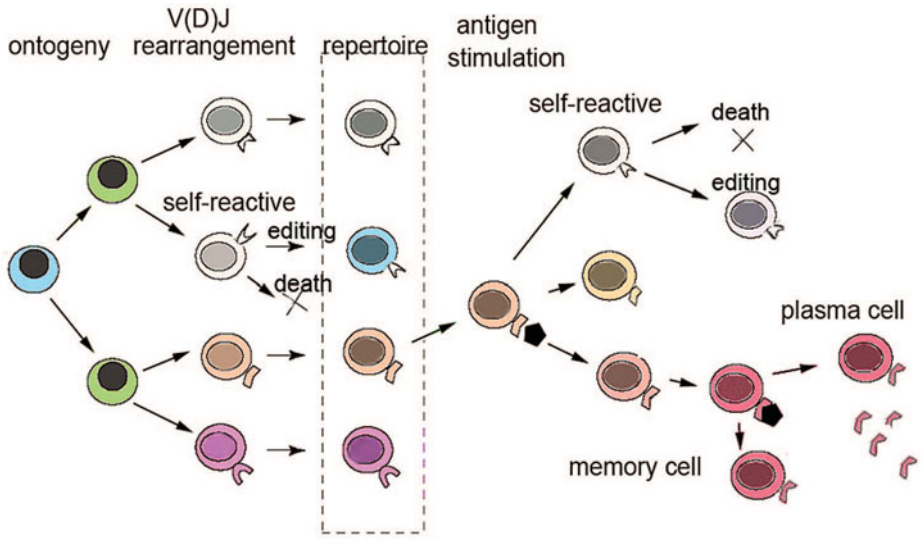

Figure 2. Highly specific antibody is a result of rounds of receptor modification and selection by antigen.

editing/revision when the V segment in a rearranged receptor is completely or partially replaced by another V segment. This process is mediated by RAGs proteins and depends on cryptic RSS sites in V segments.[32,33] Cells still bearing self-reactive receptors are removed by apoptotic death or silenced.

The variable and constant regions of lymphoid receptors evolve differently. V regions have evolved according to the "birth-and-death" model when duplication events together with lineage-specific gain and loss of individual members contribute to the rapid diversification of genes.[34] Constant regions are less constrained in the directions of their evolution, especially in immunoglobulins and have evolved to mediate various effector functions.

Interestingly, TCRα and TCRδ are expressed by the different types of T cells but are, nevertheless, encoded by a single locus with the delta DJC cluster proximal to the array of V segments followed by the alpha JC cluster.[16] This structure is preserved in evolution perhaps because it allows coordination of the expression of TCRδ and TCRα and, in this way, regulates the ratio of αβ to γδ T cells. TCRδ rearranges first and γδT cells are the first to appear during ontogeny. Combinatorial variability of the TCRδ is lower than that of the TCRα, because there are fewer DJ segments in the TCRδ cluster and because of restrictions on V regions rearrangement to TCRδ.[35] During the fetal life, the TdT enzyme is not active and junctions are not diversified, therefore early γδT cells have limited variability. However in adults, when TdT enzyme is involved in diversification, the potential number of different delta chains is very large, since the two D segments may participate simultaneously in V delta assembly and random nucleotides may be added at all three junctions.[36] This creates a sharp difference between the fetal and adult γδT repertoires, restricted in the fetus but variable in adults.

Immunoglobulins are much less conserved than TCRs with only IgM present in all species. The organization of Ig genomic loci also differs among species, especially that of immunoglobulin H chain. In cartilaginous fish, IgH are encoded in clusters with one V segment, two or three D segments and a single C region; separate clusters encode IgM, IgW and IgNAR.[37] Cluster organization of IgH is found only in cartilaginous fish, other species have IgH in translocon configuration with arrays of V, D and J segments encoded in the same locus. Light chains are encoded by the cluster type of loci in cartilaginous fish and by diverse types in other species.[38] Light chains are thought to have evolved to match heavy chains they pair up with in the BCR.

In teleost fish, three Ig classes are found, IgM, IgZ and IgD. Teleost IgH locus organization mimics the Tcrd-Tcra locus with an array of V segments followed by DJCzeta and DJCmuCdelta clusters.[39] V segments rearrange alternatively to zeta or mu clusters. Similarly to TCRδ, zeta has lower combinatorial variability and is expressed earlier in development than mu, however in adult fish, zeta chain has more N addition than mu. Therefore close functional parallels exist between pairs of IgZ/IgM B cells and δγ/αβ T cells. Teleost IgD is expressed by alternative splicing of DJCμCδ message often with inclusion of the first exon of Cμ.[40]

Coupling of mu and delta is preserved in tetrapods IgH loci. Contrary to teleost, tetrapods have several constant regions downstream from Cδ. These immunoglobulins are expressed by class switch recombination[41] (Fig. 1). This is a tetrapods-specific way of receptor diversification when CμCδ in the rearranged gene is replaced by one of the downstream constant regions. AID mediates this process through switch (S) regions located in front of each C region. Introduction of class switch recombination opened opportunities for evolution of various specialised immunoglobulins. For example IgA protects mucosal

surfaces and is adjusted for this function.[42] Even closely related tetrapod species often have different sets of specialised antibodies evolved to better perform specific tasks.

Hybrid molecules that have a double V structure with the N-terminal V domains similar to Ig V domain while the rest of the molecule is similar to the conventional TCRδ have been found recently in sharks and marsupials.[43,44] These receptors are thought to be a result of a recombination between the TCR and immunoglobulin loci. They combine the properties of Ig-like antigen binding with TCR effector function. They might have been present in mammalian ancestors but later were lost from most mammals.

T and B cells undergo V(D)J rearrangement and selection for the absence of self-reactivity in the primary lymphoid organs, which is thymus for T cells in all species and variable tissues for B cells.[45] The newly formed T and B cells migrate via the bloodstream to peripheral lymphoid tissues, where, following antigen recognition, they undergo lymphoblastoid transformation, clonally expand and differentiate correspondently into effector cytotoxic or helper T-lymphocytes or plasma cells. T and B cells cannot function without MHC proteins, which are necessary for antigen presentation to T cells and communication of immune cells. MHC is discussed in Chapter 18 of this book.[46]

ADAPTIVE IMMUNE SYSTEM OF JAWLESS VERTEBRATES

For long time, it was known that the jawless fishes (lampreys and hagfish) have lymphocyte-like cells and produce antigen-specific agglutinins after immunization (reviewed in Amemiya and Saha[47]). However neither lymphoid organs similar to higher vertebrates no genes essential to the classical adaptive immunity such as Rags, immunoglobulins, or MHC have been found. More recent studies show that jawless fish have adaptive immune system based on variable lymphocyte receptors (VLR).[17]

VLR is encoded as an empty cassette containing only 5′ and 3′-ends of the gene (Fig. 3). Hundreds of various leucine-rich repeats (LRR) modules are encoded upstream and downstream of the VLR locus and variable numbers of them are recombined into the cassette to create a functional gene. A gene conversion mechanism is thought to

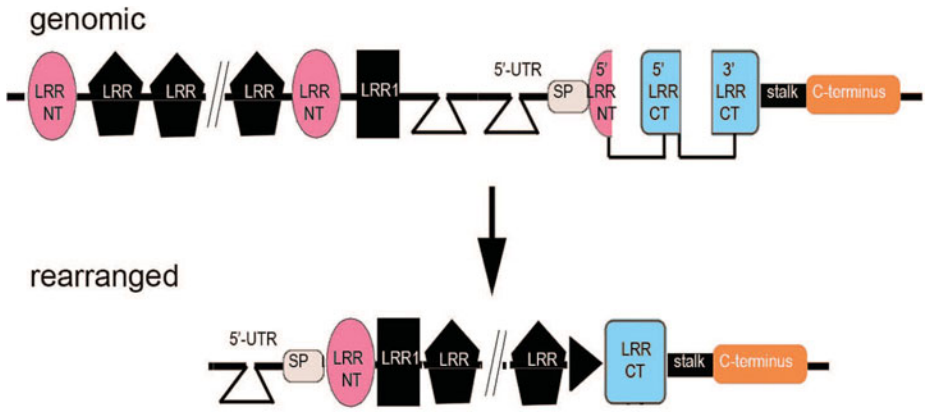

Figure 3. Adaptive immune receptors of jawless vertebrates are generated by assembly of LRR modules into incomplete VLR gene.

be responsible for copying the donor LRR sequences.[19] Lamprey lymphocytes express two putative deaminases of the AID-APOBEC family that may be involved in VLR diversification. The diversity of VLR antigen receptors is thought to be comparable to that generated in jaw vertebrates by Rag-mediated recombination.[19,48]

The structure of the VLR antibodies was predicted to resemble that of Toll-like receptors with typical LRR-containing solenoid structures.[17] Analysis of VLR sequences suggested that the concave surface of the VLR solenoid would be the binding site of the receptor since the patterns of amino acid substitutions indicated positive Darwinian selection.[48] This structure was validated after a VLR antibody was cocrystallized with the H antigen epitope from human "O" erythrocytes,[49] the same antigen that was found almost 40 years ago to elicit a specific agglutination response in lampreys.[50] Another structure resolved recently also demonstrated that an anti-lysozyme VLR was bound to the antigen by its concave surface.[51] In addition, presence of a loop that penetrated into the enzyme active site was noted.

Immunization of lamprey results in a shift in the flow activated cell-sorting (FACs) profile of lymphocytes, with increase in a population of larger, blast-like lymphocytes and a concomitant increase in *Vlr* mRNA levels.[48,52] Therefore, similar to the gnathostome lymphocytes, the agnathan lymphocytes undergo lymphoblastoid transformation following antigen and/or mitogen stimulation. Production of soluble VLRB was also noted. VLRB is bound to the membrane through glycosyl-phosphatidylinositol (GPI) anchor and the soluble protein can form via its cleavage. Immunization with several bacteria, polysaccharide antigens and some protein antigens elicited antibody with dissociation constants in picomolar range, equivalent to those of high-affinity IgG antibodies.[48,52,53]

VLRA and VLRB receptors are expressed by separate lymphocyte populations.[18] Cytosine deaminase 1 (CDA1) is expressed only in VLRA and CDA2 only in VLRB lymphocytes. Similar to receptors of the classical adaptive system, VLR receptors are expressed in monoallelic way. A surprising discovery was that VLRA and VLRB-expressing cells resemble functionally T and B cells of jaw vertebrates. Both VLRA and VLRB cells respond to antigenic stimulation by proliferation. However only VLRB lymphocytes bind native antigens and differentiate into VLR antibody-secreting cells while no direct binding of VLRA proteins to antigens could be detected and no soluble VLRA is present in lamprey plasma.[18] As a recombinant protein, VLRA is expressed exclusively as a transmembrane molecule similar to TCR. Another similarity to T cells is lymphoblastoid transformation of VLRA lymphocytes in response to phytohaemagglutinin while the response of VLRB cells is much weaker.

Gene expression profiles for VLRA and VLRB lymphocytes also resemble those of mammalian T and B cells. VLRA lymphocytes preferentially express a number of molecules characteristic of T cells in jawed vertebrates including GATA3, c-REL, aryl hydrocarbon receptor (AHR), BCL11b, NOTCH1, CD45, the IL-8 receptor CXCR2, IL-17 and MIF. In contrast, VLRB cells express CXCR4, TNFRSF14 that binds to LIGHT on T cells, two components of the BCR signaling cascade, SYK and the B-cell adaptor protein (BCAP), IL-8, the IL-17 receptor, TLR2, TLR7 and TLR10. Activated VLRB lymphocytes upregulate the expression of IL-8 while VLRA upregulate IL-17 and macrophage migration inhibitory factor (MIF).

Therefore it seems that VLRA lymphocytes recognize processed antigens and undergo selection in a manner analogous to the T-lymphocyte repertoire selection in jawed vertebrates. Lampreys lack the MHC that is used to present peptide fragments to T cells in jawed vertebrates. However, another, yet unknown molecules can perform this function in jawless fish.

The preferential expression of TLR2, TLR7 and TLR10 orthologues by the VLRB lymphocytes suggests that TLR ligands may facilitate activation of this population of lymphocytes in a manner similar to their roles in activating B lymphocytes. Some genes are expressed in a complementary way in VLRA and VLRB cells. Examples include expression of IL-17 in VLRA cells and IL-17 receptor in VLRB cells and expression of IL-8 in VLRB cells and IL-8 receptor in VLRA. Therefore VLRA and VLRB cells may communicate during immune response.

These data suggest that compartmentalization of lymphoid cells into the cellular and humoral branches have existed in ancestors of vertebrates before the appearance of different types of anticipatory receptors in jaw and jawless vertebrates and before separation of these vertebrate lineages.

ORIGIN OF THE REARRANGING IMMUNE RECEPTORS IN VERTEBRATES

Since the two vertebrate adaptive immune systems use the same type of immune cells the question arises: were these systems completely independent convergent evolutionary acquisitions,[18] did they coexist in vertebrate ancestors,[54] or did one precede the other?[55] There is currently some support for each of these hypotheses.

V(D)J recombination is very similar to the mechanisms of transpositional recombination and retroviral integration.[56,57] This led to the hypothesis that V(D)J recombination originated by integration of a bacterial transposon or a retrovirus into a genome of an ancestor of jaw vertebrates.[22] The integration resulted in cleavage of a gene encoding the V domain of an immune receptor into two recombinable fragments.[58,59] The intruder transposase evolved later into RAGs proteins. In support of this view, a fragment similar to the core region of RAG1 has been identified in transposases from Transib transposons.[60] The terminal inverted repeats of Transib transposons are very similar to RSSs used by RAGs. However, only the RAG1 core resembles Transib transposase, so it was suggested that the N-terminal domain was assembled from a different protein. One candidate is a mobile element from a mollusk.[61] *Rag1* gene is tightly linked with *Rag2* in all species. RAG2 protein bears no resemblance to transposases or any bacterial proteins, therefore it was suggested that RAG1 transposons landed in a vicinity of a primordial Rag2 gene and RAG2 was coopted by RAG1 to perform rearrangement. RAG1 core-like sequences were found in several protochordates. Strikingly, a *Rag1/2*-like linked gene pair is present in purple sea urchin genome that is similar in both sequence and genomic organization to the vertebrate *Rag1/2* pair.[22,62] Sea urchin RAGs are coexpressed during development and in adult tissues and form complex with RAG1 and RAG2 proteins from several vertebrate species. This discovery pushes the acquisition of the enzymes crucial for the origin of adaptive immunity many million years earlier than the origin of vertebrates. The role of sea urchin RAGs is unknown at present. Sea urchin RAGs are expressed in coelomocytes, which is consistent with the role of these proteins in immunity. No clusters of gene segments with similarity to the vertebrate V, D and J gene segments or RSS-like sequences have been identified within sea urchin genome. However if in sea urchin, RAGs act on diversification of a gene with only few gene segments, for example, a single V and a single J region, such a gene could be overlooked.

No RAGs have been identified in the C. intestinalis, which belongs to a group sister to vertebrates. However this species has a small genome and has likely underwent an

intensive gene loss. An alternative hypothesis is that a primordial herpes virus, rather than a transposon encoded the recombinase responsible for the origins of acquired immunity.[63] According to this hypothesis, the regulated expression of a viral recombinase in immune cells may have been positively selected for its ability to stimulate innate immunity to herpes virus infection. It provides a plausible explanation of the early and non-uniform appearance of RAGs in deuterostomata, although the sequence similarity between RAGs and herpes recombinases is low.

Another gene playing an important role in the adaptive immune system is AID.[64] AID and related APOBECs constitute a family of nucleic acid mutators.[19,64] APOBEC1 is a RNA-editing enzyme while the APOBEC3s are DNA mutators acting in defense against retroviruses. The AID/APOBECs are similar in structure and catalytic site to zinc-dependent deaminases, a large gene superfamily encoding enzymes involved in nucleic acid metabolism. The AID/APOBECs are thought to originate from tRNA adenosine deaminases (Tad/ADAT2) that edit adenosine to inosine in tRNAs in both eukaryotes and prokaryotes. The presence of two AID homologues in lamprey suggests that AID originated before the split of jaw and jawless vertebrates.[18] No full-length AID was identified in amphioxus; however, two regions exhibiting weak sequence identity to the N- and C-terminal regions of human AID have been found.[65] AID was suggested to develop in vertebrate ancestors as an antiviral protein.[19,59,66] In support, AID is induced outside the germinal centers in response to infection by certain retroviruses and contributes to antiviral defence. NF-kappaB binds the AID promoter and is required for the expression of a virus-induced AID.[67] NF-kappaB role in immune signaling is conserved in evolution, supporting the view that AID primordial role was antiviral defense. AID mutagenic activity could then become employed for somatic mutations in genes encoding immune receptors. Therefore the mechanism of deaminase-mediated diversification of antigen receptors could arise independently from V(D)J rearrangement. The AID/APOBECs family expanded in mammals and underwent complex gene duplications and positive selection.

TdT is one more molecule contributing to diversification of immune receptors by yet another unique mechanism, insertion of nontemplated nucleotides at V(D)J junctions. A closely related enzyme, polymerase μ, promotes repair of noncomplementary ends by nonhomologous end joining.[68] A gene homologous to TdT and polymerase μ is present in amphioxus and sea urchin.[65,69] Therefore many components of modern diversification machinery of the adaptive immune system are present in invertebrates.

Many candidates for a primordial receptor that was disrupted by a Rag-bearing transposon have been suggested from the members of IgSF involved in cell adhesion and innate immunity.[70] All major molecules involved in the adaptive immune system including Ig, TCR, MHC, tapasin and beta2-microglobulin have C1 type of constant domain. From other proteins with C1-like domains are signal-regulatory proteins (SIRPs).[71] SIRPs also encode V domains with a typical J motif and signal via ITAM-containing adaptor molecules. The expression of most members of this family is restricted to myeloid cells where they act as both inhibitory and activating receptors. SIRPα is an inhibitory receptor that interacts with the membrane protein CD47, which is a marker of self. CD47–SIRP interaction controls the effector functions of phagocytes protecting host cells against immune-mediated damage.[72]

In sea urchin, there are three V-C1-TM-cytoplasmic region genes.[69] Their role is currently unknown. The receptor disrupted by Rag-bearing transposon is expected to be involved in immune recognition. The somatic recombination of the receptor would

increase its repertoire and provide a selective advantage. To evolve into a typical TCR and BCR, the gene encoding this receptor would need to be duplicated to become a dimer and be duplicated again to form T and B-cell receptors. This could happen during the whole genome duplication (WGD).

WGD is likely to have played a pivotal role in evolution of the adaptive immune systems. WGD generates enormous amounts of genetic raw material available for acquisition of novel functions. Vertebrates were suggested to have undergone two rounds of WGD after the split of the urochordate and cephalochordate lineages.[21] Recent sequencing of invertebrate genomes has provided strong support for WGD hypothesis. In particular the genome of the cephalochordate amphioxus shows a high degree of synteny conservation with vertebrates. Amphioxus and the urochordate *Ciona* have a single MHC-like region that could be a precursor of the four MHC paralogons in jawed vertebrates.[65,73] Many more genes involved in modern adaptive immune system may originate as a result of WGDs.[54]

VLRs were suggested to originate from the GPIba, a part of the receptor complex GPIb-V-IX, which has a critical role in hemostasis.[19] This multifunctional receptor initiates platelet activation and thrombus formation at the sites of vascular injury and may control other vascular processes such as apoptosis, coagulation, inflammation and platelet-mediated tumor metastasis.[74] VLRs and GPIba share a unique insert between the a-helix and the first b-strand of the LRRCT module. This characteristic insert is absent from the LRRCT of other animal LRR-containing proteins.

The exact point in time when rearranging receptors originated is not known. The timing of divergence of cyclostomes from the gnathostome lineage is debated. Some molecular phylogenetic analyses suggest that the genome duplications occurred before the cyclostome-gnathostome split.[75] It can be speculated that RAG-rearranging receptor originated before the first WGD. After that, it was duplicated and became a dimer receptor. The second WGD led to duplication of the dimer receptor and created the grounds for the divergent evolution of TCR and BCR. The idea that all major components of both adaptive immune systems of vertebrates originated before the split of jawless vertebrates agrees with the presence in lamprey of such molecules as TCR-like, CD4-like, V-preB-like, CD3 epsilon etc.[76,77] Recently, two VLR-like genes have been identified in zebrafish suggesting that VLR receptors could also be in place at the time of the split.[55] This agrees with the idea that the vertebrate ancestor had both BCR/TCR and VLR precursors.[54] Their evolution might turn in opposite directions in jaw and jawless vertebrates with one system becoming dominant and the other being degenerated/lost.

Cartilaginous fishes already have a well-developed adaptive immune system. The major difference from teleost and tetrapods is the cluster organization of their IgH loci. Is cluster organization a primordial feature as often was suggested? It may be not. The structural and functional similarity of Tcrd-Tcra loci and teleost IgH loci may point to an ancestral type of organization of the locus encoding TCR and BCR that existed in jaw vertebrates before the divergence of the cartilaginous fish lineage. This scenario assumes that the IgH locus organization observed now in the teleost precedes the IgH cluster organization of cartilaginous fish.[31] Separation of IgM and IgZ loci in cartilaginous fish could occur by a translocation of one member of this pair into a different location. This would explain the origin of two ancient immunoglobulins of cartilaginous fish, IgM and IgW. Tetrapods could lose IgZ at some point in their evolution.

ORIGIN OF LYMPHOID CELLS AND ORGANS

An essential component of the adaptive immune system is a lymphoid cell able to live long to provide immunological memory and able to proliferate in response to antigen stimulation. Precisely at what time in evolution the lymphocytes had originated is unknown. Immune cells of invertebrates are mostly an unexplored area despite the long time since Metchnikoff' discovery of phagocytes in starfish larva in 1882. The oldest specialized immune cells that are found in almost all animals (metazoans) that have been studied are phagocytic cells. Moreover, immune-like phagocyte activity has been observed in the social amoebas that aggregate when starved to form a migrating slug. Cells called "S cells" engulf bacteria and sequester toxins circulating within the slug.[78] Phagocytes however, are relatively short-lived nondividing cells. Lymphocytes have emerged as a new type of immunocompetent cells. The evidence for two types of lymphocytes in lamprey and hagfish suggests that lymphocytes must have been present in the common ancestor of vertebrates. Segregation of the erythroid lineage also happened before the split of jaw from jawless vertebrates since lamprey has distinct erythroid cells.[79] Mussels have at least 3 types of hemocytes.[80] Ascidians have 5 discernible types of hemocytes including lymphocyte-like cells.[81]

Jaw vertebrates have a subpopulation of lymphoid cells, natural killer (NK) cells, which do not express rearranging receptors. Instead, they express innate receptors, activating and inhibitory.[82] Receptors recognizing MHCI on normal cells are inhibitory, those recognizing aberrant molecules are activating. NK response is the result of integration of activating and inhibitory signals. NK lyse virus infected and tumor cells. They also regulate other cell types through secretion of interferon (IFN)-γ and other cytokines. NK cells resemble adaptive cells in that they express a unique pattern of receptors creating a repertoire of specificities. Self-tolerance of NK cells must be established in each individual, which is also similar to T and B cells. Moreover, some data show that NK cells can expand in response to a specific antigen. For example, Ly49H+ NK cells expand after MCMV infection, similarly to how antigen-specific T cells proliferate after antigen stimulation.[83] Furthermore, recent data show that NK cells can produce memory response lasting at least two months after stimulation.[84,85] Therefore NK cells have characteristics of both innate and adaptive immune cells.

It is likely that cytotoxic lymphoid cells similar to NK cells developed quite early in evolution. Even the simplest animals such as corals, bryozoans and ascidians have systems for allorecognition although it is based on molecules other than MHC as "self" tags.[86] Urochordates *Botryllus schlosseri* cytotoxic cells express receptors related to mammalian NK CD94/NKR-P1 receptors.[87] Cytotoxic cells are present also in the sea urchin *Paracentrotus lividus*. The coelomic fluid from this species contains several coelomocyte types including amoebocytes and uncolored spherulocytes.[88] Cell population enriched in uncolored spherulocytes exert high cytotoxic activity against rabbit erythrocytes in the presence of amoebocytes or extracts from these cells pointing to cooperation of different cell types.

An important feature of lymphoid cells is their ability to clonally expand in response to immunological challenge. There are indications that sea urchin immune cells are a dynamic population. In response to lipopolysaccharide, they transiently increase in number.[89] The coelomocytes of individual sea urchins express scavenger receptor cysteine-rich genes from a multigene family encoding an estimated number of 1,200 SRCR domains in specific patterns unique to each individual. Their expression fluctuates up to 10-fold in

1 week and up to 30-fold over a period of 3 months.[90] It would be interesting to know is these cells can clonally expand in response to a specific antigen.

Knowledge of ontogeny of T and B cells may help to understand their phylogeny. However, the exact path from hematopoietic stem cells to lymphoid cells is not that apparent and has recently been a subject for debate. The classical model of hematopoiesis postulates early separation of lymphoid fate from erythroid/myeloid one and the existence of a common progenitor (CLP) for T and B lymphoid cells.[91] Some recent data support the existence of CLP population[92] while other data do not fit the CLP model. The thymic progenitors first lose the potential to generate B cells retaining a substantial macrophage potential as well as T, NK and dendritic cell potential.[93,94] About 30% of thymic macrophages are derived from early thymic progenitors. A close connection of B cells and macrophages was known for a while and fish and frog B cells have preserved the ability to phagocyte.[95] Recently, it was found that freshly isolated human peripheral blood γδ T cells can phagocyte, process and present antigens on MHCII.[96] It suggests a close connection of all immune cells. An alternative picture of hematopoesis suggests that both the innate (myeloid) and adaptive (lymphoid) lineages of the immune system arise from a common progenitor.[97,98] Many transcriptional factors involved in development of lymphoid and myeloid cells are shared.[99]

Until recently, it was believed that T cells are evolutionary older than B cells.[45] However B cell seems to be a default fate of lymphocyte development in absence of NOTCH signaling. NOTCH increases the frequency of multipotent progenitors; skews the T and NK potential of CLP and inhibits the differentiation of B cells.[100] Low doses of NOTCH ligands increase frequency of NK, whereas higher doses are required for increasing the frequency of T-cell clones. So it seems possible that B cells are evolutionary older than T cells.

