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Review

The ancestry and cumulative evolution of immune reactions

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The last two decades of study enriched greatly our knowledge of how the immune system originated and the sophisticated immune mechanisms of today's vertebrates and invertebrates developed. Even unicellular organisms possess mechanisms for pathogen destruction and self recognition. The ability to distinguish self from non-self is a prerequisite for recognition of sexual compatibility and ensuring survival. Molecules involved in these processes resemble those found in the phagocytic cells of higher organisms. Recognition of bacteria by scavenger receptors induces phagocytosis or endocytosis. The phagocytic mechanisms characterizing the amoeboid protozoans developed further during the evolution towards innate immunity. The scavenger receptor cysteine-rich domain SRCR is encoded in the genomes from the most primitive sponges to mammals. The immune system of sponges comprises signal transduction molecules which occur in higher metazoans as well. Sponges already possess recognition systems for pathogenic bacteria and fungi, based on membrane receptors (a lipopolysaccharide-interacting protein, a cell surface receptor recognizing β(1→3)-D-glucans of fungi). Perforin-like molecules and lysozymes are involved, among others, in defense in sponges. Reactive oxygen and nitrogen species function in the immunity of early metazoan. Genes encoding the family of reactive oxygen-generating NADPH oxidases (Noxes) are found in a variety of protists and plants. The NO synthases of cnidarians, mollusks, and chordates are conserved with respect to the mammalian NOS. The antimicrobial peptides of protozoans, amoebapores, are structural and functional analogs of the natural killer cell peptide, NK-lysin, of vertebrates. An ancestral S-type lectin has been found in sponges. Opsonizing properties of lectins and the ability to agglutinate cells justify their classification as primitive recognition molecules. Invertebrate cytokines are not homologous to those of vertebrate, and their functional convergence was presumably enabled by the general similarity of the lectin-like recognition domain three-dimensional structure. Sponges contain molecules with SCR/CCP domains that show high homology to the mammalian regulators of complement activation (RCA family). A multi-component complement system comprising at least the central molecule of the complement system, C3, Factor B, and MASP developed in the cnidarians and evolved into the multilevel cascade engaged in innate and acquired immunity of vertebrates. The adaptive immune system of mammals is also deeply rooted in the metazoan evolution. Some its precursors have been traced as deep as in sponges, namely, two classes of receptors that comprise Ig-like domains, the receptor tyrosine kinases (RTK), and the non-enzymic sponge adhesion molecules (SAM). The antibody-based immune system defined by the presence of the major histocompatibility complex (MHC), T-cell receptor (TCR), B-cell receptor (BCR) or recombination activating genes (RAGs) is known beginning from jawed fishes. However, genes closely resembling *RAG1* and *RAG2* have been uncovered in the genome of a see urchin. The ancestry of MHC gene remains unknown. Similarly, no homologue of the protein binding domain (PBD) in MHC molecules has been found in invertebrates. The pathway by which endogenous peptides are degraded for presentation with class I MHC molecules utilizes mechanisms similar to those involved in the normal turnover of intracellular proteins, apparently recruited to work also for the immune system. Several cDNAs coding for lysosomal enzymes, e.g., cathepsin, have been isolated from sponges. All chromosomal duplication events in the MHC region occurred after the origin of the agnathans but before the gnathostomes split from them. The V-domains of the subtype found in the receptors of T and B-cells are known from both agnathans and cephalochordates, although they do not rearrange. The rearrangement mechanism of the lymphocyte V-domains suggests its origin from a common ancestral domain existing before the divergence of the extant gnathostome classes. Activation-induced deaminase (AID) — homologous proteins have been found only in the gnathostomes. It appears thus that the adaptive immunity of vertebrates is a result of stepwise accumulation of small changes in molecules, cells and organs over almost half a billion years.

Keywords: antibody-based immunity, antigen presentation, complement, cytokines, evolution, innate immunity, invertebrates, nitric oxide, phagocytosis, protoza, receptors, signal transduction, sponges, superoxide, vertebrates

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Abbreviations: ABC, ATP-binding cassette; ABCB, ABC subgroup B; AID, activation-induced deaminase; AIF, allograf inflammatory factor; AIS, adaptive immune system; BCR; B-cell receptor; Bf, factor B; CCP, complement control protein; CRD, carbohydrate recognition domain; EGF, epidermal growth factor; GRP, glucose-regulated protein; HLA; human leukocyte antigen; IFN, interferon; ILD, Ig-like domain; IRAK, interleukine receptor-associated kinase; LPS, lipopolysaccharide; LRR, leucine-rich repeats; MAC, membrane attack complex; MASP, MBL-associated serine protease; MBL, mannose-binding lectin; MHC, major histocompatibility complex; NK-lysin, natural killer cell-lysin; NOS, nitric oxide synthase; Nox, phagocyte NADPH oxidase; PLC, phospholipase C; PDB, protein-binding domain; PRR, pattern recognition receptor; PSMB; proteosome macropain subunit beta-type; RAG, recombination activating genes; RCA, regulators of complement activation; RTK, receptor tyrosine kinase; SCR, short consensus repeats; SRCR domain, scavenger receptor cysteine-rich domain; TAP; transporter-associated with antigen processing; TCR, T-cell receptor; TEP, thioester protein; TGF, tumor necrosis factor; TLR, Toll-like receptor

Although immunology emerged as a modern science with the experiments on sea urchin embryos by Ilya I. Metschnikoff in 1882, and the role of B lymphocytes was recognized owing to physiological studies on the chicken *bursa Fabricii* by Bruce Glick in 1956, most what we know about immune reactions concerns mammals. This is because the main object of studies has been just a single vertebrate species, *Homo sapiens*. No wonder that for a long time it was considered self-evident that most of the complexity of immune mechanisms emerged quite recently, within the mammalian lineage. But this is hardly the case.

The immune system has developed in a stepwise way by progressive sophistication of basic functions that helped ancestral organisms to survive in their hostile environment. Even before developing the true immune mechanisms, they had to recognize their own body from the bodies of predators and parasites. Those exchanging genetic information had to develop an ability to recognize individuals of the same or different sex. This required reliable chemical sensors and routes of transferring information obtained by them to effectors acting in a way specific enough, different in respect to an enemy and to a mate.

The immune system responds to invasion using two crucial functions: sensors detect the invader, and elaborate response effectors attack it. The invader interacts with soluble or membrane-bound molecules of the host, capable of discriminating precisely between self (host) and non-self (pathogen). These molecular sensors recognize broad structural motifs (combination of sugars, certain proteins, particular lipid-bearing molecules, and some nucleic acid motifs) that are highly conserved within a microbial species but are generally absent from the host. Because they recognize particular overall molecular patterns, such receptors are called pattern recognition receptors (PRRs). The Toll-like receptors, belonging to PRRs, are the most important group of innate receptors which detect microbial products. Signals initiated at the TLRs of macrophages stimulate phagocytosis and production of chemical agents that are toxic to the phagocytosed microbes.

Detection of pathogen-associated molecular patterns by soluble or membrane-bound mediators of innate immunity triggers an action of multiple components of immunity. The soluble mediators include initiators of the complement system (mannose-binding lectin, C-reactive protein). The activation cascade of the complement leads to opsonization or lysis of the invaders. In addition, some of the byproducts of complement activation promote inflammation and thereby bring leukocytes to the sites of infection. Activated macrophages also secrete a class of molecules, known collectively as cytokines, that communicate *via* cell receptors to induce specific cell activities. For example, activated macrophages secrete cytokines such as IL-1, IL-6 and TNF-α, which induce and support inflammatory response. The cytokines released by cells involved in the innate response affect the nature of subsequent adaptive immune responses to the infection.

In vertebrates, antibodies and T-cell receptors, the sensors of adaptive immunity, recognize finer details of molecular structure. During the adaptive response, cytotoxic T-cells detect and destroy pathogens in the host's cells; wheareas antibodies produced by B-cell neutralize the capability of the invader to infect other cells. Moreover, antibodies (due to antibody-mediated uptake) increase the likelihood that the invader will be phagocytosed by macrophages and neutrophils. Antibodies also activate the complement system to bring about the lysis of microbes. After the infection is cleared, some of the B and T-cells will persist in the host in the form of memory T and memory B-cells. Further infections by the pathogen will then be met by a ready reserve of lymphocytes specific for the pathogen and capable of mounting a rapid response.

Below, an inferred sequence of events leading from the hypothetical simplest organisms able to mount immune reactions to the most advanced mammalian adaptive reactions is reviewed. Many of the ideas presented remain working hypotheses, requiring testing by more complete and reliable evidence. Research in this area is expanding steadily and this review is intended to be just an introduction to the extensive literature on the subject.

ORIGIN OF SELF/NON-SELF RECOGNITION

Without an ability to distinguish self from non-self no species of eukaryotic sexual organisms could survive, as this is a prerequisite to recognizing sexual compatibility. Moreover, this is the way to prevent feeding on individuals of the same species. Thus, the self/non-self mechanism is crucial not only for survival of individuals but also for speciation. This is achieved owing to specialized genomic regions that promote self/non-self interactions during sexual reproduction of eukaryotes. The sex-determining regions of the genome include mating type loci in protozoans and sex chromosomes in multicellular plants and animals. The mechanism of self/non-self recognition is known to function in the acrasid „amoebae" *Dictyostelium*: the self recognition to avoid cannibalism is based on a species-specific receptor which is lacking in canibalistic strains (Waddell & Duffy, 1986).

Since unicellular eukaryotes phagocytose for food and defense, phagocytosis is the most ancient and universal tool of defense against foreign material. Amoebae are distant relatives of the animals, but they already show mechanisms that allow recognition, internalization and destruction of foreign material. In fact, amoebae and macrophages share similar phagocytic mechanisms, e.g., prey-recognition by cell surface receptors (Allen & Dawidowicz, 1990) and prey-killing by oxygen radicals (Davies *et al.,* 1991). In the case of *Dictyostelium* phagocytosis, a heterotrimeric G protein and phospholipase C are involved in cell signalling (references in Cardelli, 2001). Apparently, the phagocytic mechanisms characterizing the amoeboid protozoans were inherited during the evolution towards innate immunity (Sillo *et al.,* 2008). Recently, it has been found that genes encoding carbohydrate-binding membrane proteins such as a gp138-similar protein, a putative TIR-like domain (Toll/IL-1R-like domain)-containing protein, a bystin-similar protein, or a tetraspanin family protein, are up-regulated during phagocytosis (Sillo *et al.,* 2008). In mammalian cells tetraspanins are suggested to be involved in the early steps of phagocytosis (Little *et al.,* 2004; Artavanis-Tsakonas *et al.,* 2006). TIR-like proteins are essential for interaction in the TLR signal transduction pathway. One of at least two genes encoding a cytosolic protein with TIR-like domain (*tir A*) was found to participate in an immune-like response in *Dictyostelium* (Chen *et al.,* 2007). A special type of cells, the S („sentinel") cells, carry out detoxification and immune-like phagocytosis. They thus provide a simple innate immune system for the social amoebae, being functional analogues of neutrophils and macrophages. The tirA protein performs a role of immune-related sig-

nalling system in the response of *Dictyostelium* to bacteria. This suggests that the use of TIR domain-based signalling for defense is an ancient function already present in the progenitor of all present-day groups of eukaryotes, preceding the appearance of multicellularity.