T cells in all jaw vertebrates develop in thymus. Expression of NOTCH ligands by thymic epithelial cells is necessary for T-cell development. The fact that T-like cells in jawless fish express NOTCH suggests that development of these cells may be governed by the same signaling pathways as classical T cells. VLRA lymphocytes are enriched in the lamprey's gill regions suggesting that development of this cell population may take place in the same region, where T cell develop in jaw vertebrates.[18] *Foxn1* gene is expressed in epithelial cells of thymic primordium and is crucial for thymus development.[101] The agnatha ortholog of this gene, *Foxn4L*, is expressed in pharyngeal region in lamprey. Moreover, this expression overlaps NOTCH ligand DELTA-like 4, which is necessary for differentiation of lymphocyte progenitor cells into the T-cell lineage and is a downstream target of FOXN1.[101] In the same study, pharyngeal epithelial structures have been examined by in situ hybridization for the presence of lymphoid aggregates and none were found. This led to the conclusion that lamprey does not possess a lymphoid organ resembling the thymus of jawed vertebrates. However, the probe used for in situ hybridization was from *VLRB* gene, which is expressed in B-like cells. Usage of *VLRA* probe may clarify if T-like cells develop in lamprey's pharyngeal region.

The places where B cells develop vary among species and even among developmental stages in the same species. In jaw vertebrates, sites of B-cell development are often associated with the gut.[102,103] In developing lamprey, blood forms in the typhlosole (an invagination of the intestinal epithelium) and nephric fold, while in adults, blood forms in the protovertebral arch.[47] The development of cytotoxic T and NK cell in the gill region and antibody-producing B cells in the gut region may represent the ancient division of labor in protection of the regions most exposed to pathogens.

INNATE-ADAPTIVE INTERACTIONS

Adaptive immune system evolved from the innate immune mechanisms and it cannot function without their help.[104] Innate immune cells are the first to contact pathogens and they secrete various cytokines instructing adaptive cells about the nature of pathogen. For example, bacterial infections induce interleukin-12 (IL-12), while helminthes induce IL-4 and IL-13. In response, T cells develop into different subsets of helper cells; it is T-helper 1 (Th1) in case of intracellular bacteria, Th2 in case of helminthes and Th17 in case of extracellular bacteria and fungi.[105] Innate immune cells such as dendritic cells present antigens to T cells. Although B cells can recognize soluble antigens directly, antigen presentation to them is often mediated by macrophages and dendritic cells.[106] B-cell memory response is supported by basophils.[107] Therefore, innate immune modules control the adaptive cells so that the response is tailored to the pathogen, including the intensity of inflammation, duration, isotype of antibodies produced by B cells etc.

Many features of the adaptive immune system have developed to communicate with older innate mechanisms. For example, special regions of Ig evolved to bind the complement. B cells, in addition to adaptive receptors, express innate immune receptors such as TLRs. These receptors cooperate with BCRs in antigen recognition. B cells usually need T-cell help to be activated after BCR binds an antigen but if both BCR and TLR recognize the same antigen, B cells respond without T-cells help.[108]

On the other side, innate cells in species with adaptive immunity have also been extensively modified to work with adaptive mechanisms. All innate immune cells express MHCI and II genes, which are an integral part of the adaptive immune system. The machinery for antigen presentation was introduced in the innate immune cells. New cytokines to communicate with adaptive cells and the corresponding receptors have evolved. The complement system evolved to accept antibody-antigen complexes as activators. Phagocytes evolved receptors recognizing antibody bound to antigens. Introduction of T and B cells resulted in reduction of innate receptors. For comparison, there are 10 TLR in humans and 222 in sea urchin.[109]

Therefore innate modules in species with adaptive immune system differ from analogous modules in species without such system. Rightly, there are no pure innate or pure adaptive cells in our immune system, they turned into a nonseparable blend unable to function without one another. There is always some redundancy between individual immune mechanisms. In absence/defects of adaptive immune system, innate mechanisms are upregulated.[110] The relative contribution of innate and adaptive mechanisms into defense may vary between species. For example, zebrafish with mutation in *Rag1* gene and the corresponding absence of T and B cells, are viable and can be kept at usual condition;[111] while humans develop severe disease in absence of T and B cells.[112]

Recent studies have highlighted the role that helminth infection may have played in evolution of the adaptive immune system.[113] Helminth infections are very common in vertebrates. Helminths produce high levels of tissue damage, nevertheless the infection is usually well tolerated. Helminths modulate the host immune system by suppressing inflammatory reactions and skewing T-cell response to Th2 type. Th2 response is also involved in wound healing and tissue remodeling. Helminth-infected populations show increased susceptibility to microbial infection, while helminth-free populations have increased frequency of allergy and autoimmunity. A popular hypothesis is that the immune system evolved in the presence of helminths and developed a dependence on factors produced by the parasites. Helminths-derived products are intensively studied now as potential immunomodulators.[114]

CONCLUSION

There have been many speculations why the adaptive system evolved at all and why in vertebrates. The assumption has been that there should be something special in the vertebrate life-style of physiology that made diversification of receptors necessary. One hypothesis was that the predator life-style of early vertebrates created a necessity for gut protection.[115] Another hypothesis also assumes that there was a special need for adaptive immunity in vertebrates. It suggests that the maintenance of symbiotic microbial communities, especially in the gut, was the primary force driving the evolution of the adaptive immune system.[116,117] The basis for this hypothesis is that vertebrates harbor hundreds of symbiotic bacteria species while invertebrates do not form such relationships. Another idea is that the appearance of anticipatory immunity in vertebrates might have been driven by a need to facilitate the developmental and morphological plasticity in addition to increasing the scope of pathogen recognition.[118] Fewer offspring in jaw vertebrates in comparison to invertebrates was also suggested to play a role.[54]

The danger of self-reactivity, which accompanies the diversification of adaptive receptors and the fact that some species without adaptive immune system such as mollusks still have long life span is interpreted sometimes as evidence that the origin of adaptive immunity is just an evolutionary serendipity, which left us with an overly complex, costly and self-harmful immune system.[119] Yet the finding that the two surviving branches of vertebrate radiation jawless fish—lamprey and hagfish—and jawed vertebrates both developed adaptive immune systems based on different receptors yet strikingly similar functionally suggests that adaptive immunity provided a great survival benefit. Moreover, the existence of receptor diversification and memory in invertebrates suggests that similar driving forces act on all species leading to unique mechanisms of receptors diversification.[9,69,90]

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CHAPTER 14

EPIGENETIC CODE AND SELF-IDENTITY

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Abstract: Epigenetics is a new and expanding science that studies the chromatin-based regulation of gene expression. It is achieving considerable importance, especially with regard to developmental mechanisms that drive cell and organ differentiation, as well as in all those biological processes that involve response and adaptation to environmental stimuli. One of the most interesting biological questions concerning animals, especially human beings, is the ability to distinguish self from nonself. This ability has developed throughout evolution, both as the main function of the immune system, which defends against attack by foreign organisms and at the level of consciousness of oneself as an individual, one of the highest functions of the brain that enables social life. Here we will attempt to dissect the epigenetic mechanisms involved in establishing these higher functions and describe some alterations of the epigenetic machinery responsible for the impairment of correct self-recognition and self-identity.

INTRODUCTION: EPIGENETICS

In recent decades, biology has welcomed a new science to the classical disciplines of cell and molecular biology, biochemistry and genetics; it is termed epigenetics and its relevance is extending to almost all fields. We can summarise the importance of this science in a few sentences. Each gene is expressed in a defined space and time following the dictates of the epigenetic machinery that, by altering the physical structure of the genetic information, makes it readable or unreadable. The structure of the chromatin, like the genetic information itself, can be inherited by cellular progeny, creating a new and stable level of information for the unwinding of the genetic program.

Self and Nonself, edited by Carlos López-Larrea.

A first definition of epigenetics was proposed by Conrad Waddington in 1942[1] as the study of how genotypes give rise to phenotypes through programmed changes during development. New concepts were subsequently added to this original definition: an epigenetic event would be something that affects gene expression without changing the nucleotide sequence, in a way that can be inherited through cell division and possibly through gamete formation. Today, epigenetics refers to heritable changes in gene activity and expression, as well as to stable, long-term alterations in the transcriptional potential of a cell that are not necessarily heritable (http://nihroadmap.nih.gov/epigenomics/index.asp). In this sense, epigenetics would include all mechanisms for unfolding the genetic programme in processes such as development, differentiation, stress response and pathological states. Indeed, epigenetic modifications are stable but at the same time plastic, as they can be modulated by cellular or environmental factors.[2] This nuance of plasticity is the most striking feature of epigenetics, as it enables elaboration of the genetic information and its integration with the environment.

Epigenetic mechanisms involve covalent chemical modification of DNA (methylation) or chromatin (histone modification).[3] An increasing number of additional mechanisms are being reported, mostly related to the former two, which regulate gene expression and chromatin structure (noncoding RNA, among others).

DNA, like a book, is organised into modules. All the epigenetic machinery can be seen as a complex system of enzymes or structural proteins; in response to a cell's internal and external status, these proteins are able to write the instructions in their own language for the accessibility of the basic book of each cell, i.e., the genome. Each page is represented by the nucleosome core particle, which consists of 147 bp of DNA organized in approximately two superhelical turns of DNA wrapped around an octamer of core histone proteins (two copies each of H2A, H2B, H3 and H4 or their histone variants). When associated with other components, higher-order nucleosomal structures are formed, like chapters or sections of a book. The epigenetic machinery is in charge of determining the accessibility of the pages to the readers of DNA, for example RNA polymerase. This ensures that the genetic information is stored, organized and read out in a correct spatial and temporal sequence during cell differentiation and organism development. The epigenetic code used consists of a large number of small covalent modifications on DNA or histones. Among epigenetic proteins, we recognise enzymes that perform these covalent modifications, the "writers", as well as enzymes able to eliminate them, the "erasers". Finally, other proteins, the "readers" of the epigenetic code, are able to recognise these modifications and join them to the effector function: opening or closing the chapter of the book. Variations introduced into nucleosome array structures by this machinery determine differences in chromatin compaction that correlate closely with "open" versus "closed" states, which in general coincide with "active" versus "inactive" states of gene expression.

DNA Methylation

DNA methylation is the simplest and perhaps best-studied epigenetic modification. In mammals, it consists of the addition of a methyl group to the 5 carbon of a cytosine that is followed by a guanine in the DNA sequence. The CpG dinucleotide is found at very low frequency in the genome, but is particularly concentrated in gene promoters, where it mostly regulates gene expression, blocking transcription when the methyl group is present. Most of these regions, called CpG islands, are subject to dynamic methylation

modifications that are linked to tissue differentiation and formation. Once differentiation is complete, tissue-specific methylation is established in each cell type and is generally maintained throughout the cell's life. Promoter methylation accounts for only a small part of global genome methylation: the bulk of methylated CpG in the genome is located in repetitive sequences, most of which are derived from transposable elements. Methylation keeps these sequences silent, making the event of amplification and new insertion in the genome extremely rare.[4] At least two additional genetic mechanisms rely on DNA methylation in normal cells: genomic imprinting and X-inactivation. Genomic imprinting occurs in some genes whose expression is always restricted to either the maternal or the paternal allele. It requires DNA methylation at one of the two parental alleles to ensure monoallelic expression. A similar gene-dosage reduction is involved in X-chromosome inactivation in females.[5]

The "writers" of these modifications are members of an enzyme family called DNA methyltransferases (DNMT). DNMT1 is responsible for maintaining methylation in DNA replication. When a new DNA strand is synthesized, the methyl-CpG site is copied to an antisense CpG on the other strand, creating a hemi-methylated site. DNMT1 specifically recognizes these hemi-methylated CpG and transfers a methyl group to the unmethylated cytosine ring. In this way, methylation can be transmitted to both daughter cells. These features explain the stability of the modification, which enables it to be inherited in cell division. The "eraser" responsible for the reversion of this modification has not yet been identified univocally in animals (although it is described for plants[6]); some candidates have been proposed,[7-9] but we still await clear demonstrations. Even though there are numerous exceptions, most genome methylation is not maintained during meiosis and gamete formation.[10] The entire genome undergoes global demethylation in gametes and methylation is subsequently re-established in early embryonic stages by the de novo DNA methyltransferases DNMT3a and 3b, which are expressed specifically during the first phase of embryo development.[11]

Finally, the "readers" for this modification are a family of proteins that contain the methyl-binding domain (MBD). These proteins link the CpG methyl group to chromatin remodelling machinery that turns off transcription and locks the chromatin in a condensed state.[12]

Histone Modification

A second, more complex aspect of epigenetics is chromatin modification. The close interactions between DNA and histone proteins in a nucleosome lead to a high degree of structural condensation that, by default, impedes gene transcription. Histone proteins have positively charged tails that protrude from the core structure of the nucleosome and can be modified in many amino acid residues, generating a code of histone modifications (histone marks). There are at least 30 sites of possible modification for each nucleosome, six types of modification (methylation, acetylation, phosphorylation, ubiquitylation, sumoylation and proline isomerization), some of which can occur in different configurations (for example, lysine can be mono-, di- or trimethylated); a huge number of combinations are thus possible.[13]

In eukaryotic cells, gene activation is closely associated with covalent modifications of histone N-terminal tails, which often differ between active and silenced chromatin. Acetylation is the most extensively studied modification and can effectively influence gene expression. Acetylation of lysine 14 or 9 in histone H3 (H3K14, H3K9) and/or

of H4K16 are generally associated with active gene transcription. Gene activation by histone acetylation has a biophysical explanation. The lysine side chain is positively charged and can bind tightly to the negatively charged DNA to form a closed chromatin structure that impedes access of transcription factors. Acetylation of lysine residues removes their positive charge and attenuates the charge interaction between histone tails and DNA.[14] In addition to this simple mechanism, acetylated lysines also act as docking sites for other proteins that play the role of "readers", mainly other chromatin-modifying enzymes and basal transcription machinery. One protein domain, termed the bromodomain, binds specifically to acetylated lysines.[15] The bromodomain is often found in enzymes that help activate transcription, including SWI/SNF, an ATP-dependent chromatin-remodelling complex. Acetylated lysine can recruit this complex to facilitate transcription activation. Histones are acetylated in lysines by "writer" enzymes called histone acetyl transferases (HAT) that add an acetyl group from the high-energy donor acetyl-coenzymeA to a lysineε-amino group and deacetylated by the "eraser" histone deacetylases (HDAC).

Methylation is another important histone modification, which occurs in the functional group of lysines or arginines. This modification is functionally more complex than others for several reasons: (i) within any histone, multiple lysines or arginines can be modified, (ii) individual lysine residues can be mono-, di-, or trimethylated, (iii) similarly, arginine residues can be mono- or dimethylated and these can be dimethylated in a symmetric or asymmetric fashion, (iv) all the core histones can be methylated depending upon physiological setting[16] and finally, (v) methylation of distinct residues has opposite functional consequences on gene activation. For example, trimethylation of H3K4 and methylation of H3 arginine (R)17 are associated with active chromatin, whereas H3K27 or H3K9 methylation is associated with transcriptional silencing.

In the case of methylation, global charge of the residue is unaffected by the modification; the final effect is thus determined by different "readers" that link these marks with other effectors. Proteins with a chromodomain, such as HP1, can bind specifically to methylated lysine. HP1 is a transcription-silencing protein that interacts with histone deacetylase (HDAC); its binding to methylated H3K9 results in histone deacetylation that eventually leads to gene silencing. H3K4 trimethylation is recognized by the chromodomain protein CHD1, which can further recruit HAT to activate target gene transcription.

These various methylation reactions are mediated by "writers" termed histone methyltransferases (HMT), enzymes that use an S-adenosyl methionine (SAM) high-energy methyl-donor to transfer the methyl group onto the histone residue. Each of these enzymes has its own activation pathways and its own specificity. For example, the methyltransferase Suv39h1/2 selectively methylates H3K9, whereas mixed lineage leukaemia (MLL) encodes an H3K4-specific trimethylase. Histone methylation is removed by demethylases ("erasers") of distinct classes and specificities; for example, LSD1 is an H3K4 mono- and didemethylase, whereas UTX is an H3K27 demethylase.

Other modifications also have central functions in chromatin remodelling, such as the phosphorylation of H3S10, which confers an open chromatin conformation that facilitates transcription. For a broader view of histone phosphorylation, see the review by Ito.[17]

There is increasing evidence that, in many circumstances, DNA methylation and histone modifications are interrelated in gene regulation. DNMT, for example, often associate with HDAC and other proteins to form silencing complexes, which are recruited

to heterochromatin or silenced promoters. In summary, there is extensive crosstalk and close cooperation between DNA methylation and histone modifications in the regulation of gene activity during development: histone marks and DNA methylation contribute to the generation of a code that regulates chromatin structure.

The above are only a few of the epigenetic marks and pathways involved in chromatin modification. The number of additional mechanisms, enzymes, combinations of marks and proteins that recognize them and link them to the final function continues to increase.

EPIGENETICS OF SELF

The field of epigenetics is particularly relevant in defining mechanisms of self-identity and self-recognition from different points of view. For instance, epigenetics lends a further degree of individual variability to the genetic background. In all sexually reproducing organisms, each individual is supposed to be defined by the specific genetic information contained in its DNA. As described in the introduction, epigenetic modifications orchestrate the genetic information, in the sense in that they determine which parts of the genome are to be expressed and which are to be silenced throughout ontogenic development. Recent epigenomic studies have shown that epigenetics introduces a further degree of difference between two individuals. This aspect was demonstrated in monozygotic twins, individuals with an identical genetic background, a classical model used to discern environmental from genetic factors. Epigenetics is the level at which the effects of environment and lifestyle of each person can be translated as gene expression levels into biological differences: older twins have more epigenetic differences than younger ones, indicating that an epigenetic drift can be observed during an individual's lifetime. Moreover, greater differences in lifestyles between members of a twin pair accentuate distances in the global epigenetic landscape, implying that the environment has a considerable effect on this epigenetic drift.[18]

From the cellular viewpoint, each cell is committed to a specialised function, applying a defined epigenetic pattern that drives its differentiation. The identity of each cell in the organism is thus defined by an epigenetic signature characteristic of cell type, developmental stage, spatial localisation and physiopathological state.

At the organism level, multicellular organisms need to discriminate self from nonself. First, the individual needs to distinguish self from nonself at the molecular level, to be able to set up a defence against attack by other organisms. For this reason, multicellular organisms have developed a very complex system able to recognise self and nonself and to protect the organism efficiently from external injuries. Second, each individual must recognise itself and its role to enable a social life. In this context, one of the highest functions of the brain is the ability to discriminate self identity as different from other beings.

All these complex functions have developed to distinct degrees on different branches of the evolutionary tree and we now have a considerable amount of molecular and mechanistic information about how these functions are achieved. Nevertheless, because of the extreme complexity of the self-recognition mechanisms in the immune and the nervous systems, we are far from understanding molecular details of these functions. In the following sections, we will try to see the role of epigenetic mechanisms in immune and nervous system development and that epigenetics is extremely important for the correct establishment of these functions. Although it is difficult to correlate a specific epigenetic phenomenon directly with the establishment of self-identity, we will show

that such events are fundamental in creating the complexity that makes possible the development of immune and nervous systems. We will also describe some pathologies in which disruption of the epigenetic machinery leads to alteration of the correct definition of self and nonself at all the levels considered.

IMMUNE SYSTEM RECOGNITION OF SELF AND NONSELF

The immune system is constituted by a variety of cell types circulating in the blood/lymph or residing in tissues or specialised organs. The system must be able to discriminate external and dangerous agents from the organism's own cells and molecules. Different levels of defence have evolved for this function. The most ancestral and least specialised is termed the innate immune system, which reacts to simple molecular patterns typically present in pathogens. This system is comprised of specialised phagocytic cells resident in tissues (macrophages and related cells) or in circulation (granulocytes, monocytes) as well as soluble proteins activated in cascades (complement, defensins). These elements immediately establish a primary response to destroy the invading agents. In this case, self/nonself recognition relies simply on ancestral innate molecular recognition that has evolved with each species. A more specialised set of responses, called adaptive immunity, is often required for the complete neutralization and long-term protection against each pathogen. This response is based on the recognition of specific antigens, appropriately presented on the surface of professional antigen-presenting cells (APC). An effective clone of lymphocytes must be selected that is able to react specifically to each antigen and to maintain immunological memory of the immune response. This type of immunity relies on a complex genetic strategy, termed somatic or V(D)J recombination (from the name of the gene modules that join in this process), that allows the generation of molecules (T and B-cell receptors [TCR, BCR] and antibodies) able to recognise every existing antigen and subsequent selection against the self-reactive molecules.

The key for correct immune function is thus the discrimination between self antigens, those that are physiologically present in the organism and nonself antigens. This concept followed F.M. Burnet's first proposal in the 1940s of a self/nonself model in immunology. Although this theory was found to be overly simplistic in some aspects, it is still generally accepted[19] and is helpful for understanding the functions and mechanisms of the immune system.

All immune cells in charge of the aforementioned functions are derived from a common progenitor, called the haematopoietic stem cell (HSC). HSC constitute a small cell population that is able to proliferate and maintain itself in a multipotent state and to generate more differentiated cells that will gradually convert into all the mature cells that constitute the immune system and other blood cells.

Epigenetics of Immune Differentiation and Lineage Specification

Correct distinction between dangerous external antigens and self antigens is mediated by a wide range of specialised cells. This equilibrium implies a high degree specialization of distinct cell populations that is achieved by epigenetic patterning. Just by observing the difference between the nucleus of a lymphocyte and of a neutrophil, we realize that chromatin structure is extremely important for lineage specification in the haematopoietic system. Each cell with the same genome has a specific function conferred by the particular

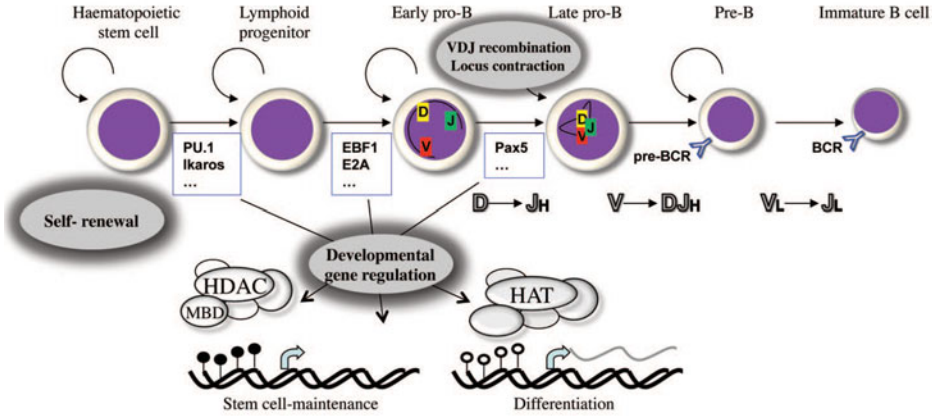

Figure 1. Epigenetics of B-cell differentiation. Steps in which epigenetic mechanisms are involved are indicated in shadowed ovals.

set of genes expressed; the epigenetic machinery is responsible for establishing these gene expression patterns during differentiation.

The lineage specification of B cells is a good example for understanding epigenetics of cell differentiation. HSC must go through many epigenetic steps before reaching the final effector function of B-lymphocytes (Fig. 1). First, a defined set of transcription factors must be expressed sequentially: the HSC relies on the presence of Ikaros and PU.1 to generate the common lymphoid progenitor.[20-21] Transcription factors generally act by recruiting the chromatin remodelling machinery (coactivators or corepressors) to turn gene expression on or off. In the case of B-cell commitment, these essential factors are E2A, EBF1 and Pax5.[22] The first two alone can activate the B-cell-specific transcription programme, but Pax5 is perhaps the most important, not only because it is indispensable for further differentiation but also because it locks the cells into the B-cell-committed lineage. In fact, $Pax5^{-/-}$ mice show B-cell differentiation arrest at the early pro-B stage and also maintain the ability to reprogram these pro-B cells to the myeloid lineage if ectopic expression of myeloid factors is forced. Pax5 activates the expression of genes specific for B-cell differentiation, such as the components of the preBCR CD79a (Igα) and the costimulatory coreceptors CD19 and CD21, while silencing the expression of genes specific for other lineages (myeloid, erythroid and T lymphoid). The promoter of the *CD79a* gene is a model of the epigenetic changes of a developmentally regulated gene. This promoter has binding sites for the basic B-lymphoid transcription factors (E2A, EBF1, Pax5 and RUNX1); it is hypermethylated in the HSC stage and undergoes demethylation during B-cell differentiation.[23] Since $E2A^{-/-}$ and $EBF1^{-/-}$ lymphoid progenitors are unable to demethylate this gene, a first demethylation step is probably necessary for gene activation; it is mediated by the action of EBF1 and Runx1, possibly through still-unidentified demethylation machinery. Chromatin structure must subsequently be remodelled to allow gene expression; Pax5 enables such changes by recruiting HAT such as SAGA and p300/CBP to open chromatin. In promoters of some genes specific for other lineages, Pax5 can silence

gene expression by interacting with HDAC of the Groucho protein family.[24] Many other mechanisms are obviously involved in molecular definition of B cells, but they generally obey these basic rules.

Epigenetics of Antibody Generation

During lymphopoiesis, B and T cells must "establish and declare their identity", i.e., their antigen specificity, to allow selection against harmful autoreactive cells. This is achieved by genetic means, through immunoglobulin and TCR gene recombination. This process in lymphocyte development is another instructive example of the importance of epigenetics in the correct definition of self/nonself recognition. V(D)J recombination occurs first in each of the IgH and TCRβ loci, only at the specific differentiation stage in which they become accessible to the recombination machinery (RAG1 and 2 and other companion proteins).[25] Chromatin surrounding these loci is normally inaccessible to the recombination machinery; in immature lymphoid-committed cells and in nonlymphoid cells, chromatin exposure to the recombination machinery does not lead to productive recombination of the Ig locus.[26] The correct spatio-temporal pattern for recombination is achieved by specific, sequential chromatin remodelling of this region. For example, when recombination of one of the IgH or TCRβ alleles is complete, allowing expression of the preBCR and -TCR complexes, recombination of the second allele is blocked by epigenetic inactivation; the IgL and TCRα loci are remodelled and exposed to the recombination machinery. Histone acetylation in the IgH locus is important for DNA accessibility: when the recombination process begins, a large region encompassing DH, JH and Cm loci is first hyperacetylated, after which DH-JH rearrangement induces histone acetylation of the DH-proximal VH genes. The distal region is subsequently activated.[27] A similar process is observed in TCR recombination.[28] Nonetheless, acetylation is not sufficient to explain the selective chromatin accessibility for the recombination complex, as many other regions are hyperacetylated in the genome but do not recombine. Domain-specific H3K27 trimethylation by the HMT EZH2 also regulates this process,[29] as does the dimethylation of H3K4,[30] whereas dimethylation of H3K9 correlates inversely with somatic recombination.[31] It appears that a specific chromatin signature is needed to recruit the recombination machinery to these sites. A first round of transcription of the unrecombined gene also seems to be important for productive recombination; it is a means to label the region with transcription-specific histone marks.[32]

Finally, other processes related to the epigenetic machinery are needed for correct recombination, including the subnuclear relocation of Ig genes and locus contraction of immunoglobulins. In non-B cells, the IgH and the IgK loci are located in an extended conformation in the nuclear peripheral heterochromatin.[33] When B-cell differentiation begins, these loci move toward the centre of the nucleus. In committed pro-B cells, this region undergoes a long-range contraction that juxtaposes the recombining segments. These processes appear to be driven by specific B-cell differentiation nuclear factors, especially Pax5, because $Pax5^{-/-}$ pro-B cells are unable to contract the locus even if chromatin is accessible for recombination.[34] EZH2 is also necessary; it might mediate physical contact between distant regions by means of the notorious Polycomb repressive complexes, PRC2, which methylates H3K27 and PRC1, which might bind to this mark and mediate contraction of this region.[35]

Epigenetic Alterations in Immune Disorders

We showed some examples of how the epigenetic machinery is important for the correct establishment of cell identity and function. It should be now easy to understand how alterations in epigenetic processes give rise to misregulation of immune system function and incorrect recognition of self and nonself. A good example can be found in a disease that affects the epigenetic machinery and leads to marked immune alterations: the immunodeficiency, centromeric instability and facial abnormalities (ICF) syndrome, a rare autosomal recessive pathology caused by mutations in the gene encoding DNMT3B.[36] It is characterised clinically (among other symptoms that include facial dysmorphism, mental retardation and centromeric instability typically in chromosomes 1,9 and 16) by severe immunodeficiency that often leads to death before adulthood due to infection. These individuals have lymphocytes, but specific immunoglobulin isotypes are not produced correctly, so the lack of DNA methylation might generate a problem in late B-cell maturation and Ig isotype switch.

We have also begun to observe the importance of epigenetics in various other aspects of immune system pathology. With regard to self/nonself discrimination, immune system activation against self underlies many common pathologies categorised as autoimmune diseases (AID). In general, these are multifactorial diseases that develop due to a combination of genetic predisposition, environmental factors and, occasionally, as the result of a microbial infection that induces an abnormal immune response to self antigens.[37] Epigenetics has recently been implicated in the pathogenesis of systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) and less directly in Type 1 diabetes and multiple sclerosis. T cells from SLE and RA patients show a loss in global DNA methylation;[38] concomitantly, specific genes are hypomethylated and abnormally expressed in these cells. In $CD4^+$ T cells from SLE patients, for example, demethylation-mediated perforin overexpression could contribute to altered monocyte killing,[39] while increased expression of CD70 in the same cells drives overstimulation of B cells.[40] In vitro experiments also confirm the role of DNA methylation in autoimmunity, since $CD4^+$ T cells become autoreactive after treatment with DNA demethylating drugs such 5-azacytidine; these cells are activated even in the absence of a nonself peptide presented in the MHC class II complex on APC.[41] This could be due to hypomethylation-mediated overexpression of the integrin CD11a,[42] which drives T helper cell:APC interactions.[43] The mechanism for altered methylation in AID remains unknown; the discovery of a "DNA demethylase" would possibly shed new light on these questions.

Other evidence shows that histone modifications are implicated in AID pathogenesis. For instance, the autoimmune regulator (AIRE) is a transcription factor that interacts with unmethylated H3K4 to activate expression of specific genes and is known to be important in the clonal deletion of self-reactive T cells; its mutations cause autoimmune polyendocrinopathy syndrome Type 1 (APS-1).[44]

Escape of Cancer Cells and Embryos from Immune Nonself Recognition

The concept of self/nonself in immunology is challenged by the immune response to cancer. In the case of cancers whose first oncogenic step is infection by an oncovirus, metaplastic cells can be regarded as infected cells and express nonself antigens. In all other cases, cancer cells are self cells that can only be distinguished by certain patterns of molecular mutation or altered expression. To eliminate the cancer, the immune system

must be able to recognise these altered patterns in a self context. From another viewpoint, a successful cancer must escape immune system recognition, basically in two ways: they hide these altered molecules by avoiding their presentation at the cell surface, or they inhibit the function of specific immune cell subsets that should recognise the malignant cell. Many tumours show impaired antigen presentation; MHC class I, the molecular complex responsible for antigen presentation in all cells and for activation of the $CD8^+$ T-cell cytotoxic response, is often downregulated in tumours. Mechanisms for this downregulation include total, haplotypic or allelic loss of the MHC heavy chain gene, as well as deletion or point mutations in β2-microglobulin (β2m) or other components of the antigen presentation machinery such as TAP1 and 2.[45] No structural gene defect is detectable in many tumours, however and gene repression is achieved by epigenetic mechanisms, generally DNA methylation or repressive histone modification. Promoter hypermethylation of the human MHC class I gene has been described in oesophageal squamous cell carcinoma[46] and treatment of melanomas with the DNA demethylating drug 5-aza-dC significantly upregulates MHC class I expression.[47] Similarly, expression of MHC class II is downregulated in leukaemic T cells; in this case, the mechanism is based on hypermethylation of the transactivator CIITA, essential for membrane expression of MHC class II.[48] Other mechanisms have been described for immune escape, which involve inhibition of cytotoxic immune function, some of which are also epigenetically regulated (reviewed in ref. 49). Epigenetic regulation of immune tolerance to cancer cells is of particular interest for its potential therapeutic applications. DNA demethylating drugs and HDAC inhibitors are currently used for cancer therapy or are in clinical trials, given their ability to restore the skewed expression of tumour suppressor genes. If a clear role is defined for these drugs in restoring tumour immunogenicity, the possibility of combining such treatments with immunotherapy could open new avenues in the fight against cancer.

A very similar process, albeit physiological, occurs in embryonic and extraembryonic tissues in pregnancy; to escape the maternal immune response, the embryo must hide its antigenic self-identity. Epigenetics is again involved in this process, as demonstrated by studies in human embryonic stem cells (hESC) and trophoblast. Trophoblast cells achieve immune tolerance by epigenetic repression of classical MHC class I (HLA-A, -B) and MHC class II[50] and by expressing nonclassical HLA genes such as HLA-G and E, which inhibit NK cell function.[51-52] hESC also downregulate expression of these genes by epigenetic repression of *TAP-1*, *TAP-2, TPN* and the *β2m* gene; in addition, hESC repress NK recognition through expression of the NKG2D ligands MICA, MICB and ULBP (Suárez-Álvarez et al unpublished results).

NERVOUS SYSTEM: SELF-CONSCIOUSNESS AND SELF-IDENTITY

The complexity of the human brain allows the development of sophisticated behavioural skills such as language, tool use, symbolic thought, cultural learning and, of course, self-consciousness. This last concept has been discussed intensely by psychologists, neuroscientists and philosophers and has been dissected at different levels and to different degrees. In general, we can consider self-consciousness as one's ability to be aware of oneself as a "subject" and of one's own states, physical and mental. In language-competent beings, it can be translated into the ability to refer to oneself in first person. There is controversy as to when and how this skill was acquired in evolution and to what extent any living organism can somehow be conscious and able to differentiate

itself from other beings. Evidence from embryology and genetics of the brain as well as from psychological studies of human behaviour and learning, especially in human infants, indicates that the capacity for cooperative imagination and joint interest in objects and tasks is determined long before birth by expression of genes and epigenetic elaboration of neural systems. These original innate mechanisms in the embryonic brain are needed later for generating intelligent exploration of the environment and for the emergence of an additional dialogic mechanism that represents the self-subject, as opposed to other-subjects. Recent imaging studies in humans[53-54] show that brain regions located in the subcortical midline form a highly wired network directly involved in self-related processing. Apart from anatomical data, however, we cannot describe any complete molecular mechanism for the establishment of self-consciousness, due to the extreme difficulty in the biological analysis of higher brain functions. Data from basic developmental biology, from animal models and from human psychiatric dysfunctions that interfere with self representation, behaviour and emotions, hint at the importance of epigenetics in these superior functions, both in cells (in neuron differentiation and generation of neural diversity) and in systems (during establishment of neural connections in the brain).

Epigenetics of Neural Differentiation

As in the case of the immune system, epigenetics plays a major role in the correct anatomical and functional development of the nervous system. All nervous functions are mediated by highly specialised cells, the neurons and by the highly wired web of contacts that they establish during ontogenic development. Epigenetics is one of the main mechanisms that mediate lineage specification and the creation of the enormous variety that characterises cells in this system (Fig. 2).

Neural precursor cells (NPC) are defined as cells with the capacity to self-renew and to generate the three major CNS cell types: neurons, astrocytes and oligodendrocytes.

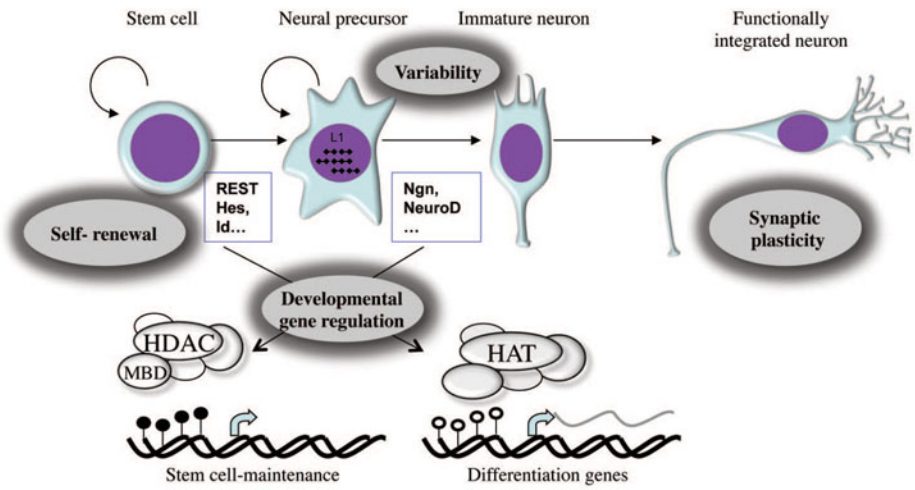

Figure 2. Epigenetics of neuron differentiation. Steps in which epigenetic mechanisms are involved are indicated in shadowed ovals.

Only small NPC populations, called adult neural stem cells, have been found to persist to adult life and are located in two principal neurogenic regions, the subgranular zone in the dentate gyrus of the hippocampus and the subventricular zone.

Terminal differentiation of neurons during development requires the simultaneous upregulation of neurogenic factors and downregulation of antineural factors, accompanied by the appropriate pattern of epigenetic modifications. For a cell at the NPC stage, the main objectives are maintenance of multipotency and the correct induction of neural or glial differentiation at the appropriate time. Stem cells are characterised by a specific epigenetic signature; in hESC, this is termed the bivalent domain and is obtained by the concomitant presence on many promoters of developmental genes of both a repression (3me-H3K27) and an activation mark (3me-H3K4);[55] these genes are silenced but poised for activation at later stages. Upon differentiation, genes essential for the specific differentiation induced are enriched in the activation mark, while those for other differentiation programs are definitively repressed. This gives an idea of the importance of epigenetic regulation in gene orchestration during differentiation; similar conditions prevail for NPC differentiating into neurons. As we described for the immune system, the process of neurogenic differentiation is driven by the stepwise activation of transcription factors that work together with the epigenetic machinery to regulate the activation of specific sets of developmental genes, both spatially and temporally.

Factors in charge of maintaining the stem cell state by inhibiting neural differentiation involve mainly bHLH (basic-helix-loop-helix) proteins, members of the Hes and Id families, which are highly expressed in NPC. The Hes proteins bind to short DNA sequences called N-boxes in promoters of neural differentiation genes such as *Mash1* and the neurogenins. They interact with HDAC directly or indirectly through corepressors such as Gro/TLE1,[56] inducing histone hypoacetylation and transcriptional repression, thus preventing precocious neurogenesis.[57-58] Another important system is that based on REST/NRSF.[59] This factor binds to its response element in promoters of neuron-specific genes including *Mash1* and *Bdnf*. It recruits a large complex of enzymes that includes HDAC, histone demethylases (LSD1), corepressors such as coREST and BRAF35, the methyl-CpG-binding protein MeCP2 and a potent chromatin remodelling enzyme called Brg1[60] and induces a strong repressive structure in chromatin. The REST complex silences neural-specific genes in both NPC and nonneural cells, although the nature of the repressive complex differs according to cell type, as does the degree of promoter inactivation. REST complex-bound promoters in nonneural differentiated cells are strongly repressed, presenting heterochromatin features such as methylated H3K9 and DNA methylation.[61] In NPC in which these genes are also silenced, these "strong" inactivation marks are absent and the coexistence of activation marks (3me-H3K4) has been observed.[59] This bivalent state could resemble that described for hESC, as these genes are repressed in NPC but poised for activation, since these precursors retain the possibility to become neurons whereas other differentiated cells cannot.[62]

When the neural differentiation program is switched on, several changes are observed in these cells, in the epigenetic machinery as well as in regulatory mechanisms such as alternative splicing, with the objective of turning the undifferentiated cell into a neuron. The basic mechanisms are again the downregulation of stem cell maintenance factors and the upregulation of neurogenic factors. Downregulation of REST is essential[63] and occurs in different ways including inhibition of synthesis and degradation. A switch is also observed in the composition of the REST complex; a noncoding dsRNA, termed NRSE, is produced at the time of neurogenesis and associates to the REST complex.[64]

This event converts the enzyme complex from a repressor to an activator; MeCP2 and HDAC leave the complex and histone acetylation increases, the corepressor BRAF35 is replaced by iBRAF,[65] which recruits histone methylase MLL, responsible for the addition of the gene activation-associated histone mark 3meH3K4 to these promoters. In this way, proneural bHLH including neurogenins, Mash1 and NeuroD are induced and can heterodimerise with the ubiquitous factor E2A to bind their response element (E-boxes). This initiates the proneural transcription program.[66-67]

The proneural transcription program is based on the same regulatory mechanism as in NPC and in fact shares many common epigenetic factors with the previous pathway, but on different promoters. Brg1, which in NPC is associated to the REST complex, in neurons is reported to interact with certain neurogenic TF such Ngn1 or NeuroD.[68] Some chromatin remodelling complexes adapt to the change of transcriptional program by a slight change in composition: the SWI/SNF-like BAF chromatin remodelling complex replaces the subunits BAF45a and 53a in NPC with BAF45b and 45c in neurons, in this way adjusting its activity and specificity.[69] These are only a few of the molecular mechanisms in early neurogenic differentiation; these examples help understand the importance of the epigenetic machinery in neural differentiation. A small defect in one of these systems can alter the correct timing and location of differentiation and is detrimental for the establishment of the neural networks that create the high functions of the human brain.

Generation of Neural Complexity

Human brain is constituted of around 10^{12} neurons, which form approximately 10^{15} specialised functional connections termed synapses.[70] The high degree of neuron heterogeneity and that of their interconnections makes each individual unique and unrepeatable; it is the product of the ability of these cells to generate this variability and organise a complex selective process. Neurons formed in embryonic life are in principle genetically equal; nonetheless, at the end of CNS formation a considerable variety of neurons can be distinguished.

It is estimated that only 15-40% of mature neurons survive to adult life,[71] indicating an ongoing selective process similar to that in the immune system. In lymphocyte V(D)J recombination and selection, diversity is created genetically and stochastically by somatic recombination, but many attempts to define a similar process in neurons have been unsuccessful. Other mechanisms, genetic and epigenetic, appear to drive the generation of this diversity. For example, roughly 33% of NPC in mouse brain display chromosomal aneuploidy,[72-73] that is, the loss or gain of at least one entire chromosome. These cells can mature correctly into neurons or glial cells and integrate functionally in neural circuits;[74] these neurons will obviously have considerable differences in gene expression compared to euploid cells. Another recently discovered mechanism that generates genetic diversity in the neuron population is the retrotransposition of the long interspersed element-1 (LINE-1) in NPC. Demonstrated in rat[75] and confirmed in humans,[76] these 6kb retrotransposons are activated by downregulation of the LINE-1 repressor factor Sox2 during neural differentiation and by the loss of methylation in the LINE-1 UTR. Widespread de novo retrotransposition is then observed in the genome, generating insertional mutagenesis or deregulation of genes. These events occur with certain specificity near neuronal genes such as ion channel-associated genes and neuron receptors. As the LINE-1 recombination protein machinery can function in trans, it can also mobilise other repetitive sequences such as Alu repeats and cellular RNA, creating

processed pseudogenes. In general, these mechanisms contribute to the constitution of what is defined as somatic mosaicism.

Other nongenetic mechanisms of gene regulation have been implicated in the generation of neural diversity. Well-known examples are alternative promoter usage, as is the case of the glutamate receptor ion channel genes[77] and the BDNF gene,[78] an important neurotrophic factor. Alternative splicing is also involved, as clearly shown by the classical example of the *DSCAM* gene (discussed elsewhere in this book). Events such as these are strictly dependent on the chromatin environment of the gene and are thus in some way connected to the epigenetic machinery. Other mechanisms such as RNA editing and posttranslational modification further contribute to enhance this diversity.

Epigenetics of Synaptic Plasticity

A general property of cells, especially of neurons, is their ability to store information over a long period of time, commonly referred to as cellular memory. This effect is achieved through the stable activation of a specific gene expression pattern that ensures maintenance of cell identity and dictates the pathways to be followed during differentiation. In neurons, this higher-level information, combined with its integration into intercellular networks, allows the more complex cognitive and behavioural functions. Yet environmental stimuli, injury and other factors show that even a committed cell can revert in its differentiation processes and to some extent establish new networks and functions. This cellular reprogramming process implies that a cell can maintain certain flexibility in its fate determination, a plasticity that enables it to react and adapt to external stimuli. This ability can be ascribed mainly to epigenetic mechanisms.

In a physiological context, many neurons must be able to reprogram their function in response to repeated patterns of synaptic transmission, creating a means of long-lasting information storage. Various forms of synaptic plasticity have been described at excitatory and inhibitory synapses: the best known are long-term potentiation (LTP) and long-term depression (LTD), whereby the efficacy of synaptic transmission is up- or downregulated, respectively. Certain forms of LTP and LTD are long-lived and are thought to be dependent on lasting changes in gene expression. Increasing evidence suggests that histone modifications might be involved in these processes. H4 acetylation at specific promoters in Aplysia neurons is altered after LTP and LTD[79] and HDAC inhibitors promote LTP in mammalian neurons.[80] In addition, during synaptic transmission, neurotransmitters trigger responses in target neurons by activating receptors such as ligand-gated ion channels and G protein-coupled receptors, which can trigger more long-lasting effects than simple synaptic transmission. These effects include changes in gene expression via control of transcription and thereby, chromatin remodelling. An example involves the transcription factor CREB which, once activated by signalling-induced phosphorylation, recruits CREB-binding protein (CBP), a coactivator with intrinsic HAT activity.[81] An increase in cytoplasmic Ca^{2+} levels also activates Ca^{2+}/calmodulin-dependent kinases, which phosphorylate class II HDAC. This phosphorylation provides a docking site for 14-3-3 protein, which mediates export of phosphorylated HDAC from the nucleus.[82]

Synaptic activity is also reported to influence DNA methylation, which undergoes rapid and dynamic regulation in the nervous system;[83] this finding was unexpected, as methylation was classically considered a very stable modification. An example is the

activity-dependent control of *Bdnf* gene expression, which correlates with reduced DNA methylation and release of a repressor complex comprising methyl-CpG-binding protein 2 (MeCP2).[84] It is proposed that neural activity, increased cell Ca^{2+} levels and activation of Ca^{2+}/calmodulin kinases lead to MeCP2 phosphorylation and release from the Bdnf promoter. This induces Bdnf expression and concomitant dendritic outgrowth.[85]

Consistent with the coupling of histone modifications with synaptic plasticity, there are numerous reports of the importance of these modifications in behavioural memory. CBP-deficient mice show memory deficits; administration of an HDAC inhibitor can restore normal long-term memory formation in these mutants and even enhance it in normal animals.[80] Contextual fear conditioning, a classical model for the study of memory formation in animals, increases H3S10-K14 phospho-acetylation levels in the CA1 area of the hippocampus without affecting H4 acetylation.[86] In addition, it induces Dnmt3A and -3B expression in CA1 neurons and administration of DNMT inhibitors blocks induction of both contextual fear conditioning[87] and hippocampal LTP.[83] It nonetheless remains unknown how these drugs, thought to regulate DNA methylation in dividing cells only, affect gene expression in mature neurons. Fear conditioning causes rapid methylation and silencing of the protein phosphatase 1 (Pp1) gene promoter,[87] important for LTP and memory formation. Interestingly, fear conditioning also induces demethylation of the reelin promoter,[88] indicating that both DNA methylation and demethylation might be highly regulated in the adult brain.

Epigenetic Alterations in Neuro-Psychiatric Disorders

A confirmation of the importance of epigenetics in the brain and its relevance for the establishment of self-consciousness in the human brain derives from molecular psychiatry. Given the involvement of epigenetic mechanisms in nervous system function, it is not surprising that a growing number of disorders, including mental retardation and autism spectrum syndromes, are linked to chromatin remodelling defects. There are well known epigenetic alterations that have profound consequences on the correct establishment of these higher functions, leading to mental retardation and behavioural problems.[89-90]

The best-studied "epigenetic disease" associated with altered neurological function is Rett syndrome, a relatively common (1:10000-15000 prevalence in the USA) X-linked postnatal autism spectrum disorder characterized by microencephaly, with motor, learning and social abnormalities that generally worsen with time. Candidate gene analyses identified *MeCP2* as the causative gene. MeCP2 is one of the MBD-containing proteins described as the methyl-CpG "reader"; it binds selectively to methylated-CpG dinucleotides in heterochromatic regions, where it interacts with corepressors and HDAC. In mouse, brain-specific deletion of MeCP2 mimics Rett symptoms. Moreover, MeCP2 directly influences cognitive functions: MeCP2 deficiency increases anxiety and, in mice bearing a truncated endogenous MeCP2 form, mild overexpression of wild-type MeCP2 enhances synaptic plasticity in the hippocampus and improves spatial memory.[94]

In Rubinstein-Taybi syndrome, a rare congenital disorder (1:100,000 prevalence in the US) characterized by mental retardation and developmental abnormalities, causative mutations map to the *CBP* gene and can result in impairment of HAT activity.[95] Mice haploinsufficient for *CBP* show impaired cognitive function, altered neuron plasticity and aberrant histone acetylation at target promoters;[96] these behavioural symptoms can be ameliorated by administration of HDAC inhibitors.[97]

Coffin-Lowry syndrome, a type of human mental retardation,[98] is another genetic disease involving Rsk2, a kinase responsible for H3S10 phosphorylation. Chromatin changes, in part brought about by H3S10 phosphorylation, have been directly demonstrated in the hippocampal neurons of these patients.[99]

Besides these genetic syndromes involving epigenetic components, a role for epigenetics is being highlighted in other psychiatric disorders. In general terms, psychiatric diseases include disorders such as drug addiction, depression, bipolar disease and schizophrenia. A common attribute of psychiatric disorders is the long-term nature of the behavioural anomalies; symptoms usually develop gradually and are long-lasting. Psychiatric drugs are unique in that chronic treatment is necessary for efficacy; their effects are not perceived for weeks or months after the beginning of treatment. These features suggest that both the pathologies and the therapeutic effect of psychiatric drugs are probably mediated by stable gene regulation changes, in all likelihood through the epigenetic machinery.

In schizophrenia, the extracellular matrix protein reelin is reported to show aberrant epigenetic repression. Reelin is a glycoprotein expressed normally by GABA-containing neurons; its promoter contains a large CpG island that regulates its expression. Postmortem studies showed that reelin expression is considerably repressed in different regions, with no association to neural loss.[100] Strikingly, drugs that enhance DNA methylation, such as SAM, can induce psychotic events in schizophrenic patients.[101] Other data that support an epigenetic role in this disease come from psychopharmacology; antipsychotic drugs such as haloperidol and raclopride (both dopamine receptor D2 antagonists) induce increased phospho-acetylation of H3 at a global level and of certain specific promoters like the c-fos gene.[102] Moreover, the well-known HDAC inhibitor valproate, used in psychiatry as an anticonvulsivant and mood stabiliser, increases reelin expression and diminishes methylation of its promoter. Co-administration of valproate with antipsychotic drugs accelerates the onset of their therapeutic action.[103] Other HDAC inhibitors have the same effect,[104] suggesting that crosstalk between DNA methylation and histone acetylation acts in the pathogenesis of the disease, even if the molecular mechanism is still unclear.