The choanoflagellates, unicellular and colonial protozoans closely related to sponges (King *et al.,* 2003), have a number of cell-signalling and adhesion protein families typical for the metazoans; these include cadherins, C-type lectins, several tyrosine kinases, and components of tyrosine kinase signalling pathways. The presence of proteins involved in cell to cell interaction in protozoans demonstrates that these proteins evolved before the origin of multicellular animals and were later co-opted for their development.

CELLS OF THE IMMUNE SYSTEM

It seems self-evident that the necessity for protection of integrity of organisms against parasites intensified dramatically with the appearence of multicellular organization and brought about further improvements of immunity. The evolutionary „arms race" resulted in sophistication of both immune reactions and ways to „cheat" the host's immune system by parasites. It is a popular, but wrong, assumption that highly specialized blood cell types involved in gas transport (erythrocytes), blood clotting (thrombocytes) and immune response/tissue repair (leukocytes) are specific to the vertebrates. Actually, freely moving cells that have properties of at least some of the vertebrate blood cells are present in all animals (Hartenstein, 2006). In animals without the coelom (epithelium-lined body cavity) these cells are named amoebocytes, interstitial cells or neoblasts. In animals that have evolved the coelom along with a vascular system, these cells are commonly referred to as coelomocytes and/or haemocytes.

Sponges represent presumably the most basal clade of multicellular animals (Metazoa). The gelatinous matrix (mesoglea) between the ectoderm and endoderm of their body contains large numbers of motile amoebocytes that carry out multiple functions, the most primitive being digestion. Cnidarians and ctenophores also have a cell-rich mesoglea with interstitial cells that may act as phagocytes (Fautin & Mariscal, 1991). Haemocytes occur in the vascular lumen and the coelomic cavity (coelomocytes) of all coelomate invertebrates. Plasmatocytes, one of four classes of haemocytes, can be compared to monocytes/macrophages/histiocytes of vertebrates. These are phagocytic cells involved in the removal of apoptotic cells during the development, as well as in the ingestion or encapsulation of pathogens (innate immune response). Granulocytes (the second of the four classes of haemocytes) are involved in developmental and metabolic functions, as well as in immune functions including wound healing, blood clotting, phagocytosis and encapsulation of pathogens. In *Drosophila*, injuries evoke a clotting response that consists of the aggregation of haemocytes, followed by plasma coagulation caused by release of clotting factors from storage granules in haemocytes. Foreign bodies (such as eggs of parasites deposited in the host body cavity) are countered by cellular capsules formed by the plasmatocytes. Phenol oxidase is involved in the clotting as well as encapsulation of pathogens. In invertebrate deuterostomes (echinoderms, hemichordates, cephalochordates, and urochordates), haemocyte classes with characteristics similar to those in other invertebrates occur. Granular haemocytes and macrophages have been described in the coelom and vascular lumen of *Amphioxus* (Rhodes *et al.,* 1982). Haematopoiesis in insects is under the control of a number of transcription factors and signalling pathways (such as GATA factors, JAK/STAT or Notch pathways) (Meister & Lagueux, 2003), most of which have homologues that participate in the control of mammalian haematopoiesis.

MOLECULES USED IN SPONGE IMMUNITY

The Porifera include the least derived of metazoans, lacking epithelial (except for some species of controversial affinities) or muscular tissues. Despite their anatomical simplicity, sponges have molecules similar in structure to those involved in the immune system in higher animals. This concerns both the self/non-self recognition and the innate immune system in mammals based on phagocytosis, the complement system, and macrophage-derived cytokines.

Self/non-self recognition

Sponges, like other animals including mammals, demonstrate immunological self-tolerance (Moscona, 1968). A strong up-regulation of integrin receptors at zones between sponge autografts suggests their functional involvement (Wimmer *et al.,* 1999). It has also been found that spherical-shaped *Suberites* cell aggregates of different species cultivated *in vitro* initiate a signal transduction mechanism resulting in the induction of a gene encoding preB-cell **c**olony-**e**nhancing **f**actor (CEF). The identity between the human and sponge genes is over 55% (Samal *et al.,* 1994). The human preB-cell CEF is a secreted protein and its gene can be induced in lymphocytes by a lectin, however, it is not tissue specific. A cytokine-responsive macrophage molecule termed **a**llograft **i**nflammatory **f**actor 1 (AIF-1) has been identified in rat cardiac allografts (Utans *et al.,* 1995). The expression of the sponge gene encoding a putative AIF-1 protein was observed both in autografts and allografts from *Suberites* (Müller *et al.*, 1999). Like the vertebrate molecules, also the sponge AIF-1-related protein has an EF-hand (Ca^{2+}-binding) motif and a motif characteristic of peptide hormones. All this strongly suggests that sponges are provided with a molecular mechanism to recognize non-self. Some components of this mechanism, e.g., proteasome genes (Pancer *et al.,* 1996b), have already been found. It was previously assumed (Humphreys *et al.,* 1994) that in invertebrates the process of allorecognition is based primarily on active recognition of "self," but it appears that, similarly as in vertebrates, allorecognition in sponges involves also reactions to "non-self" antigens. It has been suggested that during allorecognition tissue responses the melanization occurs as well (ref. in Müller *et al.,* 1999b). Melanin is a polymer formed by phenoloxidase(s) from tyrosine, which is produced from phenylalanine by phenylalanine hydroxylase (PAH). In the sponge *Geodia,* allogeneic rejection involves an up-regulation of PAH. The sponge enzyme is the phylogenetically oldest member of a group to which all metazoan PAHs belong (Wiens *et al.,* 1998).