CONCLUSION: EPIGENOME, TECHNICAL ADVANCES AND APPLICATIONS

Until only a few years ago, epigenetic analysis was limited to studying a single mark in a single genome location at a time. Recent technological innovations such as microarrays and ultra-high-throughput sequencing have enabled description of the global epigenetic landscape, multiplying the amount of information that can be obtained from a given sample. These advances enable the organization epigenome projects, which aim to describe the totality of the epigenetic marks in a cell; these data will be useful to better understand normal processes such as development and abnormal processes like pathologies with an epigenetic component. Numerous limitations still curb scientists working in this field. As we have attempted to describe in this chapter, epigenetic marks different from one cell type to another as well as within each cell type, depending on the precise developmental stage. Further technical improvements are needed to refine the analysis of individual cells or homogeneous groups of cells. Finally, new possibilities are opening up that involve the use of imaging techniques in live whole organisms.[105] It is hoped that these techniques will provide new information about the epigenetic dynamics

of the living cell. In any case, the complexity of the systems such as those that regulate self-recognition, self-sensing and self-awareness is the major limitation to exploring the molecular mechanisms that drive these processes.

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CHAPTER 15

VIRAL IMMUNOMODULATORY PROTEINS: Usurping Host Genes as a Survival Strategy

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Abstract: Large DNA-containing viruses encode a plethora of gene products that are homologous to cellular proteins and key for their success in nature. During the millions of years of co-evolution with their respective hosts, viruses have extensively captured cellular genes, frequently duplicated them and insidiously shaped them to yield optimized specific molecules capable of either mimicking or interfering with the original host function and/or of executing novel tasks. A vast number of these gene products have become an integral part of the elaborated counteracting immune evasion strategies developed by viruses to withstand with the selective evolutionary pressure imposed by the host immune system. Cytomegaloviruses (CMVs) constitute an outstanding example of the many and varied encoded proteins directed to modulate both innate and adaptive immune responses, which determine their ability to establish life-long latency with sporadic shedding in their hosts. This chapter focuses on the current understanding of those genes encoded by human CMV (HCMV) with a known homology to cell proteins of the immune system. A systematic study of these genes, in addition to unraveling specific virus-host interactions, may supply valuable tools to dissect the molecular basis of immune responses.

INTRODUCTION

Large genome DNA viruses, such as poxviruses and herpesviruses, in addition to encode proteins required for replication of the viral DNA and their assembly into virions, they also bear genes encoding a number of proteins that are not essential for in vitro replication and which are primarily involved in direct interaction with the host. Most of these genes, which can constitute more than 50% of the pathogen-coding capacity, are believed to

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have undergone capture by the viruses during co-evolution with their hosts.[1] The fact that gene capture has been a major hallmark of large DNA viruses (ranging from 100 kb to more than 200 kb genomes) evolution is apparent by the abundance of viral proteins that are homologues to cellular proteins. For example, approximately 13% of the herpesvirus proteins have clear sequence similarities to products of their host genomes.[2] The estimated evolutionary rate of large DNA viral genomes is roughly more than 10 fold higher than that of the host genomes.[3] Thus, the relative number of captured genes may be significantly higher since many of these genes have evolved a long way from their original cellular counterparts, rendering any degree of relatedness almost undetectable. Phylogenetic studies in large DNA viruses indicate that host genes have been acquired at widely different times during evolution. While certain genes may have been captured early in their evolutionary history since they are common in most members of a viral family, others appear to have been acquired much more recently, based on the fact that they are present only in viruses belonging to a unique lineage or are capable of infecting a single species.[4,5]

Various mechanisms that might account for cellular gene acquisition by viruses, also known as horizontal transfer, have been postulated. The most likely one is believed to involve the recombination of viral genomes with cDNA copies of cellular transcripts. The reverse transcriptase required for this process could presumably derive from the host cells co-infected with retroviruses.[6] This genetic mechanism is supported by the fact that, unlike their cellular counterparts, a vast number of captured genes are encoded by exons without introns, which would have arisen from insertion of intron-less cDNA copies of spliced RNAs. The presence of introns in some captured genes would indicate that the inserted cDNA may, in fact, derive from a partially spliced RNA.[7] An alternative mechanism, at least in large DNA viruses that replicate in the nucleus, involves the acquisition of cellular genes via the capture of host genomic DNA. This is supported by the observation that some virally encoded genes retain the exact intact cellular gene structure of introns and exons.[8]

In some instances, stolen gene products may maintain the same functionality of the original ancestral host. In most cases, however, they diverge and though they may retain some fundamental structural characteristics of the host homologue, they also gain one or more novel functions that confer additional advantages to ensure pathogen survival.[9] Yet another potential mechanism for generating new protein functions is gene duplication with subsequent divergence from the original captured gene. This process often results in families of related genes, which in many cases remain clustered together within the viral genome. Finally, viruses may become equipped with novel functions through genetic capture from other viruses; a process termed lateral transfer.[9] Given the strong evolutionary pressures viruses encounter, as well as their genome size constrains, any superfluous incorporated genes that do not enhance their fitness are most likely rapidly discarded.

Succesful identification of cellular gene homologues encoded by viruses greatly depends on the methods used to scrutinize and compare the viral and cellular genomes. In addition, homologies are not always found throughout the entire protein, but are instead restricted to specific domains. In general, a virally encoded cellular gene homologue will be considered as such when containing a significant amino acid sequence homology to a cellular protein or when possessing a highly conserved amino acid motif. Homologues of cellular proteins found in viruses include enzymes associated with nucleic acid metabolism and replication, proteins involved in apoptopsis regulation and cell cycle control mechanisms, although a large number of them have also been implicated in different aspects of the immune defense.[10,11]

This chapter focuses primarily on those proteins encoded by human cytomegalovirus (HCMV) that bear a known homology to cellular proteins associated with immune responses. Examples of specific animal cytomegalovirus (CMV) proteins have been included, particularly when they either provide relevant in vivo information or their mechanism of action differs from their HCMV counterparts.

CYTOMEGALOVIRUSES

CMVs, the principal members of the betaherpesvirus subfamily, are ubiquitous, highly species-specific pathogens, which generally establish asymptomatic infections in immunocompetent hosts.[12] Although CMVs elicit an immune response, this response is typically unable to thoroughly clear the infection, thus allowing the virus to persist in the host in a latent state. Recurrence from latency as well as primary infection of HCMV (also referred as to human herpesvirus-5 [HHV-5]) constitute a significant cause of morbidity and mortality in immature or immunocompromised host; e.g., transplant recipients and AIDS patients. In order to ensure their survival, particularly in the face of an active immune system, HCMV has developed various ways of modulating both the innate and adaptive arms of the immune system. Thus, HCMV encodes an elaborate array of proteins directed to block antigen presentation, interfere with NK cellular responses, disrupt cytokine/chemokine-signaling networks, evade antibody recognition and inhibit apoptosis.[13,14] Among these proteins, several have been identified as cellular homologues (Table 1).[10,11,15] Recently, a HCMV noncoding microRNA was also reported to subvert NK activation.[16] The redundant mechanisms, employed by HCMV to counteract a number of immune molecular processes, emphasize the importance of such responses in viral control.

The lytic growth cycle of HCMV is relatively slow, lasting around three days. Following cell entry, HCMV capsids travel to the nucleus where they release their genomes with viral transcription and replication then taking place.[12] HCMV genes are expressed in a temporally regulated transcription cascade, which leads to the synthesis of three classes of viral proteins designated as immediate early, early and late. The viral DNA is encapsidated and the infectious virus is then released from the host cell. HCMV particles contain the linear genome enclosed in an icosahedral capsid, surrounded by a layer of proteins termed tegument.[17] This, in turn is bound by an outer lipid envelope with glycoproteins. HCMV exhibits a broad cell tropism during natural infection, with epithelial cells, endothelial cells, fibroblasts and smooth muscle cells being the major targets for viral replication.[18] Myelomonocytic cells provide an important reservoir for latent infection. As studies examining HCMV in human subjects have obvious limitations and since the virus cannot infect experimental animals, rodent CMVs (in particular murine CMV-MCMV) have been extensively used to analyze virus-host interactions.[19]

With a complex linear double-stranded DNA genome of approximately 230 kb, encoding more than 200 predicted ORFs, HCMV is the largest of the known human viruses.[20] The genome is comprised of two regions with unique sequences (unique long [UL] and unique short [US]) flanked by repeated segments located either at the end of the complete genome (TR_L and TR_S), or internally at the joining point of the two unique components (IR_L and IR_S). Compared to the reference HCMV strain AD169, clinical isolates contain a region of around 15 kbp near the edge of the UL sequence (referred to

Table 1. Genes encoded by HCMV with known homology to cell proteins related to immunity

HCMV Gene	Cellular Gene Family	Closest Cellular Homologue	Identity*	Mechanism of Action	Immunomodulatory Function
UL18	MHC	MHC class I	28%	Binds to LIR-1	Interferes with NK recognition
UL142	MHC	MHC class I	Limited identity	Downregulates MICA	Inhibits NK cytotoxicity
UL144	TNFRSF	HVEM	33%	Binds to BTLA	Blocks T-cell proliferation
UL111A (cmvIL-10)	Cytokine	IL-10	25%	Binds to IL-10R	Inhibits lymphocyte proliferation and production of proinflammatory cytokines; downregulates MHC class I and II expression; inhibits DC maduration; stimulates B cells
UL111A (LacmvIL-10)	Cytokine	IL-10	25%	Binds to human IL-10 receptor	Downregulates MHC class II expression
UL146	Chemokine	CXCL1	25%	Binds to CXCR2	Attracts neutrophils
UL147	Chemokine	CXCL1	29%	Unknown	Unknown
UL128	Chemokine		Limited identity	Forms a complex with gH and gL in virions	Determines endothelial, leukocyte and dendritic cell tropism
US28	Chemokine receptor	CCR1	38%	Binds to a number of CC chemokines and CX3CL1; constitutive signaling	Sequesters CC chemokines; promotes smooth muscle cell migration
US27	Chemokine receptor	CCR1	25%	No ligands identified; no constituve or ligand induced signaling	Unknown
UL33	Chemokine receptor	CCR1	24%	No ligands identified; constitutive signaling	Unknown
UL78	Opioid receptor	OPRL1	23%	No ligands identified; no constituve or ligand induced signaling	Unknown
TRL11/IRL11	Immnunoglobulin SF	FcγRI	12%	Binds to IgG	Unknown
UL119/UL118	Immunoglobulin SF	FcγRI	20%	Binds to IgG	Unknown

*BLAST with ORFs in the HCMV Toledo strain (except UL33 AD169)

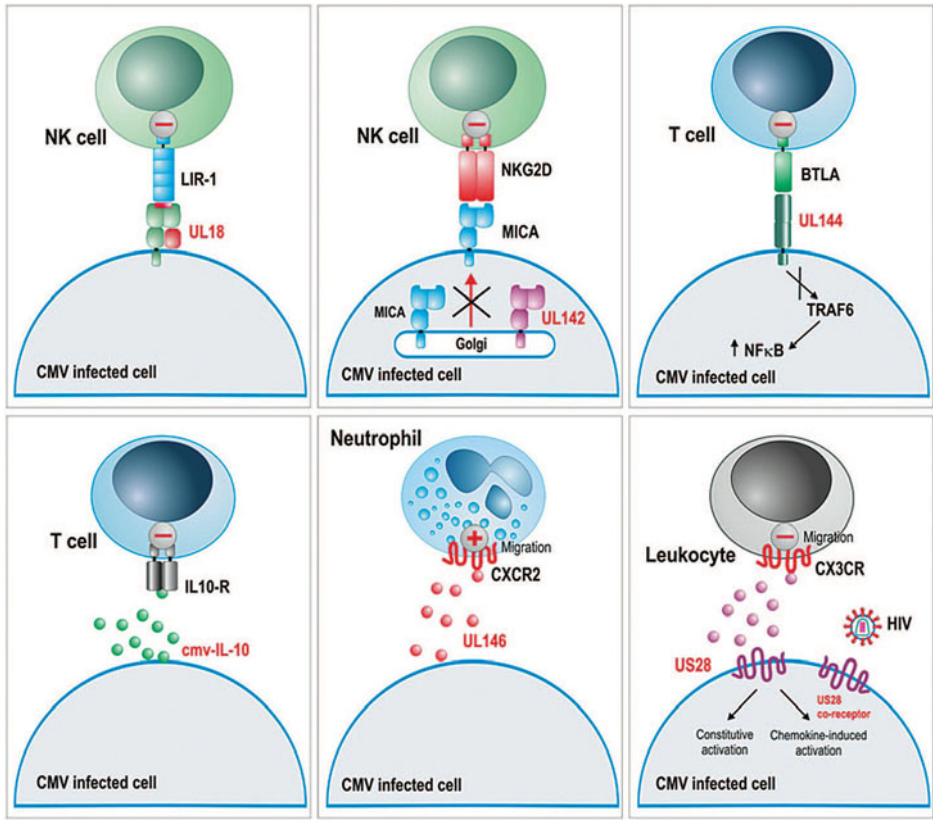

Figure 1. Immune modulation by HCMV-encoded cellular homologues. Some examples of cellular homologues expressed by HCMV infected cells that interfere with the host immune response are shown. UL18 (upper-left panel), UL142 (upper-centre panel), UL144 (upper-right panel), cmvIL-10 (lower-left panel), UL146(lower-centre panel), and US28 (lower-right panel).

as UL/b´) encoding at least 19 ORFs which are absent in the laboratory-adapted strains extensively passaged in cell culture.[21] ORFs are designated by letters indicating their location within the unique and repeated regions of the genome and are sequentially numbered. To date, the precise function(s) of a great proportion of the HCMV gene products remains unknown. A set of 46 core genes, which primarily encode essential functions conserved among all herpesviruses (e.g., those involved in DNA replication, packaging and processing), resides in the central part of the genome within the UL segment. By contrast, the terminal regions exhibit a significant divergence among different isolates, tending to harbor CMV-specific genes that are non-essential for replication in tissue culture and which are mainly devoted to interaction with the host, particularly in the modulation of innate and adaptive immune responses. Nine related gene families have been identified in HCMV, generally appearing as tandemly repeated copies as a result of gene duplication and subsequent divergence.[22]

MHC CLASS I HOMOLOGUES

MHC class I molecules play a central role in the initiation of adaptative immune responses to infection by presenting foreign peptides to cytotoxic CD8 T-lymphocytes (CTLs). Classical MHC class I molecules consist of a glycosylated transmembrane heavy chain with three extracellular domains (α1, α2, and α3) and the β2-microglobulin (β2m) light chain. Viral infected cells express on their cell surface MHC class I molecules carrying 8 to 11 viral peptides, which are recognized by CTLs through their antigen-specific receptor (TCR). After antigen recognition, activated CTLs proliferate and differentiate into cytotoxic cells, resulting in the eradication of the infected cells and hindering subsequent propagation of the virus. Thus, as with many other viruses, the evolution of HCMV has empowered it to employ a variety of strategies to down-modulate MHC class I expression in order to avoid CTL-mediated lysis.[23,24] However, NK cells, which do not express TCRs, are able to kill viral infected cells presenting low levels of MHC class I, since these molecules also act as ligands for NK inhibitory receptors. Therefore, to avoid NK killing, HCMV encodes molecules capable of directly engaging inhibitory receptors or of impeding the triggering of activating receptors on NK cells.[25-27] Additionally, specific HCMV proteins down-regulate the cell-surface expression of stress-induced ligands that activate NK cells. As part of these tactics, HCMV encodes structural homologues of MHC class I-like molecules.

UL18

UL18 is an MHC class I homologue that was identified in the HCMV laboratory strain AD169 around 20 years ago.[28] It is a Type I transmembrane protein, whose extracellular region shares 28% amino acid sequence identity, that covers the three α1, α2, and α3 domains, with the extracellular regions of human MHC class I (Fig. 1). Like classical MHC class I molecules, UL18 associates with β2m and present endogenous peptides.[29,30] This later characteristic makes UL18 unique among viral MHC class I homologues. However, in contrast to MHC class I molecules which contain one N-glycosylation site, UL18 is highly glycosylated, with 13 potential N-glycosylation sites.[28] All HCMVs analyzed thus far contain the UL18 gene and this protein is conserved in Chimpanzee CMV (chCMV).[20] UL18 has been reported to both activate and inhibit NK cell functions.[31-34] Its inhibitory properties are dependent on the interaction that occurs through its α3 domain with the leukocyte immunoglobulin-like receptor (LIR-1, also known as ILT-2 or CD85j).[35] This inhibitory receptor binds a broad range of classical and nonclassical MHC class I molecules and is mostly present on monocytes, dendritic and B lymphoid cell types and subsets of NK and T cells. UL18 binds to LIR-1 with 1.000-fold-higher affinity than MHC class I molecules.[36,37] In addition, genetic variations of UL18 in HCMV genomes result in marked differences in the avidity of binding to LIR-1.[38,39] The mechanism by which UL18 enhances NK cell responses remains to be elucidated. Finally, UL18 has also been reported to play a role in T-cell stimulation.[40] Despite the intensive research carried out on UL18, at present there is still no clear understanding of the activatory and inhibitory activities displayed by this viral protein in the context of the infection. Levels of UL18 expression, its cellular localization and the receptor repertoire of the target cell

have all been proposed as determinants of the different functions that UL18 may play in immune modulation.[41] The possibility that UL18 interacts with another unknown receptor on NK or T cells remains an open question.

UL142

HCMV encodes an additional MHC class I related protein, UL142, within its UL/b region present in clinical isolates and low passage strains.[42] UL142 possesses MHC class I-related α1 and α2 domains, while it differs from classical MHC class I molecules in that its α3 domain is truncated and that it is extensively glycosylated. UL142 inhibits NK cell cytotoxicity by downregulating the cell-surface expression of the MHC class I-related chain MICA, a stress-inducible ligand of the activating NK cell receptor NKG2D that can also be found on CD8 T cells as well as in certain T-cell subsets (Fig. 1). MICA exhibits a substantial sequence polymorphism and it has been shown that UL142 discriminates among MICA alleles.[43] A recent study indicates that UL142 acts by retaining full-length MICA alleles in the cis-Golgi.[44] The fact that UL142 is not present in HCMV laboratory-adapted strains that nevertheless retain the capacity to down-regulate MICA, reflects the functional redundancy exhibited by the virus to target specific immune pathways.

While not sharing the same relative genomic position or having a significant sequence similarity to HCMV UL18, MCMV also encodes an MHC class I homologue, m144.[45] The putative extracellular region of m144 displays a 29% aa- sequence identity to classical MHC class I molecules, with the predicted α1 and α3 domain structures of cellular MHC class I retained, but with the α2 domain containing considerable deletions. Although m144 can bind β2m, unlike UL18 it is unable to associate with endogenous peptides due to its truncated α2 domain.[46] These observations are supported by data obtained from the crystal structure of the UL144/β2m complex, the first structure of a viral MHC class I to be resolved.[47] That m144 contributes to immune modulation was clearly shown by its ability to interfere with NK recognition.[48,49] Indeed, a mutant MCMV with an m144 disruption exhibited severe attenuation during acute infection when compared to its wild-type counterpart, which could be reversed by NK cell depletion of the mouse.[48] In addition, expression of m144 in different cell types can confer partial resistance to NK cell cytotoxicity.[49,50] Although it has been suggested that m144 acts via the engagement of inhibitory NK receptors, neither the exact mechanism of action nor the nature of the putative receptor has been identified.

It is of interest that other members of the MCMV m145 family to which m144 belongs have also been predicted to contain an MHC class I structure and thus are thought to possess immunomodulatory functions.[51] Accordingly, four such MCMV m145 family members have been already reported to interfere with NK function. m157 has been shown to differentially bind to two NK receptors: to the activating Ly-49H receptor in MCMV resistant mouse strains and to the inhibitory Ly49-I receptor in sensitive murine strains.[52,53] m145, m152 and m155 down-regulate surface expression of the three distinct ligands of the activating NK cell receptor NKG2D: MULT-1, RAE-1 (which has five different isoforms) and H60 respectively.[54-57] Consequently, MCMV mutants with deletions in m145, m152, or m155 are associated with augmented virus sensitivity to NK control in vivo. An additional role for m152, retaining MHC class I molecules in the ER/Golgy compartment and inhibiting CTL lysis has also been reported.[58,59]

TNF RECEPTOR SUPERFAMILY HOMOLOGUES

TNF and TNF receptor (TNFR) molecules are two large superfamilies containing more than 30 receptors and 19 ligands.[60] Signals mediated through these receptor-ligand interactions modulate immune and inflammatory responses. Several TNFR cosignaling molecules have been shown to control T-cell activation by regulating T-cell proliferation, cytokine production, cytotoxicity, T-cell apoptosis and survival. Some TNFRs activate serine kinases, thereby promoting the expression of various survival and proinflammatory genes through the transcription factors NF-κB and AP-1, whereas other TNFRs induce apoptosis, negatively regulating T cells by cellular elimination.[61,62] Viruses can block TNF and TNF-mediated responses at multiple levels; e.g., by inhibiting the TNF ligand or its receptors, or by modulating key transduction molecules of the TNF signaling pathway.[63]

UL144

HCMV encodes a TNFR homolog, UL144, which is the only TNFR homolog identified thus far in herpesviruses. Although poxviruses encode decoy TNFRs that contain amino acid sequences similar to the extracellular TNF-binding domains of their cellular counterparts, they lack the transmembrane and cytoplasmic domains. As a result, they are secreted and are capable of neutralizing TNF-alpha activity.[64] In contrast to the poxvirus secreted TNFR homologues, UL144 encodes a Type I transmembrane protein with an ectodomain encompassing a leader peptide, a cysteine-rich region, a membrane extension region, a transmembrane domain and a short cytoplasmic tail. The UL144 ectodomain shows the highest amino acid sequence homology to the TNFR superfamily member HVEM (or Herpesvirus Entry Mediator, also known as TNFRSF14) (36%) followed by other members of this superfamily (Fas, 29%; TNFR-1, 28%; LTβR, 25%; TRAIL-R2, 15%).[65] HVEM can operate as a molecular switch that modulates T-cell activation by transmitting positive signals from the TNF-related ligands LIGHT and lymphotoxin , or inhibitory signals through the Ig superfamily member B and T-lymphocyte attenuator (BTLA).[66] Although the UL144 protein does not bind any known TNF ligands including LIGHT, similar to HVEM it does bind to BTLA (Fig. 1).[67] The incapacity of UL144 to interact with LIGHT highlights the refined adaptation process that has been attained by CMV during co-evolution with its host, usurping the negative cosignaling functions of the HVEM-BTLA interaction, while losing the positive costimulating signals of the HVEM-LIGHT interaction.[67] Binding of UL144 to BTLA blocks T-cell proliferation and reduces lymphocyte responses to HCMV, selectively mimicking the inhibitory cosignaling function of HVEM. A distinct function assigned to UL144 is to activate NF-κB by sequestering the TNFR-activator factor TRAF6, causing up-regulation of the chemokine CCL22 (MDC).[68] CCL22 is a chemoattractant of Th2 and regulatory T cells. Activation and attraction of these cells may aid HCMV in evading T-cell-mediated antiviral activity. Moreover, CCL22 also acts as a chemoattractant of those myeloid cells that may promote viral dissemination. UL144, like other genes located in the UL/b region, exhibits a high degree of amino acid sequence variation among clinical isolates and hence a number of studies have addressed the potential correlation of polymorphisms in this molecule and CMV pathogenesis.[69] In most cases, however, a clear association between genotypes of distinct HCMV strains

and disease outcome has not been observed. In this connection, it is noteworthy that despite the sequence variability found in the ectodomains, UL144 from different viral isolates binds with comparable affinities to BLTA.[70]

CYTOKINE HOMOLOGUES

Cytokines are secreted proteins that regulate a vast array of immune and inflammatory responses, of both the innate and the adaptive immune systems. Many viruses have developed strategies to antagonize cytokine activity over a range of different mechanisms. As part of these strategies, large DNA viruses—in addition to expressing cytokine receptors (as seen in the preceding section on HCMV) or cytokine-binding proteins-they are capable of secreting their own cytokines, also known as virokines.[64]

IL-10 is a cytokine with potent immunosuppressive functions, which include blocking the production of pro-inflammatory cytokines and down-regulation of MHC molecules and costimulatory molecules.[71] IL-10 is normally produced late after pathogen infection when it functions to attenuate the immune response. However, IL-10 has been shown to stimulate B-cell survival, proliferation and differentiation.[72] Herpesviruses and poxviruses encode interleukin-10 homologues, which in some instances (e.g., in the case of Epstein-Barr virus) can share over 80% identity with the cellular IL-10 molecule.[73] The fact that distinct viruses have independently captured host IL-10 genes supports the hypothesis that bearing an IL-10 molecule confers an advantage to the pathogen, plausibly steming from its ability to modulate cell-mediated immune responses and enhance viral dissemination and growth.[74]

UL111A

HCMV encompasses an IL-10 homologue, known as cvmIL-10; encoded by the UL111A gene, it is secreted during viral infection.[7] This viral gene product arises from a transcript consisting of three exons and two introns. cmvIL-10 has been shown to bind strongly to the IL-10 receptor despite sharing only 25% homology with human IL-10 (Fig. 1). In fact, cmvIL-10 binds to the IL-10 receptor with greater affinity than human IL-10 or the Epstein-Barr virus IL-10.[73] Therefore, it is not surprising that cmvIL-10 is as efficient as human IL-10 in suppressing proinflammatory cytokines, such as IL-1α, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF in peripheral blood mononuclear cells and monocytes.[75] In addition, a number of other modulatory activities (including both immunosuppressive and immunostimulatory) are shared, in a cell-type dependent manner, by cmvIL-10 and the human IL-10, some of which are outlined here. The CMV encoded IL-10 homologue is also able to alter dendritic cell maturation and prevent the up-regulation of the costimulatory molecules CD40, CD80, CD86, B7-DC and B7-H1.[76,77] Moreover, the synthesis of Type I INFs has been reported to be directly abrogated by cmvIL-10 in plasmocitoid dendritic cells. Another function assigned to this viral molecule is to induce B-cell growth and differentiation.[78] One recent study reported that cmvIL-10 suppressed CD4 T-cell recognition of latently infected myeloid progenitor cells in vitro, thus implying that this gene contributes to the persistence of HCMV in the immunocompromised host.[79]

An alternative spliced transcript from the UL111A gene, denoted as latency-associated LAcmvIL-10, has been detected during the latent phase of HCMV infection.[80] The repertoire of immunomodulatory properties of LAcmvIL-10 is however more restrictive than that of UL111A, retaining only its capacity to downregulate MHC class II expression.[81]

In evolutionary terms, it is worth noting the conservation of splicing patterns, which can even be identical, between certain virally encoded IL-10s and host IL-10. To some extent, this applies to UL111A; while bearing only three out of the five exons of the human IL-10, UL111A retains identical boundaries between exons. Homologues of IL-10 have also been identified in some primate CMV genomes (in rhesus macaque, African green monkey and baboon, but not in chimpanzee). The pronounced amino acid sequence divergence found not only between the CMV encoded IL-10 homologues and their corresponding host´s IL-10, but also among these proteins in even closely related CMVs (such as HCMV and Rhesus CMV), suggests that these specific captured events occurred early in the co-evolution of primates and their respective CMVs.