Phagocytosis

In mammals, macrophages are the firsT-cells that encounter non-self material, especially bacteria, and engulf

and subsequently degrade them using hydrolytic enzymes and oxidative attack. A variety of receptors on macrophages are used to detect infection. Recognition of bacteria and apoptic hosT-cells by scavenger receptors induces phagocytosis or endocytosis (Kindt *et al.*, 2007). Phagocytosis is the major cellular defense system in sponges. Phagocytized bacteria on which sponges feed are ingested with the help of both oxidative (Peskin *et al.,* 1998) and nonoxidative (enzymatic) mechanisms. Several cDNAs coding for lysosomal enzymes, e.g., cathepsin (Krasko *et al.,* 1997) have been isolated from sponges. Type I macrophage scavenger receptor has highly conserved cysteine-rich (SRCR) domains (Resnick *et al.,* 1994). The SRCR domain consists of approx. 100 amino acids with conserved spacings of six (group A) to eight (group B) cysteines which form intradomain disulfide bonds. Both types of SRCR domains are found in a putative sponge **a**ggregation **r**eceptor, AR (Pancer *et al.,* 1997; Blumbach *et al.,* 1998) and a putative sponge "multi-adhesive protein". The SRCR domains are found in receptors of higher metazoans, *for instance in speract (sperm-activating)* peptide receptor of see urchin egg (Dangott *et al.,* 1989), human CD6 antigen (group B scavenger receptor), macrophage M130 antigen, as well as in soluble protein complement factor I. The putative sponge „multi-adhesive protein" comprises, besides the SRCR module, a fibronectin module, and a **s**hort **c**onsensus **r**epeats (SCR) module. This latter module is present in the putative aggregation receptor as well (Blumbach *et al.,* 1998). The short consensus repeats are typical for the mammalian complement control (regulatory) protein superfamily (Reid & Day, 1989) and coagulation factor XIII-b (Nonaka *et al.,* 1993). The sponge SCR repeats region (370 amino-acids) reveals a similarity to mammalian selectins, mammalian complement receptors, and invertebrate SCR proteins (*Limulus* clotting factor C), all of which are characterized by four conserved cysteine residues in each repeat.

Sensing of pathogens

Sponges possess recognition systems for pathogenic bacteria and fungi. The lipopolysaccharide (LPS)-interacting protein on the sponge cell surface is a receptor for Gram-negative bacteria. Upon binding to LPS (endotoxin), the receptor dimerizes and interacts with MyD88-like protein. A perforin-like molecule (with high sequence similarity to the mollusc macrophage-expressed protein) is up-regulated as result of LPS binding (Wiens *et al.,* 2005). Additionally, *Suberites* responds to LPS with up-regulation of tachylectin, a D-GlcNAc-binding lectin (Schröder *et al.,* 2003). In higher metazoans, LPS-interacting proteins bind the endotoxin in concert with TLRs. This association leads to a signal transduction cascade, resulting in an increased expression of genes coding for cytokines (e.g., interferon or tumor necrosis factor) (Pålsson-McDermott & O'Neill, 2004; Wiens *et al.,* 2007). In the sponge immune response to bacterial lipopeptides, three major elements, TLR, the IL-1 receptor associated kinase-4 like protein (IRAK-4l), and a novel effector caspase from *Suberites*, have been identified. *Suberites* recognizes Gram-positive bacteria through binding to the bacterial proteoglycan (ref. in Wiens *et al.,* 2005); consecutively, the sponge cells respond with increased synthesis of lysozyme and endocytic activity. With respect to fungi, sponges are provided with a cell surface receptor recognizing β(1→3)-D-glucans (Perović-Ottstadt *et al.,* 2004); after binding, a signal transduction pathway is initiated that results in the expression of genes encoding a fibrinogen-like protein and epidermal growth factor. These anatomically least derived extant metazoans already possess the signaling pathways typical for the antimicrobial immune systems of other metazoans. Like in mammals, after LPS binding to sponge cells (ref. in Wiens *et al.,* 2005) the mitogen-activated protein kinase pathway is activated (p38 kinase and c-Jun N-terminal kinase) (ref. in Wiens *et al.,* 2005; Böhm *et al.,* 2000) and the NF-*κ*B pathway (Wiens *et al.,* 2005; 2007) are activated. The *NF-κB* gene of the sponge *Amphimedon queenslandica* has been found to be expressed during *A. queenslandica* embryogenesis, suggesting a developmental role, but it is possible that *NF-κB* also has an immune function in sponges (Gauthier & Degnan, 2008).

Molecules involved in signal transduction in sponges show sequence similarity to molecules found in higher metazoan phyla. The LPS-interacting protein is related to the aggregation factor known from the sponges *Microciona* (Fernàndez-Busquets & Burger, 1997) and *Geodia* (Müller *et al.,* 1999a). The LPS-interacting protein has, for instance, a threonine/proline-rich motif that is also found in other metazoan receptors (Gan *et al.,* 2004). Perforin-like molecules are involved in defense against bacteria in humans (Ambach *et al.,* 2004) and molluscs (Mangel *et al.,* 1992), as well as in the sponge system (Wiens *et al.,* 2005). The sponge perforins show considerable sequence similarity to the mammalian macrophage-expressed protein, as well as the mollusc *Haliotis* protein (abMpeg1); it is especially high within the perforin domain (Mah *et al.,* 2004). Since the sponge MyD88 (in higher metazoans, MyD88 is an adapter protein for TLR) has a TIR domain but lacks a clear death domain, and the predicted extracellular domain of the sponge TLR lacks true **l**eucine-**r**each **r**epeats (LRRs) the presence of canonical Toll/TLR pathway in sponges is, according to Hemmrich *et al.* (2007), not evident.