CHEMOKINE AND CHEMOKINE RECEPTORS HOMOLOGUES

Chemokines and their receptors play a key role in immune homeostasis regulating leukocyte migration, differentiation and function. More than 50 chemokines and 20 chemokine receptors have been described thus far.[82-85] Chemokines are small cytokines (8-10 kDa) that induce chemotaxis in leukocyte populations. They are divided into four subfamilies (CC, CXC, C and CX3C) based on the conserved cysteine motifs they possess. Chemokine receptors, which belong to the large family of 7-transmembrane G-protein-coupled receptors (GPCRs), propagate intracellular signals, such as calcium mobilization and phosphorylation of serine/threonine kinases, in response to chemokine binding. Chemokines not only mediate inflammatory processes by triggering the arrest and firm adhesion of leukocytes to the vascular wall, but they also stimulate the effector mechanisms of lymphocytes.

A large number of experiments using specific gene-deleted mice strains or blocking antibodies of chemokine system members have demonstrated that these are essential mediators of inflammation and important for the control of viral infections.[86] The existence of more than 30 known virally encoded chemokine and chemokine receptor mimics is the best indication that chemokines and their receptors are crucial for antiviral defense.[87] Among herpesviruses, genes encoding for proteins with homology to chemokines or chemokine receptors have been identified only in the β and γ herpesviruses subfamilies and not in the herpesviruses. HCMV encodes two known CXC-like chemokines, UL146 and UL147, as well as one CC-like chemokine, UL128. In addition, it harbors four chemokine receptors homologues: UL33, UL78, US27 and US28.

CHEMOKINE HOMOLOGUES

Viral-encoded chemokine homologues may exhibit two distinct properties: they can mimic the activity of the host chemokine by attracting selected leukocyte subsets through host chemokine receptors, thereby facilitating dissemination of the virus; or/and they can act as chemokine receptor antagonists.[88]

UL146 AND UL147

UL146 and UL147 share important characteristics with cellular CXC chemokines, including the spacing of the four conserved cysteines and the ELR-like motif known to be relevant in receptor binding and activation of neutrophils.[89] Consequently, the protein encoded by UL146 is a potent CXC-chemokine that binds with high affinity to the host CXCR2 receptor, efficiently stimulating calcium mobilization and chemotactic properties in a manner similar to those of the cellular CXCL1 (also known as GRO-alpha) (Fig. 1).[90] UL146 is able to attract neutrophils in vitro and inhibit apoptosis. The function of the closely related UL147 has not been independently studied and it is not known whether the two genes are functionally redundant. Work with HCMV mutants lacking both UL146 and UL147 indicates that either one or both of the proteins is necessary for efficient viral transmission to polymorphonuclear leukocytes, but not to monocytes.[91] Although UL146 and UL147 sequences from clinical HCMV isolates present high variability, no significant correlation between the polymorphisms in these molecules and disease outcome has as yet been appreciated.[92]

Phylogenetic studies suggest that HCMV UL146 and UL147 arose from a gene duplication event that most likely occurred subsequently after gene capture. UL146 and UL147 are present in chCMV together with another CXC chemokine-like protein (UL146A) and chCMV UL146 has been reported to have biological properties similar to that of its HCMV counterpart.[93] Interestingly, CXC chemokine-related genes appear to be restricted to simian CMVs. Moreover, detailed analysis of a number of primate CMV genomes indicates that they contain tandemly repeated gene clusters encoding a number of divergent CXC chemokine-like proteins along with GPCR–like protein clusters. These gene clusters appear to have considerable variation in CXC chemokine-like and GPCR-like protein copy numbers among the different CMVs. Based on the number and organization of these two cluster types in viral genomes (e.g., two, six and eight genes compose the CXC chemokine-like cluster in the owl monkey, rhesus and green monkey CMV respectively), the emerging picture is that the CXC chemokine-like gene cluster evolved from a CXCL chemokine (probably CXCL1) that was captured in an incomplete spliced form by an ancestor of Old and New World primate CMVs (more than 42 Mya), subsequently evolving via complex duplication and deletion events.[94] This same process of duplication and deletion that appears to be associated to the evolution of the viral CXC chemokine-like cluster may also have played a role in the generation of the human CXC cluster that comprises the nine human CXCL genes (including CXCL1 and IL-8) on chromosome 4.[94]

UL128

Although UL128 exhibits limited amino acid homology to CC-chemokines, it does display hallmarks of these molecules, containing four conserved cisteine residues.[95] The activities of this protein, including its potential chemotactic function, are not yet known. Independently of its possible role as a chemokine, however, UL128 has been implicated, together with the HCMV proteins UL130 and UL131A, in determining endothelial cell tropism, viral transmission to leukocytes and infection of dendritic cells.[91,95,96] UL128, UL130 and UL131A proteins form a molecular complex with the glycoproteins H (gH) and L (gL) and localize in virion particles mediating entry into cell types such as epithelial

and endothelial cells, and are not required for infection in fibroblasts. UL128 is highly conserved among clinical HCMV isolates, although it is disrupted in certain strains.[97,98]

The mouse CMV also bears a gene, m131/m129, encoding a protein with potent chemokine activity.[99] It has been found to induce calcium signalling and adherence in murine peritoneal macrophages.[100] In addition, human chemokine receptor CCR3-expressing cells as well as the human macrophage cell line THP1, have been shown to be responsive to this viral CC-chemokine homologue.[100] Moreover, using an MCMV with alterations in m131/m129 it has been demonstrated that the viral protein promotes not only inflammation at the site of inoculation, but also monocyte-associated viremia and subsequent dissemination of the virus to the mouse salivary glands.[99-101] Interestingly, a homologue of the CC chemokine MIP (macrophage inflammatory protein) has been found in guinea pig CMV, which is capable of signaling and inducing chemotaxis in cells expressing the human chemokine receptor CCR1.[102]

CHEMOKINE RECEPTOR HOMOLOGUES

Upon the onset of agonistic binding, the chemokine family of G-coupled receptors regulates a number of physiological processes by inducing signal transduction networks via activation of heterotrimeric G proteins. Chemokine receptors (CCR, CXCR, CR, or CX3CR) interact with a set of chemokines belonging to their respective subfamilies. A number of viruses encode cellular homologues of chemokine receptors which can alter host cell signaling pathways for the benefit of viral replication, dissemination and/or immune evasion. In contrast to their host counterparts, a remarkable trait of virus-encoded chemokine receptors is their capacity to bind a broader spectrum of chemokines. However, some of these viral molecules remain orphan receptors, as yet no chemokines have been reported to bind or modulate their activity. Nevertheless, such orphan receptors might be functional, since in many instances virally encoded chemokine receptors work in a ligand-independent manner by being constitutively activated.[103-106] Among the four chemokine receptor homologues encoded by HCMV, UL33 and UL78 appear to be present in all sequenced CMVs, presumably having being pirated from an ancient host by an ancestral CMV, while US27 and US28 are only encoded by primate CMVs, pointing to a more recent incorporation event.

US28

US28 is closely related to receptors for CC chemokine; indeed, it can bind a number of pro-inflammatory CC-chemokines (CCL5, CCL-3, CCL4 and CCL2), as well as to the C3X-chemokine CX3CL1 (Fig. 1).[107-110] Despite this broad spectrum of ligands, US28 presents high levels of constitutively activated signal transduction cascades, including phospholipase C, MAP kinase pathways and various transcription factors, such as NFκB and CREB. The intracellular carboxi-terminal tail of US28 is constitutively phosphorylated (a modification that requires ligand binding in most chemokine receptors) resulting in G protein stimulation and subsequent signaling.[104] In addition, ligand binding to US28 can also activate cell-type and ligand-specific signaling pathways. Therefore, a combination of agonist-dependent and agonist-independent signaling activity appears to be important for the biological effects of this molecule. In vitro studies have demonstrated roles played

by US28 that could potentially contribute to both immune evasion and viral dissemination in the host. US28 prevents leukocyte recruitment by degrading chemokines through constitutive endocytosis and recycling thereby acting as a chemokine decoy and scavenger receptor preventing the activation of endogenous host receptors.[111,112] It has been shown that ligand stimulation of US28 promotes smooth muscle cell migration.[113] This might lead to enhanced viral spread and reveal a molecular mechanism implicating HCMV in the development of vascular disease, such as arteriosclerosis.[114] In addition, expression of this viral protein has been shown to influence tumor formation by functioning as a viral oncogene.[115] Interestingly, US28 may serve as a coreceptor for HIV entry into cells and mediate cell fusion.[116,117]

US27

Considerably less is known about US27, a gene related and adjacent to US28. Although the similarities between these two proteins have led to the hypothesis that US27 is also a functional chemokine receptor no constitutive and/or ligand-induced signaling has been reported for this viral molecule.[118,119] US27 is expressed with late kinetics in the infected cell and is present in enveloped virus particles which suggests that this protein may play a role during the early stages of infection.[120,121] Dimerization of chemokine receptors has been documented to be a relevant event for signaling.[122] Hence, one possibility is that some of the viral encoded-chemokine receptor homologues might exert their actions by interacting with specific host G-coupled receptors.

UL33

Despite its sequence similarity to CC-chemokine receptors (with a high conservation of both individual amino acids and secondary structure distributed throughout the entire protein), UL33 does not appear to interact with chemokines. Thus, potential ligand(s) for this viral protein still need to be identified. However, UL33 has been shown to alter cellular signaling, including inositol phosphate production and activation of both NF-kB and the nuclear factor of activated T cells (NFAT), in a constitutive manner.[106] UL33 is expressed with late kinetics in infected cells and forms part of the viral envelope.[123] This protein is highly conserved among beta herpesviruses. In this regard, the murine and rat homologues of UL33, M33 and R33, respectively, have also been shown to display constitutive activity, although significant differences in the signaling mechanisms (mainly associated with the activation of distinct G protein types) between UL33 and its rodent homologs have been observed. In fact, M33 and R33 appear to activate signaling pathways similar to those activated by US28. Hence, it has been hypothesized that primate UL33-like proteins have lost some signaling properties as similar functions may have become redundant due to the presence of US28 genes.[124] Disruption of both R33 and M33 in their respective viruses results in severely attenuated viruses in vivo, unable to replicate in and disseminate to the salivary glands.[125,126] Thus, these results provide evidence that these proteins play a significant role in the pathogenesis of the infected host. Interestingly, it has been shown that HCMV UL33 was able to partially compensate for the lack of M33 in vivo, suggesting that the biological roles of these genes have been conserved.[127] It is worth noting, however, that M33 has been reported to bind to mouse

chemokine CCL5, inducing vascular smooth muscle cell migration and activating the small G protein Rac1, as well as the extracellular signal-related kinase.[128]

UL78

In contrast to the HCMV UL33, US27 and US28 proteins, UL78 has no significant sequence identity to chemokine receptors or any other GPCR, but does share some general conserved GPCR features. However, no constitutive and/or ligand-induced signaling has been reported for UL78. Hints as to the role of HCMV UL78 during viral infection come from UL78 homologues existing in other animal CMVs. In this regard, gene knockout experiments of the rodent CMV homologues M78 and R78 have shown that these genes contribute to the cell–cell spread of these viruses in vitro and to viral growth in target organs in the in vivo context.[129,130] In addition, the fact that M78 is present in virions has lead to the postulation that M78 may facilitate immediate-early mRNA accumulation.[130] It is noteworthy that UL78 counterparts in two other β-herpesviruses, human herpesvirus (HHV)-6 and HHV-7, which are encoded by U51, have been shown to bind a number of CC-chemokines.[131,132] Indeed, they are capable of activating or regulating signal transduction although this has not been demonstrated with any other UL78 viral homologues.[133] Thus, the most plausible scenario is that UL78-viral homologues might have evolved into disparate functions in their respective β-herpesvirus members.

Fc RECEPTOR HOMOLOGUES

Fc receptors (FcR) are molecules of the immunoglobulin superfamily present on the cell surface of most immune cells. They bind the Fc region of antibodies, triggering a number of effector mechanisms including NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, cytokine-release and/or the regulation of lymphocyte proliferation.[134] By connecting cell-mediated and humoral immune responses, FcRs participate in the host defense against viral infections. Thus, viruses have evolved mechanisms to modulate antibody responses via FcRs. In the case of HCMV, both infected cells and virions display Fc-binding activity and HCMV has been shown to encode proteins with homology to FcγRs.[135,136]

TRL11/IRL11 AND UL119-118

Two predicted Type I transmembrane glycoproteins encoded in the HCMV genome, TRL11/IRL11 and UL119-118 share structural characteristics with cellular Fcγ receptors, including the Ig-like domains with conserved key amino acid residues.[137,138] The closest cellular homologue of UL119-118 is FcγRI (20% identity with the third domain), while TRL11/IRL11 shows the highest level of homology to the second domain of the Fcγ receptor III (12% identity).[138] The lack of homology (7% identity) among both viral genes, as well as their disparate positions in the HCMV genome, most likely might indicate the occurrence of independent capture events during co-evolution with the host. Both gene products have been shown to bind human IgG with differential affinities depending on the

distinct isotype, but not IgA or IgM. Potentially, these viral Fc receptors could function by eliminating circulating anti-CMV antibodies transporting them from the cell surface to the endolysosomes for destruction. In addition, CMV Fc receptor homologues could be used by antibody-coated viral particles to enter cells contributing to virus dissemination. However, despite all these speculations, the functional role of these proteins still remains to be elucidated.

More enlightening thus far has been the IgG Fc-binding protein expressed by MCMV, m138.[139] m138 is a Type I glycoprotein that exhibits the same structural properties as cellular FcγR, although it does not share any homology with its counterparts in the HCMV genome. The in vivo relevance of this protein is supported by the impaired replication exhibited by an MCMV containing a mutation in m138. Surprisingly, however, m138´s mechanism of action has been shown to be independent of the humoral response due to the fact that reduced MCMV titers in different organs in the absence of m138 were seen in both wild-type and B-cell deficient mice.[140] Interestingly, m138 downregulates the cell-surface expression of three ligands of the activating NK receptor NKG2D: H60, RAE-1epsilon (one of the five RAE isoforms) and MULT-1. This is in agreement with the observation that the m138 MCMV mutant grows to levels comparable to that of wild-type MCMV in NK cell-depleted mice.[141,142] The downregulation of MULT-1 and H60 appears to involve two different domains at the N-terminal region of m138 and in the case of MULT-1 is caused by a faulty trafficking mechanism that leads to degradation of the protein in lysosomes.[141] m138 is also able to reduce cell-surface trafficking of the costimulatory molecule B7-1 (CD80) on dendritic cells, intercepting the capacity of these cells to activate CD8 T cells.[143] Thus, m138 is another excellent example of a CMV-encoded gene that exerts various independent immune evasion activities.

MOLECULAR MIMICRY AND AUTOIMMUNITY

The concept of molecular mimicry in viral infection was introduced in the 80´s by Fujinami and Oldstone.[144] They proposed that antigen-specific lymphocytes primed by microbe antigens could cross-react with self antigens, thereby achieving the potential to cause autoimmunity. A number of epidemiological, clinical and experimental studies now support an association between viral infections and autoimmune disease through the mechanism of molecular mimicry (for recent reviews see refs.145, 146). This could be of particular relevance for viruses such as HCMV or other herpesviruses due to their inherent capacity to remain life-long in their hosts. In that respect, HCMV has been implicated in the pathogenesis of a variety of autoimmune disorders. In theory, viral homologues of cellular genes may represent potential candidates for triggering autoimmunity via a process of molecular mimicry. Direct evidence for a molecular mimicry mechanism, by which antibodies against the HCMV protein (UL94) that recognize the cell-surface molecule NAG-2 cause endothelial cell damage and fibroblast activation in patients with systemic sclerosis, has been reported.[147,148] Moreover, in atherosclerosis, it has been shown that antibodies against the HCMV proteins UL122 and US28, which are capable of cross-reacting with the human heat-shock protein 60, can induce the apoptosis of nonstressed endothelial cells.[149] It must be pointed out, however, that molecular mimicry alone may not be capable of causing clinical disease and may do so only in conjunction with other factors, which could be provided by some nonspecific immunostimulation or by virally induced immune stimulation in genetically susceptible individuals.[150]

CONCLUSION AND FUTURE PROSPECTS

As reviewed here, part of the immune evasion strategies employed by CMVs are based on gene products with homology to host proteins, skilfully tailored during co-evolution to become sophisticated weapons with unique properties. The possibility to undertake more elaborated bioinformatic searches, together with the availability of increasing numbers of CMV sequences, might allow researchers to uncover camouflaged molecules that may have considerably diverged from the original captured gene, expanding the list of the identified host cell homologues encoded by these viruses. In this regard, additional products ressembling host proteins involved in immune responses have been distinguished in the HCMV genome although these await to be explored.[2,151] It must be noted however, that only a few of the CMV-encoded cellular homologues have been characterized with respect to their biological functions and mechanisms of action. Frequently, the properties of these viral molecules have been assessed using recombinant proteins and therefore the role they play during the viral life cycle remains unclear. Moreover, the strict species specificity associated with CMVs has hindered, in many cases, any analyses during viral pathogenesis. While in some instances this will prove a complicated undertaking, the combination of detailed clinical studies together with the availability of small animal models and advances in viral genetics, may aid future attempts to decipher their functions in vivo. The study of these immunomodulatory proteins is crucial not only for obtaining a better understanding of viral pathogenesis, but also for fully comprehending the critical host immunological pathways, goals which may well contribute to an elucidation of previously unknown cellular functions. Finally, serious consideration must be given to the potential use of these viral factors in the treatment of disease.

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CHAPTER 16

THE EMERGENCE OF THE MAJOR HISTOCOMPATILIBILITY COMPLEX

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Abstract: The Major Histocompatibility Complex (MHC) is a genomic region that contains genes that encode proteins involved with antigen presentation and, therefore, plays an important role in the adaptive immune system. The origin of these genes was probably an ancestral MHC that appeared before the emergence of the adaptive immune system and contained genes related to immunity. The organization of MHC genes varies in different groups of vertebrates; although, there are some characteristics that are maintained in all groups, which indicates that they confer some evolutionary advantage: Organization of the genes to form clusters and genetic polymorphisms. The study of how the MHC appeared during evolution and how it is organized in different species can help us clarify what features are essential in their participation in self-nonself recognition.

INTRODUCTION

One feature of the adaptive immune system is that it appears to have originated suddenly in a short period of time, during the emergence of the jawed vertebrates. All of the elements that define the adaptive immune system appeared during this period, including the T-cell receptor (TCR), Immunoglobulins (Ig) and the major histocompatibility complex (MHC). The MHC is a genomic region that contains genes that encode for the polymorphic class I and class II proteins, as well as other proteins related to the generation and transport of the peptides presented by class I molecules: Transporters associated with antigen processing (*TAP1* and *TAP2*), immunoproteasome components (*LMN2* and *LMP7*) and tapasin (*TAPBP*).

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T cells recognize foreign antigens found in infected cells, which are presented to them by polymorphic MHC molecules (class I or class II), by using TCRs that are also polymorphic. Apparently, there have been evolutionary pressures that, compared with other genes, made MHC genes evolve rapidly. The polymorphisms of these genes cause a diverse genetic susceptibility to infection; thus, individuals with different class I genes select different peptides to activate the immune system. This represents a population advantage by avoiding the possibility that a pathogen could kill an entire population. To elude their recognition by the immune system, many pathogens have developed strategies to interfere with class I presentation and, in this way, not be detected by T cells.[1]

To avoid this immune evasion, natural killer (NK) cells have receptors that recognize the altered expressions of class I molecules and, thus, allow them to distinguish infected from uninfected cells. In humans, this recognition occurs via the use of two types of class I receptors that are found in the NK cell membrane: CD94/NKG2A, which belongs to the C-type lectin-like family and killer-cell immunoglobulin-like receptors (KIRs), which belong to the immunoglobulin superfamily (IgSF). KIRs are polymorphic and bind directly to class I molecules.[2,3] The study of the KIR and MHC genes in apes has revealed that the interaction of these two polymorphic molecules was another mechanism that triggered the rapid evolution of the MHC in this group of animals.[4]

WHAT IS THE MHC?

We can describe the human leukocyte antigen (HLA) system, the human MHC, by considering the characteristics it shares with the MHCs found in other animals. The HLA system is a 4-megabase (Mb) region located on chromosome 6. It contains more than 200 genes, many of them related to the immune system and the remainder having roles not related to immunity.[5,6] In humans, the MHC can be divided into class I, class II and class III regions. The class I region contains three class I genes that encode for the heavy-chains of the class I molecules and several nonclassical class I genes. The class II region includes the genes for three class II α- and β-chains. These genes are arranged in pairs, which together comprise each class II molecule α- and β-chain loci. The class II region also includes the *TAP*, *LMP* and *TAPBP* genes that encode molecules involved with the production of peptides that will be loaded onto the class I molecules and the HLA-DM gene that encodes a protein involved with loading peptides onto the class II molecules. The class III region also contains genes related to the immune system, such as the complement factors C2, C3 and Bf and the tumor necrosis factor (TNF) gene.

The function of the class I and class II molecules is to capture peptides from pathogens and display them on the cell surface. T cells specific for these peptides can recognize the peptide-MHC complex and initiate an adaptive immune response. The class I proteins capture peptides derived from endogenous antigens located in the cytoplasm, particularly those derived from viruses, while the peptides presented by the class II molecules originate from exogenous antigens.

Some features characteristic of the MHC are worth mentioning here: Class I and class II polymorphisms, the existence of genes that encode proteins with similar functions and genetic linkage. The amazingly high amounts of polymorphisms found in class I and class II genes are generated, basically, by two mechanisms: Point mutations and recombination.[7] Point mutations appear due to mistakes that are made during

DNA replication. More extensive changes are produced by recombination, sometimes producing the interchange of an entire exon between two alleles. Positive selection, driven by pathogens, promotes the survival of new alleles with differences in their antigen presentation properties, whereas the sequences in other regions tend to be conserved to maintain the structure of the HLA molecules.

Another mechanism for generating polymorphism is gene duplication. This duplication generates loci that produce similar proteins. Thus, in humans, there are three class I genes, *HLA-A*, *-B* and *-C*, which produce proteins with similar functions. Therefore, the existence of several class I or class II genes also increases the level of polymorphism and, for this reason, their presence would be advantageous for an individual. However, there is an apparent limitation to the number of functional HLA genes in an organism and, perhaps, an incremental increase in this number could increase the possibility of the appearance of autoreactive T cells. Gene duplication also produces genes that eventually acquire new functions, as has happened with the nonclassical class I molecules that are produced from the classical class I molecules when they acquire a certain number of changes that cannot maintain their function.