Molecules of vertebrate adaptive immune system (AIS)

Some precursors of the adaptive immune system of mammals have also been traced in sponges. The expression of a protein similar to mammalian lymphocyte-derived cytokine is up-regulated during non-self-recognition in *Suberites*. In *Geodia*, two classes of receptors with Ig-like domains have been identified: **r**eceptor **t**yrosine **k**inase (RTK) and the non-enzymic **s**ponge **a**dhesion **m**olecules SAM. They contain two polymorphic Ig-like domains. The expression of these molecules is also up-regulated during grafting. Amino acid substitutions within the Ig-like domains in *Geodia* are restricted to „hot spots" (Pancer *et al.,* 1996a). The *Geodia* RTK molecule comprises (1) an extracellular part with a Pro/Ser/Thr (P/S/T)-rich region and two complete immunoglobulin (Ig)-like domains, (2) a transmembrane domain, (3) a juxtamembrane region, and (4) a catalytic tyrosine kinase (TK) domain (ref. in Müller *et al.,* 1999a). The intraspecies polymorphism of Ig-like domains suggests that RTK is probably a key molecule involved in recognition in *Geodia* (Pancer *et al.,* 1998). Two sponge adhesion molecules (SAM) have been isolated, differing in size (Blumbach *et al.,* 1999). The Ig-like domains are present in both species. Only the long form of SAM contains a Pro-Ser-Thr-rich domain. It may be noted that the T-cell receptor has a structure similar to that of the sponge adhesion molecules from *Geodia*: two extracellular Ig-

like domains and a short cytoplasmic tail (Müller *et al.,* 1999a). To conclude, it seems that at the moment of splitting off of the evolutionary branch of sponges many of the key molecules that are used in higher vertebrates for their innate and adaptive immune systems were already developed. It is likely that some of those molecules acquired a dual function during subsequent evolution, first as cell adhesion receptors or growth control factors and then as immune molecules required for self/non-self-recognition. At present however, it cannot be stated with certainty whether the sponge proteins showing high sequence similarities with the immune proteins of other lower invertebrates or mammals also have similar function.

RECEPTORS IN INNATE IMMUNITY

Various kinds of pattern recognition receptors (PRR) are involved in the identification of foreign factors in vertebrates and invertebrates (Akira *et al.,* 2006). They seem to have originated independently of each other by recruiting molecules or pathways earlier performing different physiological or developmental functions in ancestral organisms. Among such receptors of different evolutionary origins are scavenger receptors and Toll-like receptors. Also cytokine-like receptors and lectins may have originated in invertebrates independently from their vertebrate counterparts and may be unique to a particular taxonomic group.

Scavenger receptors

Scavenger receptors have high affinity for a broad array of polyanionic ligands (bacteria, modified lipopolyprotein, for instance acetylated low-density lipopolyprotein, and apoptotic cell debris). The ability to bind polyanions appears to be a widely conserved feature and, as a consequence, various classes (A–H) of scavenger receptors have little structural and weak functional homology (reviewed in Peiser *et al.,* 2002). The scavenger receptor cysteine-rich domain (SRCR) is an ancient and highly conserved protein module of about 100 amino acids. In sponges that do not have specialized immune cells, the scavenger receptors appear to be fairly evenly distributed in the sponge body. They may have various roles, such as adhesion and aggregation mediated through cell–cell interactions (Blumbach *et al.,* 1998; Pahler *et al.,* 1998), owing to the presence of the fibronectin domain. The sea urchin genome contains approx. 150 genes encoding proteins with of one or more SRCR domains; however, the functions of these genes are not clear. Many of these 150 proteins containing SRCR domains have multiple splice variants (Pancer, 2000). Unequivocal class C scavenger receptors have been found up to date in *Drosophila* (Lazzaro, 2005), amphioxus (Huang *et al.,* 2008), and lamprey (Mayer & Tichy, 1995). This lamprey scavenger receptor may be involved in intercellular contacts and cell activation or differentiation in the immune system as it is a type-I integral membrane glycoprotein containing two SRCR domains flanking five epidermal growth factor (EGF)-like repeats. In the teleost fishes, scavenger receptors have been found to be expressed on subsets of non-specific cytotoxic cells (equivalent to natural killer cells) (Kaur *et al.,* 2003) and on phagocytic cells. Whether the SRCR domain originated from a single ancestral gene early in the evolutionary history or arose several times independently is not clear. Although it appears to be present in the genome of both lower metazoans, such as sponges, and higher ones, such as mammals, there are organisms (for example nematodes) in between that do not have proteins containing SRCR domains.

Toll-like receptors

Toll-like receptors (TLRs) are type I integral membrane glycoproteins possessing extracellular domains with varying numbers of leucine-rich repeats (LRR) motifs, and a cytoplasmic signalling domain homologous to that of interleukin 1 receptor (IL-1R). This domain, TIR, which denotes **T**oll, **i**nterleukin-1 receptor, and plant disease **r**esistance genes (Beutler & Rehli, 2002) is the most conserved protein motif within the Toll-like receptors. A TIR-like domain was suggested to be present in prokaryotes (Beutler & Rehli, 2002) and has been found in amoebae (Sillo *et al.,* 2008). In plants, the N gene of tobacco is related in sequence to Toll and many additional plant disease resistance genes encode Pelle homologues such as Pto (Whitham *et al.,* 1994; Tang *et al.,* 1999). Pelle is a homologue of mammalian IL-1R-associated kinases (IRAKs). The plant LRR-TIR and Pelle-like R proteins most likely function in signalling cascades quite divergent from their *Drosophila* and human counterparts, as no plant Rel homologues (transcription factors) have been identified. This suggest that Pto could participate directly in pathogen recognition (Rathjen *et al.,* 1999; Tang *et al.,* 1999).

The Toll/TLR pathway in animals predates the cnidarian-bilatelarian divergence. The canonical Toll/TLR pathway is present in the anthozoan cnidarians (Miller *et al.,* 2007). A Toll/TLR protein closely resembling *Drosophila* Toll in both domain structure and amino-acid sequence is found in these animals (Hemmrich *et al.*, 2007). Toll like receptors are confined to the metazoan phyla occurring in most of them, except for flatworms and annelids (Kanzok *et al.,* 2004). Based on molecular phylogenetic interpretations, these groups of animals are closely related as members of the spiralian (Lophotrochozoa) clade, the flatworms (Platyhelminthes) being located at the tip of the tree, above the Annelida and Mollusca. One secondary loss of TLR is thus enough to explain this pattern of occurrence (but TLR are known in bivalve mollusks). The two nematode TLRs (from *Caenorhabditis* and *Strongyloides*) form a separate cluster basal to all those of arthropods (both phyla belong to the Ecdysozoa clade) but are distinct from the vertebrate ones. *Tol-1* (the only *Tlr* gene) expression in adults *Caenorhabditis* is restricted to the nervous system and is implicated in embryonic development and pathogen recognition. *Tol-1 Caenorhabditis* mutants are susceptible to bacterial infection (Akira *et al.,* 2006) and it was shown that the *Toll* gene is involved in chemosensory perception of pathogenic bacteria, thus contributing indirectly to the host defense (Pujol *et al.,* 2001). Results of Tenor and Aballay (2008) indicate that Tol-1 has a direct role in response to certain Gram-negative bacteria and is required for the correct expression of ABF-2, which is a defensin-like molecule, and heat-shock protein 16.41, which belongs to the HSP family of proteins required for *C. elegans* immunity. These results show that a part of the TLR-mediated immunity is evolutionarily conserved. However, the role of Tol-1 in *C. elegans* immunity is not central and another candidate for the immune receptor has been proposed from LRR-containing receptors (Irazoqui *et al.*, 2010).

Drosophila Toll1, the most important member of its family, cannot be considered a homologue of mammalian TLRs. In contrast to mammalian TLRs, expression

of *Drosophila* Tolls is not restricted to immune-responsive cells. Rather, Tolls show complex stage- and tissue-specific expression patterns during embryonic and larval development. Almost all arthropod TLRs (with the exception of Toll9 which cluster with mammalian TLRs) form a separate cluster from the mammalian counterparts although they share a common ancestor (Luo & Zheng, 2000). This leads to the suggestion that TLR-mediated immunity developed independently in arthropods and mammals. In both pathways (immune and developmental) of insect the Toll protein first binds the extracellular protein Spätzle (a protein with structural similarities to mammalian neutrophins). The generation of distinct Spätzle fragments by two distinct proteolytic cascades is thought to orientate the Toll pathway towards developmental or immune regulation (Beschin *et al.,* 2001). A bivalve TLR was found to be homologous to *Drosophila* Tolls; moreover, expression of this receptor was regulated by LPS (Qiu *et al.,* 2007). In the horseshoe crab tToll does not function as an LPS receptor on granular haemocytes. It seems possible that the clotting protein coagulin may induce dimerization or oligomerization of tToll, leading to the activation of intercellular signalling (Inamori *et al.,* 2004). Some animal species appear to have many more TLRs than others. For instance, in a see urchin 222 genes encoding Toll-like receptors were found (Rast *et al.,* 2006). The *Amphioxus* genome contains 71 TLR genes (Huang *et al.,* 2008). Mammalian Toll-like receptors (12 members) are grouped into six families on the basis of sequence similarities. All these families are conserved in vertebrates from fish to mammals (Akira *et al.,* 2006).

According to the predominant view, a signalling pathway has been coopted from the developmental role for innate immunity function (Akira *et al.,* 2006). In view of the presence of a canonical Toll/TLR pathway in cnidarian immunity, the opposite view should be considered as well. Although TLR-mediated immunity developed independently in arthropods and mammals, Toll signaling is highly evolutionarily conservative, as similarities between proteins taking part in mammalian and *Drosophila* signal pathways are obvious. In *Drosophila*, infection with fungi or Gram-positive bacteria activates the Toll pathway similarly to the mammalian Toll pathway. In mammals, however, TLRs interact with microbial pattern molecules, whereas in the fruitfly the invading Gram-positive bacteria are recognized by peptidoglycan-recognition proteins, followed by the activation of the Toll ligand Spätzle *via* a protease cascade. Spätzle binds Toll, initiates signaling pathways by recruiting *Drosophila* MyD88 and Pelle (which has a serine-threonine kinase domain and is homologous to mammalian IRAKs) and induces nuclear translocation of the NF-κB-related transcription factors Dorsal and DIF. The end effect of Toll signalling is the dissociation of transcription factors from the protein Cactus, a homologue of mammalian IκBs (Hoffmann, 2003). In *Drosophila*, Gram-negative bacteria activate the IMD pathway. The putative receptor, **P**eptido**g**lycan **r**ecognition **p**rotein (PGRP)-LC, containing a transmembrane domain is responsible for the activation (Gottar *et al.,* 2002). The IMD protein contains a death domain highly homologous to that of mammalian RIP (TNF-receptor-interacting protein) (Hoffmann, 2003). *Drosophila* homologues of mammalian FADD (which associates with IMD), TAK1 (TGF-β activated kinase 1) and IKK-β (an equivalent of mammalian signalosome) induce phosphorylation of a NF-κB-like protein (Relish), which results in its cleavage and subsequent nuclear translocation. Relish induces the expression of genes encoding antimicrobial peptides against Gram-negative bacteria. It is worth to note that the nematode *C. elegans*, unlike other animals, has genes of the *Pelle*, *IκB,* and *TRAF* families (Tan & Ausubel, 2000) besides *Tlr*, a single gene of each. *C. elegans* lacks *NF-κB* and *MyD88*. NF-κB and MyD88 are part of Toll signalling in the inflammatory response (Irazoqui *et al.*, 2010). These genes have apparently been lost by the nematodes, as they are present in the cnidarian genome (Miller *et al.,* 2007).