Another characteristic of the MHC is its genetic linkage, which is the consequence of the presence of the class I, class II, *TAP*, *LMP* and tapasin genes on the same chromosome that form a cluster; although, this is not a constant feature in all animals, as, in bony fishes, class I and class II are not linked (see below). The organization of the MHC as a gene cluster has allowed the co-evolution of different genes involved in the same function to form haplotypes. Thus, genetic studies have revealed that alleles at different loci can form nonrandom combinations of alleles, probably because the loci proximity facilitates the preservation of a given combination of alleles when it is effective in a response to a particular pathogen. Linkage disequilibrium has been found in different groups of animals.[8-10]

ORIGIN OF THE MHC

The origin of the MHC was probably a "proto-MHC", an ancestral MHC that appeared before the emergence of the adaptive immune system in the jawed vertebrates (gnathostomes), as all jawed vertebrates possess a complete MHC region.[11] The hypothesis for the existence of an ancestral MHC is supported by the presence of MHC-like regions in nonvertebrates, such as amphioxus[12] and also by the presence of three MHC paralogous regions in humans. Thus, the human genome contains regions paralogous to the MHC on chromosomes 9q32-q34, 19p13.1-p13.3 and 1q21-q25/1p11-p32.[13,14]

The term paralogous indicates that a gene (or region) has arisen from a duplication of another gene, to distinguish it from the relationship of genes that arise from a common ancestor (orthologous).[15] Therefore, paralogous genes appear within a single species, while orthologous genes arise in different species as a result of the divergence in genes with the same ancestor. The MHC paralogous region is located on the 6p21.3 chromosomal region and comprises the extended HLA,[5] an 8 Mb fragment consisting of the traditional HLA region and four additional telomeric Mbs, including the *HFE* gene. This region contains over 40 genes with paralogous counterparts in one, two, or three of the paralogous regions on chromosomes 1, 9 and 19.[16] The extended HLA also has paralogous genes outside these three regions and, in total, about 80 genes within the extended HLA are paralogous throughout the human genome.[17]

A cogent theory to explain how these MHC paralogous regions were produced is the "block duplication hypothesis"; that is, large-scale duplications of genome fragments.[18] According to this hypothesis, the MHC paralogous regions were produced from a common ancestral region by duplication. Because there are four paralogous regions, they should have appeared by two cycles of duplications. This process of duplication could have exclusively involved the ancestral MHC region or could have been part of a more general process in which all of a genome was involved. This latter theory is based on the 2R hypothesis formulated by Susumu Ohno in 1970 (ref. 19; reviewed in ref. 20). He proposed that the vertebrate genome underwent two rounds of whole genome duplication during its evolution. This would have occurred by two rounds of polyploidization (in different species).

The MHC paralogous regions strongly support the 2R hypothesis and allow for the determination of when the two rounds of polyploidization could have occurred, considering the number of paralogous clusters that are present in each vertebrate/prevertebrate group. Studies of *Ciona* (urochordate) and amphioxus (cephalochordate) indicate that, in these animals, there is only one copy of the genes represented in the MHC paralogous regions.[11,18] Thus, the duplications would have taken place after the divergence of cephalochordates and vertebrates and before the jawed vertebrate radiation; the first duplication would have occurred in an ancestor common to all vertebrates, before the emergence of jawed vertebrates and the second would have occurred after the separation between the jawless and jawed vertebrates because, apparently, jawless fish only have two paralogous regions.[16,20]

The 2R hypothesis remains a matter of controversy and, indeed, there are those who have proposed only a single round of entire genome duplication.[21] However, whatever the mechanism (entire genome duplication or more limited duplication), large-scale genomic duplications have a great potential for generating evolutionary processes,[22] as the presence of a large amount of gene duplicates would allow for redundant copies of a gene to acquire new functions. This probably promoted vertebrate evolution and the emergence of the genes for the adaptive immune system.

The gene families present in each paralogous region and their comparisons with certain animals' MHC-like regions, like amphioxus, have provided for reconstruction of the ancestral proto-MHC region.[23,11] To carry this out, anchor genes have been particularly useful; these genes are highly conserved in the MHC, its paralogous regions and in MHC-like regions in cephalochordates[18,11,24] (Fig. 1). The order and number of paralogous genes are not conserved among the four paralogous regions in humans and between these regions and the MHC-like regions in prochordates. The human paralogous region on chromosome 9 contains a higher number of genes derived from the proto-MHC than the other three and could be structurally closer to the ancestral region.[23] The other three have undergone more modifications in their organization, including gene losses, translocations to other chromosomal regions, inversions and deletions; indeed, the paralogous region on chromosome 1 has been divided in two, with one part in each arm, while the remainder of the paralogous regions are exclusively in one of the chromosome arms. For all of these reasons it is difficult to determine the precise order in which the genes were located in the ancestral proto-MHC.

It is not known why the genes that comprised the proto-MHC were together and what their functions could have been, although the fact that they formed a group would indicate some kind of co-ordinated purpose not necessarily related to the immune system. If the latter was true, the immune function of the MHC would have appeared after the

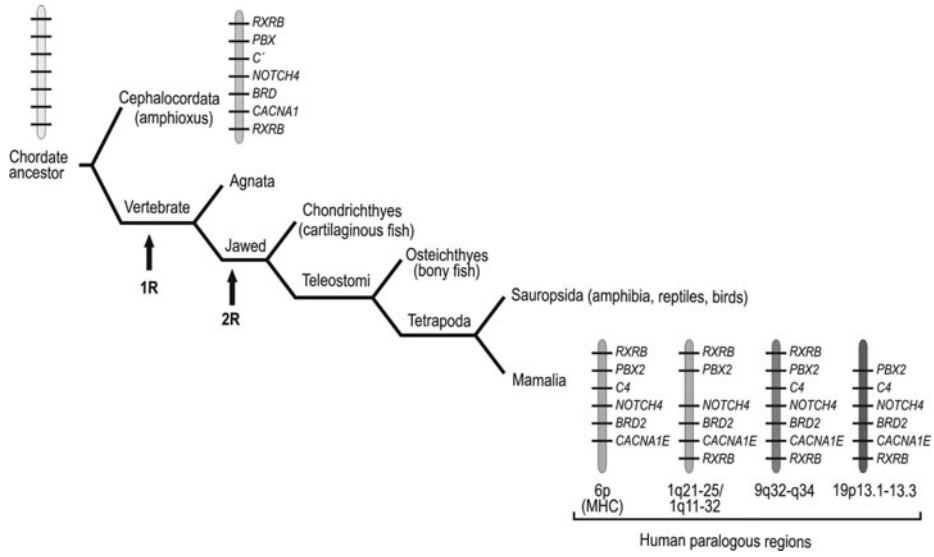

Figure 1. Large-scale duplications during vertebrate evolution. The MHC paralogous regions suggest that two large-scaled duplications of the genome took place after the divergence of cephalochordates and vertebrates and before the jawed vertebrate radiation (indicated with arrows). The comparison of the paralogous regions with the cephalochordata MHC-like regions has allowed the reconstruction of the ancestral proto-MHC by using anchor genes (genes conserved that are located in these regions). We have indicated only anchor genes that have at least three copies in the MHC paralogous regions.

duplications. An alternative theory is that the proto-MHC contained genes that encoded for proteins related to an innate immune system. The presence of these genes in the proto-MHC, which would have appeared before the development of the adaptive immune system, would suggest that there may have been a "primordial immune complex" in this area. The maintenance of this cluster of genes involved with the same immune function had to occur because it would have to entail some evolutionary advantage.[16]

THE MHC AND EMERGENCE OF THE ADAPTIVE IMMUNE SYSTEM

Whether or not there were genes involved with an innate immune system in the proto-MHC has not yet been elucidated. Regardless, genes encoding for receptors that could be ancestors of Igs and the TCR have been identified in the urochordate *Ciona intestinalis*[25] and in the cephalochordate *Brachiostoma floridae* (amphioxus).[26] The Igs and the TCR are members of the IgSF, a family of genes involved not only with immune function that are extensively distributed in the animal kingdom, plants and bacteria.

The IgSF domains can be classified into V domains (resembling Ig Variable regions), I domains (for intermediate) and C1 and C2 domains (resembling Ig Constant regions). The antibody heavy and light chains and the TCR α and β chains (or γ and δ chains) contain V domains and C1 domains, while MHC class I and class II contain C1 domains. C1 domains are found in few other proteins. The more widespread C2 domains are present, for example, in CD8 molecules. We have limited knowledge for

the existence of these domains in vertebrate ancestors. Analysis of the *Ciona* genome has revealed four genes with a "V-C" structure; two of them have C1-like domains plus C2 domains (V-C1-C2-TM-CY) and the other two have C2 domains only (V-C2-TM-CY and V-V-C2-C2-C2-TM-CY).[25] Because the C1 domains are present in Igs and the TCR, it is probable that the basic structure of the molecule that provided an origin for these receptors was V-C1.

Adaptive immunity appeared within a short period of time, 450 million years ago, in some ancestor of the jawed vertebrates after the division of this vertebrate ancestor into jawed and jawless vertebrates, as the molecules that form the adaptive immune system are present in all jawed vertebrates, but not in other groups. It is believed that adaptive immunity appeared after the insertion of a RAG transposon into the germ line of a vertebrate ancestor,[27] presumably into an existing gene with a V domain. This transposon could have been a DNA fragment with a terminal inverted repeat (recombinase signals) carrying the ancestral RAG recombinases.

This insertion would have interrupted the gene, which later would have suffered the loss of its RAG genes. Subsequent duplication events may have led to the multi-segmented immunoglobulin and T-cell receptor genes. Another RAG transposon integrated elsewhere in the genome could have acted on the recombinase signal flanking these gene segments (Fig. 2). At the same time, the T-cell receptor specific ligands must have appeared: MHC class I and class II molecules. There is no information about how these molecules appeared because there are no intermediate molecules that may give us information about their evolution. It is also unknown whether class I or class II arose first in evolution.

MHCs IN FISH

The ancestral MHC presumably had class I and class II genes that originated from a common ancestor and closely linked to genes like those of the immunoproteasome (*LMP*) genes and the *TAP* genes; these would have evolved in a co-ordinate manner. In cartilaginous fishes, the first vertebrates that diverged from the common vertebrate ancestor, class I, class II and class III genes are genetically linked[28] (Fig. 3). This association is lost in the teleosts, where class II genes underwent a translocation and are in different linkage groups. This allowed for class II genes to evolve independently,[16] while class I genes remained linked to *LMP*/*TAP* (Fig. 3). The close association of these genes forms a true class I region in which the genes co-evolved in a concerted fashion.

This linkage had a significant influence on the evolution of the class I and class II genes and, therefore, because the class II genes are not linked to other MHC genes in teleosts, they could have evolved faster (e.g., by infection pressures). This has been illustrated by studies of the MHC in two salmonid species in which the class II genes had different evolutionary histories in each species. The class II genes did not form old lineages (i.e., class I or class II genes with sequence homologies and a common ancestor) and had evolved recently and rapidly, due to differential exposures to pathogens.[29] In this respect, class II genes behave like class I genes in mammals. A higher evolutionary potential could also explain the appearance of "nonclassical" class II molecules in certain bony fishes,[30] which were probably generated as nonclassical class I genes in mammals due to the lack of linkage of class II genes with other MHC genes. Teleosts comprise about one-half of all vertebrates and although there are some differences in the MHCs among the species,[31-35] as for example the number of class I and class II loci, the lack of

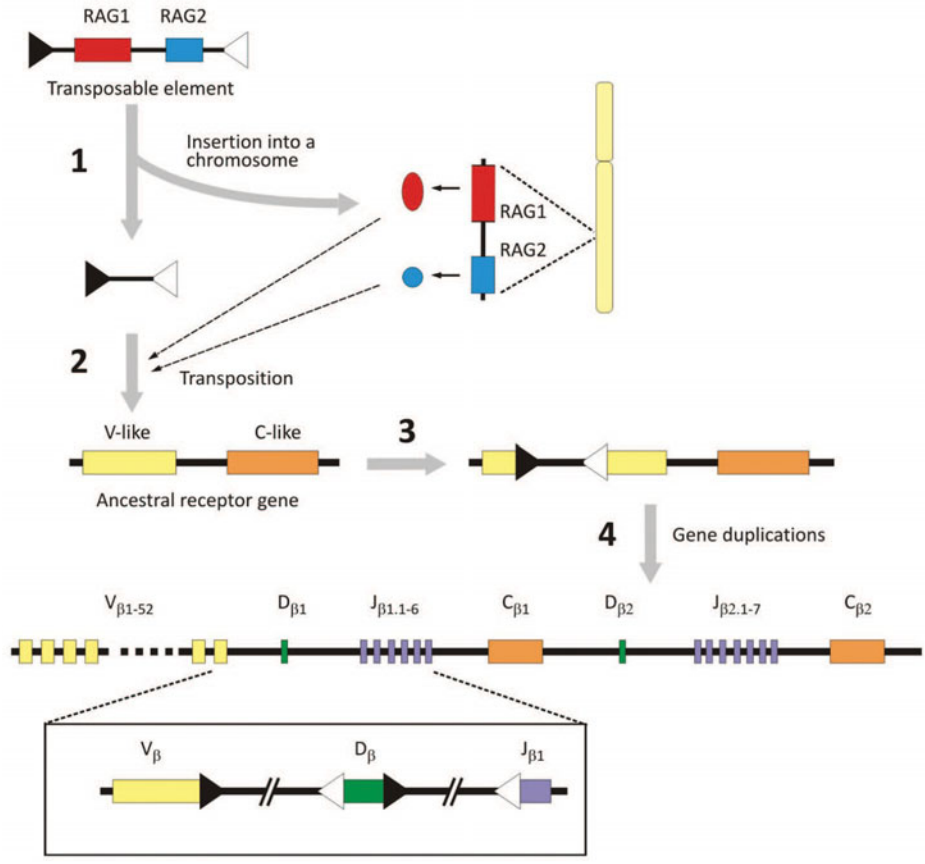

Figure 2. Model for the origin of the receptor genes implicated in the adaptive response. 1) A transposon containing the ancestral RAG recombinases could has been inserted in the germ line of any ancestor of the jawed vertebrates. 2) Then, the transposon could have produced defective copies of this element: A copy with the RAG genes and a copy with the recombination signals sequences (indicated as triangles). 3) The RAG proteins could have produced the excision of the recombination signals and their insertion into the sequence of a variable-like domain of a primordial receptor gene, giving gene segments flanked by the recombination signal, as happen with the V, J elements. Alternatively, the transposon could have inserted into the receptor genes and later could have lost the RAG genes (not shown in the figure). 4) Subsequent gene duplications could have risen to the structure characteristic of the immunoglobulin or the T-cell receptor genes. We have represented the structure of T-cell receptor gene.

linkage appears to be a common bony fish characteristic.[8] This indicates that this linkage did not necessarily provide an evolutionary advantage in all cases.

AVIAN MHCs

The chicken MHC, known as the B locus, is a small 92-kb region that contains only 19 genes.[36] This region is quite compact with no repetitive elements. There are genes with only a few introns and, in some cases, genes that are only separated by 30 nucleotides. The

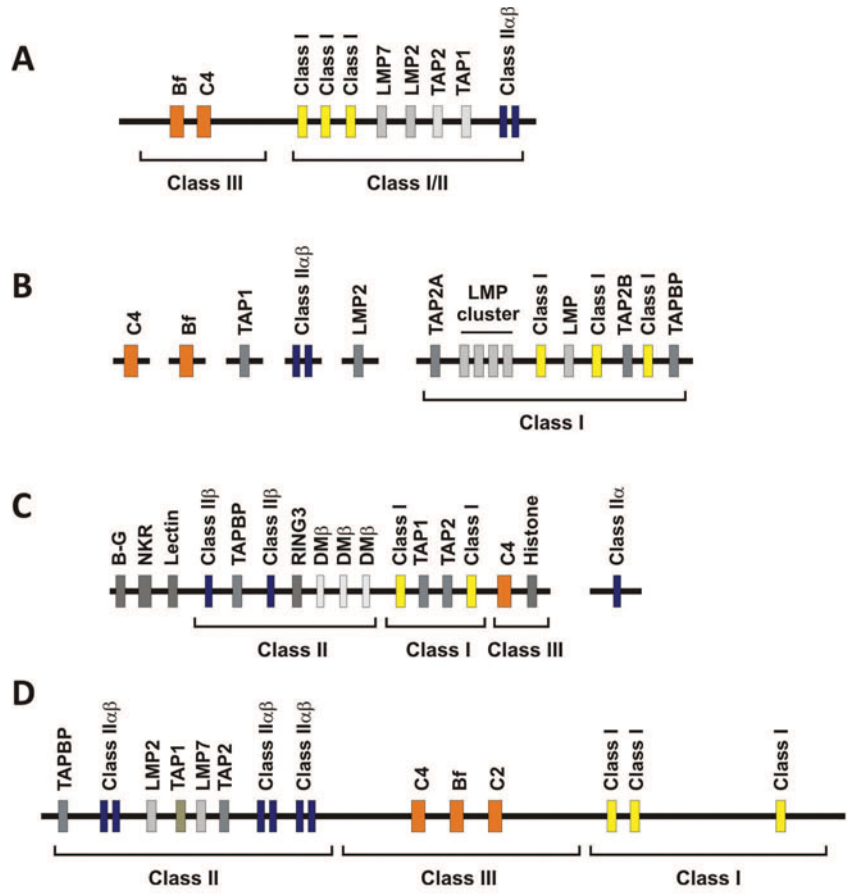

Figure 3. Comparative maps of different MHCs. A) Ancestral MHC. B) Teleosts. C) Chicken. D) Human.

chicken B locus has two class I genes, with the *TAP1* and *TAP2* genes located between them. These four genes comprise the class I region. There are two class IIβ genes surrounding the tapasin gene. The class IIα gene is on the same chromosome, but located far from the B locus. The class IIβ and tapasin genes, along with the RING3 and three DM genes, constitute the class II region. The proteasome genes (LMP) appear to be absent in the chicken genome, which would explain the atypical peptides bound to class I molecules in this species (Fig. 3). The compact structure of the chicken MHC dictated infrequent recombinations among genes and allowed for a functional evolution of the polymorphic genes. This could generate haplotypes that were able to translocate and present specific peptides and, therefore, provide lowered susceptibility to certain infections.[37]

Although the chicken MHC is the best studied, it is not representative of the avian MHC.[38] Other species that have been studied have higher numbers of genes, which do not form the compact structure of the chicken MHC, although, at least in some cases, the overall organization is similar.[39]

MAMMALIAN MHCs

The linkage of *LMP/TAP* with class I genes is missing in mammals. The translocation of the class I locus to an area with lower gene density allowed for duplication of these genes, while in other groups in which the linkage is maintained, class I genes do not undergo important duplications/deletions.[40] The MHCs are quite similar in all mammalian species.[41]

The MHC is generally divided into a class I region, which contains the classical (Ia) and nonclassical (Ib) class I genes, a class II region, which contains the class II genes, two proteasome genes, *TAP* and *TAPBP* genes and a class III region containing a large density of immune and non-immune genes (Fig. 3). However, although the organization and genes of the mammalian MHCs are quite conserved, the genomic regions that contain the class I and class II genes are the least conserved parts. It is only possible to find orthologous relationships among the class I genes within the same mammalian order (although not always), but not among different mammalian orders. Thus, comparing the maps of the class I regions in mice and humans, the nonclass I genes are orthologous; however, the class I genes in this region are not related.[41]

The sites at which the class I genes are located have undergone duplications, lost genome fragments and had other modifications during evolution. These processes have produced new class I genes (paralogous amplification) and has eliminated others, resulting in a replacement of the ancestral genes.[42] This rapid evolution of the class I genes is the reason for the difficulty in finding orthologous relationships among class I genes; indeed, even in related species.

With regard to the primates, loci orthologous to the human *HLA-A*, *-B* and *-C* have been found in the great apes. Some of these genes have also been detected in lesser apes (gibbons) and old world monkeys (rhesus macaques, baboons).[43] Thus, *HLA-A* related loci have been found in apes and old world monkeys, but not in the New World monkeys. However, orthologies with *HLA-A* only can be found in chimpanzees, gorillas and humans.[44,45] Phylogenic analyses for these latter three species have made it possible to group the *HLA-A* related genes into six lineages. However, due to the ability of class I genes to generate new alleles, as well as duplication and loss of genes, it is not possible to find orthologous relationships among related genes in other cases. Thus, the *HLA-B* related genes, the most polymorphic of the class I genes, cannot be grouped into well-defined lineages.[43]

It is remarkable that orangutans and old world monkeys have multiple B loci.[46,47] It is interesting for the interaction of KIR receptors with MHC-B molecules that the human epitope Bw4 is found in chimpanzees, gorillas and gibbons, while variants of this motif are found in orangutans and macaques. As for *HLA-C* related loci, these appeared due to a duplication of a *MHC-B* precursor gene after the divergence between apes and old world monkeys.[48] This gene has been found in chimpanzees, bonobos, gorillas and orangutans.[43] In contrast to the other species in which the *MHC-C* is always present, the orangutan *MHC-C* locus is only present in approximately 50% of the MHC haplotypes.[49]

The class II genes are more conserved in mammals than are the class I genes. The *DQA1* and *DQB1* genes have been found in most mammals.[50,51] *DP* genes are less conserved and in mice and cats they are pseudogenes. In primates, the *DP* region is quite conserved.[52] The number of *DR* genes varies among different mammals and, indeed, in the same species. Thus, in humans, the *DRB* locus can contain from two to six genes.

KIR GENES AND MHC EVOLUTION IN PRIMATES

Although *KIR* genes can be found in a great number of mammals, from mice to humans, the expansion of these genes has happened almost exclusively in primates. In these animals, *KIR* genes are encoded by gene families that form clusters. In humans they are on chromosome 19q and form part of the Leukocyte Receptor Complex (LRC).[53,54] This family of genes probably appeared from a unique primordial *KIR* gene as a result of multiple cis-duplications. Because *KIR* genes have considerable sequence similarities and are arranged in the same orientations, these first duplications could easily have been followed by other genetic processes, such as sequence exchange by gene conversion, domain shuffling, or nonreciprocal crossover, which contributed to a rapid evolution of these genes.[55]

KIR genes encode Type I transmembrane glycoproteins with Ig domains. These proteins are formed by 2 (KIR2D) or 3 (KIR3D) extracellular Ig domains, a stem sequence, a transmembrane region and a cytoplasmic tail. Depending on the cytoplasmic tail, they can be subdivided into Long-tailed (L) and Short-tailed (S). The inhibitory KIRs generally possess long cytoplasmic tails, whereas those with short tails typically generate activating signals. The function of these receptors is to specifically recognize class I molecules, which generates activating or inhibitory signals. As has occurred with the MHC, the KIR cluster is very polymorphic; thus, there are more than 100 different haplotypes distributed among different populations.[56] Each individual possesses two different haplotypes, each having a variable number of genes. There is also an allelic variability, as *KIR* genes are polymorphic.

Part of the MHC class I molecules are the only known ligands for KIR receptors. KIR genes interact specifically with certain HLA molecules. Thus, HLA-B molecules with the serological Bw4 motif are ligands for *KIR3DL1*. These interactions are stronger when the Bw4 motif has an isoleucine residue at position 80. No HLA-B belonging to serological Bw6 is known to be a ligand for any KIR. In contrast, both groups in which HLA-C can be divided (C1 and C2) can be ligands for KIR genes: Group C1 binds to KIR2DL2 and KIR2DL3 and group C2 binds to KIR3DL1. Some *HLA-A* alleles and the nonclassical HLA-G molecule can also be ligands for certain KIR receptors.

Phylogenetic studies of the *KIR* gene family indicate that they have diversified rapidly in primates in comparison to other genes and that this diversification happened in a co-ordinate manner with the MHC. Comparing the gene organizations of *KIR* haplotypes described thus far (rhesus macaque, orangutan, chimpanzee and human), it is possible to observe a similar structure.[57,58]

The rhesus macaque (*Macaca mulatta*) *KIR* genes are characterized by a considerable number of *Mm-KIR3DL* genes, two *Mm-KIR2DL4* genes and two novel families of KIR genes, *Mm-KIR3DH* and *Mn-KIR1D*.[59] The increase in the number of *Mm-KIR3DL* genes was probably a consequence of the co-evolution of *KIR* genes with their class I ligands. The class I gene family in the rhesus macaque consists of multiple A and B loci and the MHC haplotypes do not contain a fixed number of class I genes. In addition, the *MHC-C* gene is not present in this species.

The diversity of the class I genes could have resulted from a need for a higher number of *KIR* genes.[60] In the orangutan (*Pongo pygmaeus*), three *KIR* lineages have already been defined that are present in chimpanzees and humans (denoted lineages I, II and III), although *Popy-KIR2DL4* is the only ortholog of the *KIR* genes present in these two species.[61] The evolution of the *KIR* genes in the orangutan has also been influenced by

the evolution of the MHC genes. In this species, appearance of *KIR* genes coincided with the appearance of the *MHC-C* gene, the most important class I ligand and an increased number of lineage III genes, which included the *MHC-C* specific *KIR*.

This co-evolution is also evident in that the orangutan apparently only has *KIR* genes that are specific for MHC-C1 alleles, the only existing alleles in it. The rapid evolution of *KIR* genes is also evident upon analyzing the *KIR* genes in the common chimpanzee (*Pan troglodytes*). Although the chimpanzee is the most closely related animal to humans, there are only three genes that are orthologous in both species: *KIR2DL4*, *KIR2DL5* and *KIR2DS4*.[62] In the chimpanzee and humans, the *MHC-C* gene is fixed since it is present in all haplotypes. On the other hand, the two categories of *MHC-C* alleles, C1 and C2, which define the corresponding groups of KIR ligands, are also present in chimpanzees. It has been determined that the *Pt-KIR2DL6* gene is the specific ligand for the C1 group, whereas *Pt-KIR3DL4* recognizes the C2 group. The Bw4/Bw6 motifs in the *MHC-B* alleles are also present in chimpanzees.

CONCLUSION AND FUTURE PROSPECTS

The analyses of the MHC paralogous regions in humans, as well as the studies of urochordates and cephalocordates, have allowed for the reconstruction of a proto-MHC, an ancestral region that was a precursor of the MHC. It is not known how this ancestral region acquired the genes involved with the adaptive immune system and whether it contained genes implicated with the innate immune system. However, the synteny of their genes indicates that they should have a co-ordinate activity and is suggestive of functions possibly related with innate immunology.

The study of the MHC region in different vertebrates suggests that, in the ancestral MHC, the class I genes, class II genes and the genes related with antigen presentation were linked. This linkage has been lost in some vertebrates. However, the MHC has only been analyzed for a very small proportion of species. Thus, it will require more studies to determine, with greater precision, the characteristics of the proto-MHC, the organization of the ancestral MHC and how their evolution occurred in the different groups. The sequencing techniques that have been recently developed will be a great aid in this task.[63]

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CHAPTER 17

MHC SIGNALING DURING SOCIAL COMMUNICATION

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Abstract: The major histocompatibility complex (MHC) has been known to play a critical role in immune recognition since the 1950s. It was a surprise, then, in the 1970s when the first report appeared indicating MHC might also function in social signaling. Since this seminal discovery, MHC signaling has been found throughout vertebrates and its known functions have expanded beyond mate choice to include a suite of behaviors from kin-biased cooperation, parent-progeny recognition to pregnancy block. The widespread occurrence of MHC in social signaling has revealed conserved behavioral-genetic mechanisms that span vertebrates and includes humans. The identity of the signal's chemical constituents and the receptors responsible for the perception of the signal have remained elusive, but recent advances have enabled the identification of the key components of the behavioral circuit. In this chapter we organize recent findings from the literature and discuss them in relation to four nonmutually exclusive models wherein MHC functions as a signal of (i) individuality, (ii) relatedness, (iii) genetic compatibility and (iv) quality. We also synthesize current mechanistic studies, showing how knowledge about the molecular basis of MHC signaling can lead to elegant and informative experimental manipulations. Finally, we discuss current evidence relating to the primordial functions of the MHC, including the possibility that its role in social signaling may be ancestral to its central role in adaptive immunity.

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INTRODUCTION

MHC (also known as HLA in humans and H-2 in mice) signaling mediates both immune recognition during the adaptive immune response (discussed in the previous chapter) and social signaling that enhances both the recognition of optimal mates and kin-biased behaviors.[1] Social signaling meditated by the MHC was first discovered in regards to mate preferences in laboratory mice (*Mus musculus*),[2] a full three decades after the histocompatibility functions were described by George Snell.[3] Thirty years later, social signaling via MHC has been described throughout vertebrates including mammals, birds, reptiles, amphibians and teleost fish (see Table 1). MHC social signaling has been identified in over 20 species of vertebrates and is likely the basis for a vertebrate-wide chemosensory communication system. The original observation of MHC disassortative mating preferences seems to be common, but not omnipresent in vertebrates;[4] it by no means is the only behavior facilitated by MHC, nor is it the only type of observed MHC-based mate preference. MHC signaling also facilitates cooperative behavior with kin, parent-progeny recognition and pregnancy block. In the following sections we will present the current evidence for MHC as a signal of relatedness, individuality, genetic compatibility and quality. MHC-mediated behaviors are diverse and though general patterns exist within vertebrates, the exact function of MHC-based social signaling will be species specific and highly context dependant.

SIGNALING OF MHC GENOTYPE: MOLECULAR MECHANISMS

For three decades after the discovery of MHC-mediated social singling in laboratory mice,[2] the actual mechanism of how MHC genotype was perceived in conspecifics remained a mystery. Early on it was discovered that MHC genotype could be discriminated by chemical cues detected by the olfactory system. These studies showed that mice could discriminate MHC odortypes either through training[5] or in the absence of training.[6] However, the nature of the signaling odorants remained elusive. This mystery was at least partially solved by the discovery that peptides known to bind MHC molecules also bound receptors in the vomeronasal organ (VNO).[7] It was later shown that a similar process was working in the main olfactory epithelium (MOE).[8]

The critical role of MHC-presented peptides during adaptive immune recognition is well established.[9] MHC-bound peptides are presented at the cell surface for interrogation by T cells; when the peptides are of foreign origin (e.g., from a pathogen) an immune response is initiated. The majority of MHC alleles encode unique structural aspects of the peptide binding region of the molecule and these variants provide great specificity in the peptides they present. Because there is physical correspondence between MHC allelic variants and the anchor positions of the amino acid sequence of their bound peptides, it was hypothesized that MHC peptides could serve as ligands for odorant receptors that had similar binding specificity, thus allowing information about MHC genotype to be conveyed. Physiological recordings from vomeronasal sensory neurons (VSNs) stimulated with synthetic peptides proved this to be the case.[7]

Table 1. Summary of studies investigating MHC-genotype signaling in social communication

Species	MHC-Based Mate Preference	MHC-Mediated Cooperative Behavior	Phenotype Matching System	Sources
Mammals				
House Mice (*Mus musculus*)	MHC disassortative	Female Communal Nesting	Familial imprinting	Yamazaki et al 1976,[2] 1988,[20] 2007;[21] Manning et al 1992;[22]
Bank voles (*Clethrionomys glareolus*)	MHC disassortative	Unknown	Unknown	Radwan et al 2008[23]
Malagasy giant jumping rat (*Hypogeomys antimena*)	MHC assortative	Unknown	Unknown	Sommer 2005[24]
Humans (*Homo sapiens*)	MHC disassortative	Unknown	Unknown	Wedekind et al 1995;[25] Havlicek and Roberts 2008[26]
Mandrill (*Mandrillus sphinx*)	MHC disassortative	Unknown	Unknown	Setchell et al 2009[27]
Fat-tailed dwarf lemur (*Cheirogaleus medius*)	MHC supertype-disassortative and maximal diversity	Unknown	Unknown	Schwensow et al 2008[28]
Grey mouse lemur (*Microcebus murinus*)	MHC disassortative (cryptic)	Unknown	Unknown	Schwensow et al 2008[29]
Domestic sheep (*Ovis aries*)	No MHC preference			Paterson and Pemberton 1997[30]
Birds				
Savannah sparrows (*Passerculus sandwichensis*)	MHC disassortative	Unknown	Unknown	Freeman-Galant et al 2003[31]
House Sparrow (*Passer domesticus*)	MHC assortative and optimal diversity	Unknown	Unknown	Bonneaud et al 2006[32]
Seychelles warbler (*Acrocephalus sechellensis*)	MCH maximal diversity	Unknown		Richardson et al 2005[33]
Great reed warbler *(Acrosephalus arundinaceus*)	No MHC preference			H. Westerdahl 2004[34]
Red jungle Fowl (*Gallus gallus*)	MHC disassortative (cryptic)	Unknown	Unknown	Gillingham et al 2009[35]
Peafowel (*Pavo cristatus*)	MHC maximal diversity (cryptic)	Unknown		Hale et al 2009[36]

(continued on next page)

Table 1. Continued

Species	MHC-Based Mate Preference	MHC-Mediated Cooperative Behavior	Phenotype Matching System	Sources
Reptiles				
Sand lizards (*Lacerta agilis*)	MHC disassortative	Unknown	Unknown	Olsson et al 2003[37]
Tuatara (*Sphenodon punctatus*)	MHC disassortative	Kin avoidance during territory acquisition	Unknown	Miller et al 2009[38]
Amphibians				
African clawed Frog (*Xenopus laevis*)	Unknown	Tadpole schooling	Self reference	Villinger and Waldman 2008[39]
Tiger Salamanders *(Ambystoma tigrinum*)	MHC assortative	Unknown	Unknown	Bos et al 2009[40]
Fish				
Zebrafish (*Danio rerio*)	Unknown	Unknown but kin groups grow faster than non kin groups	Familial imprinting	Gerlach et al 2007[41] and 2008[42]
Three-spined stickleback (*Gasterosteus aculeatus*)	Optimal MHC diversity	Unknown	Self reference	Aeschlimann et al 2003;[43] Reusch et al 2001;[44] Milinski 2006[45]
Atlantic Salmon (*Salmo salar*)	MHC disassortative	Schooling with kin	Self reference	Rajakaruna et al 2006;[46] Consuegra and de Leaniz 2008[47]
Chinook salmon (*Oncorhynchus tshawytscha*)	MHC disassortative	Unknown	Unknown	Neff et al 2008[48]
Arctic Char (*Salvelinus alpinus*)	Unknown	Schooling with kin	Self reference	Olsen et al 2002[49]
Brown Trout (*Salmo trutta L*)	Optimal MHC diversity	Unknown	Self reference	Forsberg et al 2007[50]
Brook Trout (*Salvelinus fontinalis*)	Unknown	Schooling with kin	Self reference	Rajakaruna et al 2006[46]
Whitefish (*Coregonus sp.*)	No MHC preference (cryptic)			Wedekind et al 2004[51]

Sources are limited to first reports and reviews. Blank boxes indicate no finding would be expected given the observed result.

Detection of Peptides in the Olfactory System

The olfactory system of mammals is anatomically divided into two regions: the main olfactory epithelia (MOE) and the vomeronasal organ (VNO). Traditionally these two organs were viewed as functioning in largely non-overlapping modalities with the VNO being specialized for detection of nonvolatile small molecules and proteins that typically signaled the sexual and social status of conspecifics (pheromones), while the MOE was thought to specialize as a general detection system for volatile substances.

The initial experiments to determine if the olfactory system was capable of detecting peptides were conducted in the VNO of mice. Leinders-Zufall and coworkers (2004) tested the hypothesis that dissociated MHC class I peptides could be detected in the VNO Two peptides known to be presented either by the H-2D^{b} haplotype (AAPDNRETF) or H-2K^{d} haplotype (SYFPEITHI) were synthesized. These peptides were applied individually to ex-vivo preparations of mouse VNO. Both peptides activated a relatively specific subset of V2R-positive neurons in the basal zone of the VNO as revealed by extracellular field potential recordings and fluorescence imaging. The VSNs responded with high sensitivity at concentrations down to 10^{-12}M.

As predicted by the hypothesis that peptides can signal MHC genotype, the peptide binding by the VSNs responded in an MHC allele-specific manner. Not only was the VSN response specific to the amino acid sequence of each peptide, but the pattern of specificity mimicked the binding properties of MHC molecules. Amino acid substitutions (underlined) at non-anchor positions (e.g., SYIPSAEKI) usually continued to stimulate the same neurons. In contrast, substitution of peptide anchor residues (underlined) with alanine (e.g., AAPDARETA or SAFPEITHA) abolished stimulation of these neurons. These VSN binding properties provide a neurophysiological basis for identifying the MHC genotype of individuals, because peptides are reverse-image "molds" of the antigen-binding site of MHC molecules. Thus, sensory receptors that detect peptides in an MHC-like fashion could in principle function as an MHC genotyping system.[10] These results point to the structural importance of peptide anchor residues in binding VSN receptors and, given the similar binding properties of MHC molecules, reveal the convergent ligand-binding properties of these unrelated molecules.

The same lab group applied the same hypotheses to the MOE sensory neurons, traditionally viewed as generalist receptors of volatile chemosignals.[8] Contrary to conventional wisdom, they discovered that nonvolatile, fluorescent tagged MHC peptides gain access to the MOE without direct nasal contact to the peptide containing fluid. Most importantly, these peptides activated neurons at subnanomolar concentrations in an allele specific fashion, similar to the patterns found in the VNO. There are, however, some important physiological differences in peptide detection between the two olfactory organs.[11] First, a different transduction mechanism is used in the MOE during recognition of peptides.[12] Second, when anchor residues are substituted with alanine (eg. AAPDARETA and SAFPEITHA), olfactory sensory neurons (OSNs) cease firing at normal stimulation concentrations, but firing resumes at higher concentrations. Third, MOE-dependent peptide recognition does not induce pregnancy block,[13] despite normal MHC odor (mating) preferences. These experiments show that discrimination of MHC genotype by the two olfactory systems is achieved with separate neurological, physiological and behavioral response pathways.

If peptides are the odorants that allow MHC genotype to be discriminated, then experimental manipulation of peptides should alter behavioral responses in a fashion

consistent with MHC-mediated behaviors. The findings that the MOE and the VNO can detect peptides in an MHC-like fashion stimulated research confirming that both mouse and stickleback fish (*Gasterosteus aculeatus*) behavior is manipulated by the addition of peptides. The experimental addition of peptides to an MHC similar odor source causes animals to respond as if it were an MHC dissimilar odor source for both mating- and odor-preferences[8,14] and pregnancy block.[7]

Signaling of MHC Genotype without Peptides

Due to the general nonvolatility of peptides,[15,16] the question has remained whether peptides can explain all of the observed MHC-mediated behavioral patterns. This question was recently addressed by experimentally removing all of the peptide components from the urine of two MHC-congenic strains of mice. Mice that had been trained to discriminate between the urine odors of these two strains could continue to discriminate using the peptide-free urine.[17] These results suggest that nonpeptide volatile odorants also provide signals conveying MHC genotype information. However, odor-training experiments can introduce confounding behavioral artifacts[18] and this result should be confirmed in a paradigm that does not use training. If these results are confirmed, making yet a third independent mechanism for identifying MHC genotype, it underscores the functional importance of this olfactory ability and the importance of the associated behavioral responses.

Though it has been shown that peptides signal MHC genotype in mammals (mice) and fish (sticklebacks), the utilization of peptides in other vertebrates is undocumented. It has been questioned weather olfaction can explain MHC-mediated behavior in birds whose olfactory prowess has long been questioned.[19] No other mechanisms have been as thoroughly tested as peptide and volatile olfaction signaling of MHC genotype and more work is needed to test whether these mechanisms drive MHC mediated behavior in other taxa.

MHC AS A SIGNAL IN INDIVIDUAL RECOGNITION

Individual recognition is an important component of social behavior. Traits that specifically signal individual identity are predicted to be genetically determined, highly variable, cheap to produce (i.e., not condition-dependent) and signal variants are expected to have equal fitness at equilibrium (reviewed in Tibbetts and Dale 2007 ref. 52). MHC is an ideal candidate gene for understanding the mechanistic bases of individual recognition because it is a genetically determined trait associated with social behavior and is extremely variable (there are 10^9 MHC phenotypes in mice[53]). MHC was hypothesized to contribute to individual recognition as early as 1975.[54] Since then, the concept of individual recognition has been invoked in many studies addressing MHC-associated cues in social signaling (e.g., ref. 17). However, many authors tacitly use different definitions of this term and do not distinguish between individual recognition in the strict sense[52] and other forms of social recognition, which can include discrimination of familiar vs unfamiliar conspecifics, kin vs nonkin, same-genotype vs different-genotype and genetically compatible vs incompatible mates. We define individual recognition as being characterized by individual specificity in three elements of social communication: signaling; signal perception and template matching by the signal receiver; and a functional response by the receiver.[55] This definition includes any case where receivers have a template of a specific individual

based on a learned signal and differs from kin-recognition where the template is based on phenotype matching (see below). Here, we review studies that have sought to characterize individual-specific MHC odortypes, which have mainly focused on MHC-correlated volatile profiles and their relation to pregnancy block and scent marking.

MHC congenic strains of mice, which share the same background genome, but have unique MHC haplotypes, are a model system with which to understand behavioral responses to individuals of same- or different-haplotype at a single locus. One extrapolation from studies demonstrating MHC haplotype-dependent behavior[17] in congenic strains is the possibility that, in outbred populations where MHC allelic polymorphism is likely to be very high, MHC phenotypes would be key mediators of individual recognition. For example, it has long been understood that MHC congenic strains have unique volatile organic compound signatures that are used in chemical communication.[15] More recently, several groups have identified suites of volatile organic compounds that are regulated by MHC odortypes.[15,16,56] As predicted by a model of individual recognition, some of these suites are unaffected by environmental variation;[57] furthermore, volatile profiles from MHC congenic mice activate overlapping but distinct subsets of neurons in the mouse main olfactory bulb.[58] The authors of such studies in congenic strains often conclude that the physiological machinery is in place for volatile profiles to mediate individual-specific behaviors (e.g., ref. 57). However, counter-part experiments using outbred wild mice in a more ecologically realistic setting are lacking. Given that some genotypes will inevitably be shared between individuals, more naturalistic work is needed to understand how these volatile signatures function as signals of individuality (as defined above) or as signals of relatedness or genotype.

Pregnancy Block

Pregnancy block, also known as the Bruce effect, occurs when recently mated female laboratory mice are exposed to the odors of an unfamiliar male.[59] Upon exposure to an unfamiliar male odor, prolactin release from the anterior pituitary in the mated female is suppressed, resulting in pregnancy failure, reabsorption of the fetus and the onset of estrus.[60] The signal responsible for pregnancy block is considered to be individual specific because the unfamiliar male and the mate both express odors capable of inducing pregnancy block. Thus, females have to learn the identity of their mate (i.e., form a memory) in order to suppress pregnancy block upon perception of the mate's odors.

Pregnancy block can be induced by the presence of an unfamiliar male or simply his soiled bedding or urine and direct physical contact with the odorant seems necessary.[60] However, in at least one case volatiles alone (i.e., no direct contact) can induce pregnancy block.[61] The memory developed during pregnancy block is dependent on activation of sensory neurons in the VNO; however, the specific chemical constituents that bind receptors in these neurons have proven difficult to find. Three different classes of molecules associated with individual odors have recently been investigated: MHC and MHC peptides, major urinary proteins (MUPs) and volatiles. Peele and colleagues recently investigated the relative roles of MUPs and volatiles.[62] They found that low molecular weight fractionations (which excludes MUPs) from urine were more effective in blocking pregnancy than those of high molecular weight, suggesting a role of volatile compounds in the odor. However, the low molecular weight fraction from the unfamiliar male resulted in only 50% pregnancy block, as opposed to 90% pregnancy block via unfamiliar male whole urine. Moreover, a recent study called these findings into doubt by showing that,

contrary to Hilda Bruce's original finding, urine from castrated or juvenile males was sufficient to induce pregnancy block. These results suggest that, although volatiles can contribute to the occurrence of pregnancy block, they are not necessary to induce it.[63]

MHC-associated odors have also been shown to be sufficient to induce pregnancy block in several studies, implicating it's involvement during individual recognition. These odors were originally observed to block pregnancy when unfamiliar males differing only at the MHC could induce pregnancy block.[61] Since then, searches for an MHC-odortype mechanism have targeted MHC molecules themselves, MHC peptides and possible associated volatiles. MHC peptides were the first specific odorant found to induce pregnancy block[7] (see above).

The finding that sensory neurons in the VNO respond selectively to MHC peptides was biologically validated by demonstrating the role of peptides in producing pregnancy block.[7] As predicted, it was found that pregnancy block upon exposure to MHC peptides from an unfamiliar, MHC-dissimilar male was equally effective as exposure to whole urine from an unfamiliar, MHC-dissimilar male. In this case, the peptides had to be delivered on a urinary background (regardless of whether the urine was from a familiar or unfamiliar male). A more recent study, however, found that peptides alone (administered more frequently than in ref. 7) were sufficient to induce pregnancy block.[63] These studies show that the suite of peptides presented by an individual's MHC molecules can, when excreted in urine, be used as odorants in chemical signaling. Because of the large diversity of MHC haplotypes in a population, there is potential for individual specific odortypes simply in excreted MHC peptides. Such odortypes are detectable by VSNs that have binding specificity for these peptides similar to that of MHC molecules.[7] Where these peptide signals originate, however, remains to be found. Surprisingly, there is disagreement about whether peptides can be found in mouse urine.[7,60,64] Peptides have not been reported in other mediums of chemical communication such as saliva, tears, or skin excretions, but we are not aware of any directed searches for peptides in these secretions.

Although MHC peptides are clearly sufficient to induce pregnancy block in inbred mice, it should be noted that the experiments described above do not demonstrate individual recognition in a strict sense. Because peptides from an unfamiliar male with the same MHC genotype as the female's mate would not be expected to induce pregnancy block, MHC peptides in the context of pregnancy block might be more likely to signal the presence of an unfamiliar male. If individuality is perceived during pregnancy block, it would likely be conveyed via coupling with sensory neurons activated by the urinary background and neurons in the VNO have been found to be capable of discriminating individual mice of the same laboratory strain.[64] Finally, while pregnancy block provides an attractive system in which to test hypotheses concerning social signaling and behavior, the system is ultimately hindered by the fact that the adaptive significance of pregnancy block, which is only observed in certain laboratory strains of mice, has not been determined for natural populations. It has been suggested that the Bruce effect functions to prevent infanticide from males who have recently displaced the dominant, territorial male.[4,65]

Scent-Marking

In addition to the MHC, growing evidence indicates that major urinary proteins (MUPs) are another chemical signal critical to social communication and individual recognition in mice. MUPs are protein pheromones encoded by a polymorphic, multi-gene family. In a series of experiments, the laboratory of Jane Hurst has tested the relative

roles of MUPs and MHC in individual recognition in mice using a scent-marking behavioral paradigm. First, it was shown that wild-derived males presented with a scent mark from another male expressing a different MUP-type will investigate and counter-mark the marks significantly more than the control.[66] Second, it was shown that scent-marks associated with MHC haplotype (in MHC-congenic strains) were not necessary or sufficient to influence investigation time of male mice of congenic MHC strains. Rather, investigation time was increased only when the stimulus odor differed from the genomic background of the test animal.[67] A third experiment tested whether wild female mice could discriminate between scent marks from congenic males whose MHC and MUP genotype were controlled. Results showed that females could discriminate between individual males only when the males differed with respect to MUP haplotype; females could not discriminate between individual males that had the same MUP haplotype and could not discriminate between males that had different MHC haplotypes.[68] These three experiments indicate that, in the context of scent-marking and countermarking, MUP genotype and not MHC genotype, is the greatest determinant of individuality in urinary odors. However, it should be noted that in light of previous research, it is anomalous that the mice in these experiments did not discriminate between urinary odors that differed with respect to MHC genotype.[67,68] Previous studies have documented the ability of either MHC-congenics (e.g., ref. 5) or wild-derived mice[69] to distinguish urinary odors that only differed genetically at the MHC.

Because MUPs are likely to be polygenic, polymorphic signals only in a few rodent species it is unlikely that the functions discovered in *Mus* will have generality across vertebrates. The results from the Hurst group studies suggest that there are key differences in signals that are conserved across taxa (e.g., MHC) and signals that are species-specific (e.g., MUPs) for the identification of individual conspecifics.[68] They also reveal the curious finding that signals of individuality are limited to specific behavioral interactions.

Taken together, the individual recognition studies reviewed above show that MHC may play an important role in individual recognition in certain instances (for example in pregnancy block), but also indicate that they may not be used for individual recognition in the strict sense. Many of the studies focusing on individual recognition and the MHC have utilized congenic strains of mice, which provide a unique opportunity to study the role of a single locus or haplotype in chemical communication. However, the use of inbred stains of animals may limit our broader understanding of behavior and ecology, as 60 years of domestication has modified their behavior.[70] So, more studies will be needed to determine the role of MHC in individual recognition in outbred populations; we know of no such examples except for the aforementioned examples from the Hurst lab.