Lamprey lymphocyte-like receptors

Lymphocyte-like cells of the agnathan see lamprey *Petromyzon* have variable lymphocyte receptors (VLR) with leucine-rich repeat (LRR) segments (known from extracellular domains of TLR) as counterparts of the immunoglobulin-based receptors that jawed vertebrates use for antigen recognition (Pancer *et al.,* 2004a). The highly diverse VLR genes are somatically assembled by the insertion of variable LRR sequences into incomplete germline variable lymphocyte receptor genes. Each germline receptor gene is flanked by hundreds of LRR-encoding sequences, and these are randomly used as templates to add the missing receptor segments needed for completion of the mature gene (Alder *et al.,* 2005). Variable lymphocyte receptors are expressed by separate monoallelic lymphocyte populations A and B, ensuring that each lymphocyte expresses a receptor of unique sequence (Guo *et al.,* 2009). The presence of LRR in receptors on A-like and B-like lymphocytes in lampreys suggests a way of the evolution of adaptive immunity different than in other vertebrates. Similarities with the antigen recognition system of jawed vertebrates are evident, as only VLRB lymphocytes bind native antigens and differentiate into VLR antibody-secreting cells. Conversely, VLRA lymphocytes respond preferentially to a classical T-cell mitogen and up-regulate the expression of the pro-inflammatory cytokine genes interleukin-17 (IL-17) and macrophage migration inhibitory factor (MIF).

INVERTEBRATE IMMUNE RESPONSES

The invertebrate innate immune responses include both cellular and humoral components. The cellular responses are mediated by haemocytes (blood cells) which typically phagocytose microbial pathogens and form melanotic capsules around metazoan parasites (Nappi & Ottaviani, 2000). Humoral immunity includes activation of proteolytic cascades that initiate haemolymph clotting, synthesis of lysozyme and various antimicrobial peptides, generation of reactive oxygen and nitrogen species, and synthesis of proteolytic enzymes (Nappi & Ottaviani, 2000). Several studies have demonstrated striking parallels between the innate immune responses of insects and other invertebrates on one hand and mammals on the other hand. These parallels indicate that the innate systems of vertebrates and invertebrates have common ancestry (Ottaviani & Franceschi, 1997)

Recognition of a foreign object or pathogen leads to its elimination. This is performed by cells with **n**atural **k**iller-like activity found in many invertebrate organisms: crayfish, snails, earthworms, urochordates, and molluscs (Cooper *et al.,* 1996). In earthworm coelomocytes, a protein similar to perforins, very ancient self-assembling

proteins, has NK-like activity. The sequence analysis of this protein shows that it contains a cysteine-rich domain like mouse perforin and lytic complement component C9 (Kauschke *et al.,* 2001).

Clotting

In invertebrates, haemolymph coagulation (Muta & Iwanga, 1996) and melanin formation are responses to microbial surface antigens (Söderhäll *et al.,* 1994). Two types of clotting mechanisms are known there. One of these is found in crustaceans (lobster and crayfish) and insects (cockroach and grasshopper) (ref. in Muta & Iwanga, 1996) where the gel is formed through polymerization of clottable protein(s) catalyzed by a Ca^{2+}-dependent transglutaminase. The transglutaminase is released from the haemocytes or muscle cells through an unknown mechanism. It is worth to note that in mammals, homopolymers of fibrin are stabilized by intermoleculary cross-links made by plasma transglutaminase (Osaki & Kawabata, 2004). The clottable proteins isolated from crustacean haemolymph plasma are large dimeric lipoglycoproteins consisting of subunits of about 200 kDa (Fuller & Doolittle, 1971; Kopacek *et al.,* 1993). Their amino-terminal sequences suggest that these are vitellogenin-like proteins (Doolittle & Riley, 1990). Vitellogenin is the major yolk protein found in most egg-laying non-mammalian vertebrates and inveterbrates (Smolenaars *et al.,* 2007). In insects, another abundant plasma protein, lipophorin (the major lipid carrier protein in insects) (Ryan & Van der Horst, 2000) seems to be cross-linked upon gelation (Brehelin, 1979; Barwig, 1985). In the moth *Manduca*, an inducible glucose-specific lectin possesses haemocyte-coagulating activity (Minnick *et al.,* 1986).