MHC AS A SIGNAL IN KIN RECOGNITION

Kin recognition using polymorphic genetic systems allows individuals to engage in behaviors specific to kin or nonkin. An individual's fitness is a product of both its own reproductive success (i.e., direct fitness) and the reproduction of close relatives (i.e., indirect fitness); thus, proper identification of kin facilitates cooperation (or at least decreased antagonism) with relatives and promotes behaviors that increase fitness.[71] Additionally, recognition of kin allows for the prevention of inbreeding, and therefore reduces the homozygous expression of deleterious recessive alleles. In order for a genetic system to be used accurately to recognize kin, it must contain enough allelic polymorphism to allow

discrimination between related and unrelated individuals. Kin recognition systems that can discriminate among a range of different-degree relatives have been reported.[72] MHC is the most polymorphic genetic system in vertebrates[4] and has long been considered to play a role in kin recognition by mediating cooperation,[1] parent-offspring identification[73] and mating preferences that prevent inbreeding.[74]

Two major phenotype matching mechanisms exist for MHC-based kin recognition within vertebrates (Fig. 1). The first is a self reference system in which individuals use their own MHC odortype as a template to recognize other individuals as kin.[39,43,46,49,50] The second is familial imprinting where individuals imprint upon the MHC odortypes of kin early in development and afterwards apply the learned MHC signals to unfamiliar individuals.[20,42,75] The degree to which familial imprinting and self reference systems identify kin differ remarkably (Fig. 1). Only familial imprinting systems can identify kin that do not share odortypes with a focal individual. However, the ability to recognize kin that do not share odortypes also allows for the false recognition of unrelated individuals as relatives; this could occur in mixed litters where odortypes produced by half siblings are based on haplotypes from an unrelated individual. Both phenotype matching systems can be used to identify kin through odortypes based on either specific MHC haplotypes (both haplotypes providing a specific odor) or by odortypes based on a blended odor of both haplotypes. For example self reference systems recognize either 25 or 75% of full siblings depending on whether specific haplotypes are recognized or only the genotypic odor of the blended haplotypes[76] (Fig. 1). Currently few studies have been conducted to determine the specifics of phenotype matching systems used in nature and more research is needed to determine the relative prevalence of familial imprinting vs self reference systems and the nature of the odortypes (specific haplotypes or blended genotypes) used. Interestingly, the two systems most described in nature are familial imprinting on haplotypes and self reference based on blended genotype odors which are the best and worst of the theorized kin recognition systems respectively (Fig. 1). Regardless of the phenotype matching system used, kin recognition is likely one of the major functions of MHC-mediated signaling and the very existence of familial imprinting is evidence supporting this hypothesis because kin recognition is the only function that is enhanced by familial imprinting; self reference will be superior for functions involving genetic compatibility, individuality, or quality.[65]

Phenotype matching systems can identify more kin if multiple polymorphic unlinked loci are used, presuming a match at any locus is a signal of relatedness.[77] Though the impact of multiple unlinked loci has minimal impact on familial imprinting systems it has profound consequences on self reference systems, where multiple loci dramatically improve kin recognition (Fig. 1). Within both teleost fishes and amphibians, taxa where self reference systems are common, the MHC is not inherited as a single unit but rather as two or four separate unlinked loci.[78,79] Whether this is coincidence or represents evidence that the inefficiency of self reference systems favors translocations that breakup the MHC linkage group will await more phylogenetic data. Within both teleost fishes and amphibians it has been shown that MHC Class II genes are sufficient, but not necessary, for kin recognition. It has been proposed that other unlinked MHC genes provide additional information used in kin recognition.[46,49] Likewise, in house mice it has been observed that when MHC signals of relatedness are controlled for, signals from a different polymorphic locus (MUPs, see below) can also be used as signals of relatedness. In nature, it is highly likely that both MUPs and MHC are utilized for kin recognition in tandem.[80]

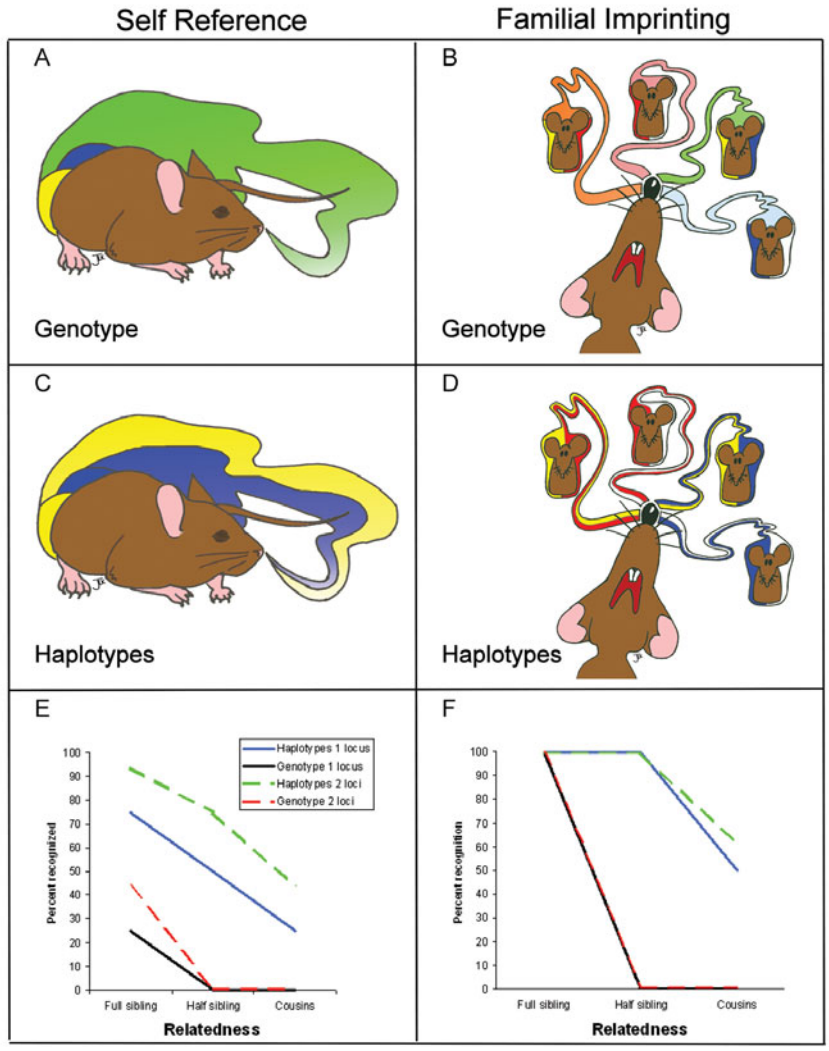

Figure 1. Possible phenotype matching systems using MHC-based odors and their effectiveness for the recognition of kin. Two kin recognition mechanisms that exist in nature are self reference (A, C, E) and familial imprinting (B,D,F). Phenotype matching can be based on genotypes (i.e. blended haplotype odor), or on haplotypes (i.e. allele-specific odors). Self reference is based on odors associated with an individual's own genotype (A) or both haplotypes (C). Familial imprinting is based on odors associated with the genotypes (B) or haplotypes (D) present in the natal nest (e.g. parents or siblings). The prevalence of these systems in nature is largely untested; current evidence suggests that the primary phenotype matching system in mice is haplotype-based familial imprinting (D). The effectiveness of each phenotype matching system for recognizing three classes of kin are plotted for one or two unlinked polymorphic loci (E & F). MHC haplotypes are inherited as a linked locus or as multiple unlinked loci depending on taxa. Each point represents the percentage of full siblings, half siblings, and cousins an individual would be able to recognize (points are connected by lines to help visualize patterns). Haplotype-based mechanisms are almost always superior to genotype mechanisms for kin recognition. Adding loci to self reference systems improves kin recognition more than in familial imprinting systems. Familial imprinting (F) generally allows an individual to recognize more kin than self reference (E). Models assume that all individuals are heterozygous, that no alleles are shared between unrelated individuals and that all combinations of parental genotypes are found within litters. (Illustrations by J.L.K; graphic design by Linda Morrison).

Cooperative Behavior

Proper identification of kin can result in cooperative behaviors between relatives; MHC mediated signaling has been shown to both promote cooperation and deter antagonism between individuals (Table 1). Schooling is an important cooperative behavior in fish and tadpoles that results in enhanced foraging and predator avoidance. Several salmonid species along with the African clawed frog (*Xenopus laevis*) have been shown to preferentially form schools with relatives that share MHC haplotypes[39,46,49] and it has been previously shown that kin-based schools have higher survival rates and larger territories.[81] A second MHC-mediated cooperative behavior has been documented in house mice; female mice communally nest and nurse offspring and it has been demonstrated that females preferentially nest with familiar sisters. When no familiar sisters are available, they preferentially nest with MHC-similar females.[1] Finally, competition over territories is fierce in many species of vertebrates and can result in serious injury; evidence suggesting that MHC signaling prevents territorial competition between kin has recently been demonstrated in tuataras (*Sphenodon punctatus*).[38] Scores of other kin-based cooperative behaviors have been documented within vertebrates and it is quite probable that we have only just begun to document those that are mediated by MHC signaling; however, it is not our intent to imply that all cooperative behaviors will be MHC-mediated. In fact, the precision of kin recognition systems will be enhanced as more polymorphic systems are used in signaling.

Parent-Progeny Recognition

Parent-progeny recognition prevents the expense associated with parental investment into unrelated individuals. This is especially true under conditions of communal living or in systems that involve extra-pair matings. Under these circumstances an identification system that could ensure parental care was only provided to genetic offspring would be highly adaptive and many such systems have been documented.[82] Female house mice nest communally and are therefore at risk of providing parental care to unrelated pups. Yamazaki and others[73] showed that female house mice can identify pups with which they share an MHC haplotype from congenic pups (genetically identical individuals with the exception of MHC type). Pups at the age of 15-21 days were also capable of recognizing and preferring their parents bedding to that of a MHC dissimilar congenic individual. This preference was reversible by cross-fostering, again showing the role of familial imprinting within MHC signaling in house mice. Currently this study offers the only evidence that MHC-mediated signaling is involved in parent-progeny recognition and though it was conducted with inbred strains of mouse, it reveals the potential of MHC signaling in nature.

Inbreeding Avoidance

Degradation of fitness due to inbreeding is a result of increased homozygosity of deleterious recessive alleles that are identical by descent. These alleles combine more frequently when related individuals reproduce compared to outbred matings. Early assessment of the fitness costs of full-sibling level inbreeding within vertebrates (mice) have been conducted and early studies showed a 10% decline in litter size.[83,84] However, these experiments only measured litter size reductions and they failed to assess the fitness

consequences of the inbred progeny in their natural context. In an experiment where the fitness impacts of a single generation of full-sibling inbreeding were assessed under seminatural conditions, it was found that outbred male mice had five-fold higher fitness than inbred males, with the consequences effectively approaching lethality for inbred sons. Daughters suffered an additional 20% reduction in fitness compared to previous assessments.[85] Likewise cousin-level inbreeding was shown to reduce male fitness by 34% and when the infectious agent *Salmonella* was present in the populations, the fitness decline in males was 57%.[86] Since the true negative consequences of inbreeding were only revealed under direct competition within a seminatural environment, we now refer to this experimental system as a phenotron because it allows the observers "to see" the true fitness consequences (phenotype) of a treatment. Disassortative MHC-based mating preferences function as a mechanism of inbreeding avoidance due to their highly polymorphic nature. Only closely related individuals are likely to share MHC haplotypes; thus a mating preference for MHC-dissimilar individuals will decrease the likelihood of inbreeding. The extent to which inbreeding can be avoided is dictated by whether a self reference or familial imprinting mechanism is utilized by a particular species.[76]

An indirect piece of evidence supporting MHC haplotype based familial imprinting and inbreeding avoidance within house mice has come from a study by Sherborne and colleagues.[80] This experiment investigated the relative importance of MHC and MUPs in mediating inbreeding avoidance behavior and its conclusion was that MHC is not involved in inbreeding avoidance behavior. House mice were released into seminatural enclosures with only full-sibling and half-sibling counterparts; inbreeding avoidance was assessed by the proportion of full-sibling vs half-sibling matings and genetic analysis was used to determine if there was either an MHC or MUP-based signal mediating inbreeding avoidance. The data showed that although no full-sibling inbreeding avoidance occurred, mice sharing exact MUP genotypes avoided mating with each other. This led the authors to conclude that MUPs are exclusively responsible for inbreeding avoidance in house mice and that MHC plays no role. However, this conclusion is unwarranted due to a flaw in the experimental design. Specifically, prior to testing in seminatural enclosures, test animals had been caged (since birth) with other individuals that possessed MHC haplotypes that were present in the enclosures. This design unintentionally allowed MHC familial imprinting to occur on all of the tested haplotypes; thus, animals upon entering the enclosures found themselves surrounded by individuals that would all be recognized as relatives by MHC-based systems. This situation forced the mice to make mate choices based on other non MHC cues and they utilized MUPs, preferring to mate with individuals that did not share exact genotypes. These results suggest MUPs are utilized in mate choice, but contrary to the conclusions of the paper, the design does not allow for the exclusion of a role for MHC. Furthermore, MUP-based mating preferences are based on self reference and not familial imprinting,[80] thus they do not offer the same protection against inbreeding that familial imprinted MHC preferences do.

MHC AS A SIGNAL OF GENETIC COMPATIBILITY IN MATE CHOICE

Genetic compatibility, broadly defined, refers to the degree to which an organism's genes, (both within and between haploid genomes), interact to increase or decrease fitness. Consequences of genetic incompatibility include inviable offspring (e.g. between species mating), severely reduced fitness (e.g. inbreeding), and incremental degradation of fitness

associated with the combination of incompatible alleles (e.g. MHC homozygosity). The fitness consequences of genetic compatibility might be so severe that finding a mate with the "right genes" to compliments one's own genome provides more indirect benefits than finding the "best genes" within high quality individuals.[87] In order to make MHC-based mate choice (or gamete fusion[88,89]) decisions in regards to genetic compatibility, individuals must possess the means to assess their own MHC types (see section on phenotype matching systems above). MHC-mediated odors readily signal information about the genetic compatibility between mates, and MHC-disassortative mating preferences (Table 1) lead to the production of offspring with compatible genotypes both at the MHC and throughout the genome.[76] The mechanisms of MHC-mediated genetic compatibility described below are MHC heterozygote advantage, offspring harboring different MHC genotypes than their parents (moving target) and the avoidance of inbreeding.

Heterozygote Advantage/Superiority

MHC-disassortative mate preferences by their very nature produce MHC heterozygous offspring, which are hypothesized to have superior immunocompetence.[90,91] Multiple lines of evidence now support the fitness-enhancing role of MHC-heterozygosity.[92-98] It was initially argued that MHC heterozygotes would have an advantage (overdominance) because they could present a wider variety of peptide antigens to the immune system making them more likely than MHC-homozygotes to recognize and mount an immune response against disease-causing agents. However, this mechanistic hypothesis has largely been rejected since experimental infections with single pathogens reveal that heterozygotes do not generally have an advantage over both homozygotes.[99] An alternative mechanism postulated that heterozygote advantage emerges over multiple infections because resistance is generally dominant and heterozygotes will benefit from the resistance profile of each allele, which masks some of the susceptibilities of each allele. This hypothesis was experimentally confirmed by laboratory-based experiments using coinfections with parasites having opposite MHC resistance/susceptibility profiles, which demonstrated that heterozygotes are more fit than either homozygote.[99] Recent studies on wild salmon[100] and vole[101] populations demonstrate that MHC heterozygotes have increased fitness under natural conditions of multi-parasite infection as well. The fitness enhancing nature of MHC heterozygote advantage in laboratory and natural settings is an example of the adaptive significance of MHC mediated signaling.

Moving Target

In addition to heterozygote advantage, selection could also favor MHC-disassortative mate preferences if the offspring genotype provided a moving target against pathogen adaptation, causing pathogens adapted to either parent to be at a disadvantage in progeny that are MHC-dissimilar to both parents.[102] This hypothesis predicts that pathogens evolve to partially escape MHC-mediated immune recognition and that MHC-dissimilar offspring are more fit than their parents when challenged with parent-adapted pathogens. Like heterozygote advantage, mate choice decisions driven by moving target processes function to maximize genetic compatibility and are thus most effectively achieved using an MHC, self reference phenotype matching system.

Numerous examples highlight the capacity of pathogens to rapidly adapt to escape MHC-mediated immune recognition.[103-110] There has been one experimental study designed

to test the other prediction of the moving target hypothesis—that MHC-dissimilar offspring will be more fit than their parents when challenged with parent-adapted pathogens. MHC did influence the trajectory of adaptation by a fungal pathogen (*Cryptococcus neoformans*), but the large virulence increase in postpassage pathogen lines showed no specificity for the host MHC genotype of passage.[111] The most likely explanation is that this pathogen is a generalist that infects most birds and mammals. The passages in mice therefore selected for adaptations to "mouseness", which likely swamped any adaptations to MHC. Future passage studies should use pathogens specialized on the host of passage.

There is anecdotal evidence from human studies demonstrating the importance of offspring genetic diversity in reducing the probability of mother-to-child-transmission of chronic infectious disease agents (e.g., HIV-1[112,113]), and suggest that there would be a significant selective advantage to mate choices that promoted genetic diversity in offspring. There is also evidence linking increased HLA dissimilarity between mother and offspring with significantly reduced chances of vertical transmission of HIV-1[114,115]). The extent of pathogen adaptation during chronic infection of the parent and its impact on mother-to-child transmission dynamics was not addressed in the above studies. Despite this, they do support the possible role of MHC- disassortative mate preferences in producing offspring of higher quality that are more resistant to infection by chronic parasites of their parents.

Optimal MHC Heterozygosity

MHC-disassortative mate choice may carry a cost if maximal MHC diversity in offspring is not optimal. For instance, during the process of negative selection in the thymus, T cells with high affinity for MHC-peptide complexes are instructed to terminate themselves via apoptosis.[9] It follows then that MHC diversity may have an upper limit beyond which the fitness benefit of having multiple ways to present peptides from foreign invaders is offset by the cost of an increasingly limited T-cell repertoire.[116] If such a fitness cost exists, then it will have important implications on the evolution of MHC disassortative mating preferences. Indeed, it has been observed that individuals with intermediate versus maximal MHC diversity harbor lower parasite burdens in experimental infections.[117] Additionally, it was recently shown that intermediate and not maximal levels of MHC diversity lead to significantly higher lifetime reproductive success in stickleback offspring.[118] Thus, it seems that maximum MHC diversity can be a costly trait.

If intermediate rather than maximal MHC diversity is optimal then an MHC-typing system could allow individuals to "optimize" the MHC diversity within their offspring. Studies with sticklebacks have shown that females are in fact capable of such quantitative estimates of MHC diversity (also known as allele counting).[119] Additionally, by estimating the extent of intra-individual MHC class IIB allele diversity within a population, it was also demonstrated that individuals with intermediate rather than maximal MHC diversity were most frequent, indicating selection for intermediate levels of MHC diversity. Subsequent experimental findings in sticklebacks[43] and brown trout[50] suggest that much of the selection for individuals with intermediate MHC diversity derives from female preference for MHC-dissimilar mates. Together, these studies indicate that maximal MHC diversity is not always optimal and that female preference for MHC-dissimilar mates is a primary driving force behind selection for the production of individuals with intermediate rather than maximal MHC diversity.

Inbreeding Avoidance

Though inbreeding avoidance has already been covered within the kin recognition section it is important to stress that it also falls under the umbrella of MHC as a signal of genetic compatibility. In fact, inbreeding avoidance may be the single most adaptive result of MHC-disassortative mating preferences in many species of vertebrates, as both sibling and cousin level inbreeding have been found to have devastating effects on the fitness of offspring.[85,86] In addition, as covered in the evolution of MHC section below, growing evidence suggests that MHC mediated kin recognition to avoid inbreeding may have been the ancestral function of MHC molecules, which were later co-opted for use in the adaptive immune system.[120]

MHC AND SIGNALS OF QUALITY IN MATE CHOICE

In contrast to MHC-mediated signals that directly convey MHC genotype information (relatedness, compatibility or individuality), the disease resistance functions of MHC can also influence social signalling by modulating the expression of secondary sexual characters. Only high-quality, disease-resistant individuals should be able to invest in costly, sexually selected advertisements,[121] thus creating a correlation between MHC genotype and these condition-dependent traits (Table 2). By endowing an individual with genetic resistance to parasites, MHC genotype can indirectly influence signals of quality by allowing more physiological resources to be devoted to signaling rather than to the immune response.[122] von Schantz and colleagues[123] were the first to report an association between MHC and a sexually selected trait; they found that spur length in male pheasants (*Phasianus colchicus*) was correlated with fitness and dependent on MHC genotype. In a study on great snipes (*Gallinago media*), females preferred males carrying specific MHC allelic lineages. Males with these genotypes were also larger and females of this species are generally known to favor larger males.[124] A study in white-tailed deer (*Odocoileus virginianus*) found that MHC divergent heterozygous males had larger antlers and body size, which was correlated with lower abundance of abomasal nematodes.[122] Finally, a study

Table 2. MHC correlations with secondary sexual traits and mating preferences

Species	MHC Correlation with Mate Preference	MHC Correlation with Traits of Quality	Sources
Great snipe (*Gallinago media*)	MHC allele-specific preference	Body size	Ekblom et al 2004[124]
Peafowl (*Pavo cristatus*)	MHC heterozygosity	Train length	Hale et al 2009[36]
Pheasants (*Phasianus colchicus*)	MHC genotype	Spur length	von Schantz et al 1996[123]
White-tailed deer (*Odocoileus virginianus*)	MHC divergent heterozygotes	Antler and body size; reduced parasitism	Ditchkoff et al 2001[122]

on a canonical sexually selected trait, trains in male peacocks (*Pavo cristatus*), showed that the train length reflects genetic diversity at the MHC.[36] The above examples show that MHC-genotype can influence the expression of secondary sexual traits that are used as signals of quality. However, MHC-genotype itself is not necessarily used in the signal.

An alternative way that MHC-genotype can indirectly influence the expression of secondary sexual characteristics is if MHC social signals are themselves costly to produce. This hypothesis has recently been tested by the laboratory of Manfred Milinski, which identified the first example of condition-dependent MHC signaling.[125] They had previously shown that female three-spined sticklebacks prefer males with optimal, rather than maximal, MHC allelic differences (relative to her own genotype) and that this mate choice is mediated by excreted MHC peptides (discussed above).[14,44] Now, they show that females do not send this signal at all and that, remarkably, males only send this signal when they are in the reproductive state. These data suggest that MHC signaling is not simply a byproduct of MHC-peptide presentation, but that it is actively regulated in a fashion consistent with it being a costly signal. The authors suggest that shedding MHC-peptide complexes will create localized deficiencies of this critical immunological component and therefore represents a trade off between immune defense and social signaling.[125]

MHC-mediated signals of quality may allow an individual to gain either direct benefits for themselves or indirect genetic benefits for their offspring. Avoidance of parasitism is perhaps the most likely direct benefit of MHC-mediated mate choice. Social behaviors are an opportunity for parasites to transmit to new hosts; in turn, hosts will gradually develop behavioral mechanisms to avoid parasites.[126] Individuals of a particular MHC-genotype may be resistant to local parasites at any given time and choosing such an individual as a mate would provide a direct benefit of reduced risk of parasitism. Although there are several examples of mate choice for parasite-free individuals,[127-129] there are surprisingly few examples of studies that link MHC-dependent resistance to pathogens and subsequent mate choice.[117]

MHC EVOLUTION: WHAT ARE THE PRIMORDIAL FUNCTIONS?

Since the immune recognition function of MHC genes in adaptive immunity was discovered far earlier than MHC-mediated behaviors, and since it was so central to the complex system of vertebrate adaptive immunity, it was initially assumed that MHC-mediated behaviors were a derived function. However, Brown argued that since kin-selected behaviors (inbreeding avoidance and kin-biased cooperation) are present in the ancestral lineages leading to vertebrates and that adaptive immunity is a derived character in vertebrates, it is most parsimonious to hypothesize that MHC-mediated kin recognition functions were primordial.[74] This controversy continues to this day.

Boehm has recently written a tour-de-force, synthetic review that evaluates self and nonself recognition systems that exist across plants, fungi and animals, with a special emphasis on how quality recognition is maintained in the face of the rapid diversification of these highly polymorphic systems.[120] Quality control (the ability to accurately discriminate between self and nonself) is of particular importance in immune recognition systems that must achieve self tolerance to protect against auto-immune disease.[120,130]

Jawless fish are the one lineage of vertebrates that appear to have a non MHC based adaptive immune recognition system.[131,132] A high diversity of lymphocyte receptors in this group is created by combinatorial assembly of receptor modules, but the critical difference

from other vertebrates is that there is no junctional diversity created by mutagenic joining mechanisms.[133] Thus, the lymphocyte receptor repertoire for jawless fish is predictable and self tolerance could be achieved by Darwinian selection for self-compatible receptor modules.[120] In contrast, jawed vertebrates achieve higher lymphocyte receptor diversity by the mutagenic VDJ combinatorial joining process, which creates the problem of unpredictable receptor specificities that can lead to auto-immunity. These potentially harmful receptors are eliminated during the evaluation of lymphocytes receptors in the thymus of jawed vertebrates. Boehm argues that it seems unlikely that an MHC-peptide presentation system could emerge de-novo to create the modern jawed vertebrate immune recognition system, which allows self-tolerance in the face of somatic generation of unpredictable lymphocyte receptors. It would be far more likely that a pre-existing MHC-peptide kin recognition system could be co-opted for immune recognition.[120] Discovery of the MHC homologues and their function in jawless fish offers one of the most promising approaches for discriminating between these two hypotheses and identifying the primordial function of MHC genes. Tunicates (a close relative of vertebrates) have a highly polymorphic histocompatibility-type (fusion) locus that functions both in allo-recognition to control colony fusion and gamete fusion,[134] at least in some species.[135] It was thought that identifying the nature of this locus might clarify the early history of MHC genes. After a two-decade search the locus was identified to be a member of the immunoglobulin super family, but it appears to not have homology to MHC genes.[136-138] These findings further focus the search for primordial MHC functions towards jawless fish.

The facts that within vertebrates there are completely different mechanisms controlling adaptive immune recognition and that in tunicates histocompatibility functions are controlled by genes unrelated to vertebrate histocompatibility genes, highlight the evolutionary flexibility of how similar functions can be achieved through different genetic systems. It is currently difficult to discriminate between the different proposed primordial function of MHC genes. However, the initial assumption that immune recognition must be the primordial function of MHC genes, should no longer be the default assumption.

CONCLUSION

In this chapter we have demonstrated the significance of MHC signaling in regards to four aspects of social communication. First, studies in mice show that MHC peptides and to a lesser extent MHC-associated urinary odors, signal individuality in the context of pregnancy block. MHC does not signal individuality during mouse scent-marking, rather, a species specific signal (MUP) is used. Second, MHC as a signal of relatedness is found across vertebrates (Table 1) and plays a role in cooperation, parent-offspring identification and inbreeding avoidance via two different phenotype matching mechanisms: self reference or familial imprinting. Third, MHC signals are used to determine the genetic compatibility of a potential mate and can result in the production of heterozygous offspring. In some animals, mate choice for MHC compatibility is so finely tuned that they can optimize the degree of MHC heterozygosity in their offspring. Fourth, information regarding MHC genotype can be signaled indirectly through correlated characters (Table 2) and a recent study demonstrated that, at least within one species, MHC signaling itself may be condition-dependent and therefore a signal of individual quality. Taken together, these studies suggest that MHC-mediated signaling is conserved across vertebrates, but takes on unique functions depending on the life-history of a given species.

Appreciating the distinction between both modes of phenotype matching (self reference and familial imprinting) is paramount in understanding the role MHC-mediated signaling plays in social communication. Though substantial overlap in functionality exists between these phenotype matching systems, there are tradeoffs. Self reference systems facilitate mating preferences that generate offspring with an immunological advantage by allowing the assessment of genetic compatibility. Familial imprinting systems of phenotype matching facilitate the identification of siblings, half-siblings and cousins; in species where either cooperative behavior or avoiding inbreeding is important (e.g., communal nesting species or species that live in high-density populations), a familial imprinting system provides an advantage over a self reference system because self/nonself discrimination is not required to increase indirect fitness. That these two systems are differentially utilized by different groups of vertebrates highlights the highly context-dependent nature of social signaling. It is important to note, however, that phenotype matching mechanisms have been described in a relatively small number of species (Table 1) and more studies are needed.

The remarkable fact that a single genetic system controls major components of both immune recognition and social recognition begs the question of which recognition system constituted the primordial function of MHC genes. The convergent evolution of similar peptide binding properties of MHC, VSN and OSN receptor molecules provides the molecular basis by which MHC genotype influences both immune and social recognition; it also implies that these distinct receptor families have responded to selective pressures that required information regarding MHC genotype (bound peptides) be associated with discriminatory sensory systems. Finally, the ubiquitous presence of various modes of self versus nonself discrimination across all three domains of life, coupled with the derived nature of the adaptive immune system in vertebrates, further suggests that MHC-mediated social signaling evolved for the purpose of discrimination between conspecifics and could represent the ancestral state. Tracing the function of MHC molecules across vertebrate evolution holds the greatest promise of resolving the relative importance of immune versus social communication in MHC evolution.

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