In the marine chelicerate horseshoe crab *Limulus*, coagulation involves haemocyte serine protease zymogens that trigger a coagulation cascade in response to LPS and β-1,3-glucan (Muta & Iwanga, 1996). The haemocyte detects LPS molecules on their surface, and then releases to the haemolymph the contents of its granules. Among granular components, coagulation factor zymogens have been found. In the presence of LPS, the autocatalytic activation of factor C triggers the coagulation cascades (factor B, clotting enzyme) resulting in the conversion of coagulogen to an insoluble coagulin gel. The factor G zymogen is activated in the presence of β(1→3)-D-glucan (Muta *et al.,* 1995). The resulting active factor G activates the proclotting enzyme directly, which brings about coagulin gel formation. This β-glucan-mediated coagulation pathway is activated on the surface of fungi. As a result of the clotting cascade, the invaders into the haemolymph are engulfed or immobilized by the clot. The coagulation cascade is regulated with the help of three types of serpin-type serine protease inhibitors exocytosed upon activation of the haemocytes to prevent diffusion of active coagulation factors, which could cause unnecessary clot formation. Several components of this cascade have been found to contain various protein domains. One of three chains of factor C, chain H that binds LPS, contains five SCR also called CCP domains, an EGF-like domain, and a C-type lectin-like domain (Muta *et al.,* 1991). The SCR domains are found mainly in mammalian complement factors. The amino-terminal L chains of factor B and the proclotting enzyme contain a small compact domain with three disulfide bonds called "clip" domain (disulfide-knotted domain) (ref. in Muta & Iwanga, 1996). The „clip" domain has been found in the *Drosophila* Snake and Easter protease precursors (Smith & DeLotto, 1992). These clotting mechanisms are homologous to similar mechanisms known in vertebrates.

Melanization

Biosynthesis of melanins occurs in a wide range of organisms, from bacteria and protozoans to mammals, indicating high evolutionary conservation and also fundamental importance of melanogenesis, even though its primary function is still obscure (Płonka & Grabacka, 2006). It may be argued that melanization of an infected tissue is either just a side-effect of the protection against the ongoing inflammatory processes or an active defense against the pathogen (Marmaras *et al.,* 1996). No doubt melanin protects from UV light and ionization, and supports resistance to heat or cold (Nosanchuk & Casadevall, 2003; Płonka & Grabacka, 2006). In arthropods, melanization is associated with multiple host defense mechanisms leading to the sequestration and killing of invading microorganisms as well as with the sclerotization of the new postmolt exoskeleton (Terwilliger & Ryan, 2006). The arthropod melanization is controlled by a cascade of serine proteases (not yet characterized) that ultimately activates the enzyme phenol oxidase which, in turn, catalyzes the synthesis of melanin (Cerenius & Söderhäll, 2004). The prophenoloxidase-activating enzymes cloned from insects are homologous to the horseshoe crab clotting enzyme and Factor B (ref. in Nagai & Kawabata, 2000). These two host defense systems of haemolymph clotting and prophenol oxidase activation have evolved from a common ancestral protease cascade (Nagai & Kawabata, 2000).

The melanin pathway results in the formation of cytotoxic pigment precursors (quinones, quinone methides, and semiquinones) as well as ROS and RNS (Nappi & Ottaviani, 2000). Insect phenol oxidase shows a close phylogenetic relationship with the arthropod haemocyanins, the copper-binding proteins involved in oxygen transport (Decker *et al.,* 2007). Haemocyanin can function as a phenoloxidase under certain conditions (Terwilliger & Ryan, 2006). In the mosquito *Armigeres* phenol oxidase is transcriptionally upregulated during the melanization of parasitic microfilariae, and is synthesized only in the mosquito haemocytes (Cho *et al.,* 1998). In *Drosophila*, phenol oxidase activity in immune reactive larvae was markedly augmented during the early stages of melanotic encapsulation of eggs of the parasitic wasp *Leptopilina*. The recruitment and site-specific aggregation and adhesion of large numbers of haemocytes that form melanized capsules around eggs of *Leptopilina* coincides with an augmented production of reactive oxygen and nitrogen species (Nappi *et al.,* 1995), which appear to represent an evolutionarily conserved innate immune response.

Reactive oxygen and nitrogen species

Reactive oxygen and nitrogen species are produced by members of all metazoan phyla. Sponges produce superoxide during consumption of bacteria, so it can be viewed as an antibacterial agent (Peskin *et al.,* 1998). Mollusc haemocytes produce superoxide in response to specific stimulators of respiratory burst (Garcia-Garcia *et al.,* 2008). Production of reactive oxygen species by haemocytes has been found in the cattle tick *Boophilus* (Pereira *et al.,* 2001). An immune response resembling the respiratory burst was found in the haemolymph and haemocytes of the cockroach *Blaberus* (Whitten *et al.,* 1999). The source of the reactive oxygen species could be the process of melanization or activity of NADPH oxidase, a key enzyme of the innate defense found in phagocytes of mammals (Dzik *et al.,* 2006; 2010).

The reactive oxygen-generating NADPH oxidases (Noxes) function in a variety of biological roles, besides host defense, e.g., in signal transduction and hormone synthesis. Genes encoding a family of these enzymes are found in a variety of eukaryotes, including plants, but there is no evidence for the presence of NADPH oxidases in prokaryotes (Sumimoto, 2008). Nox enzymes reduce molecular oxygen to superoxide in conjunction with oxidation of NADPH. The superoxide is further converted to various reactive oxygen species (Dzik, 2006). The prototypic Nox, gp91phox/Nox2 is a membrane-associated catalytic subunit of the phagocyte NADPH oxidase with a bis-heme binding motif. This suggests a possible evolutionary and functional relationship with the prokaryotic (or organellar) *b* type cytochromes. The oxidase C-terminal cytoplasmic region is homologous to the prokaryotic and organellar enzyme ferredoxin reductase (Sumimoto, 2008 and references therein). In animals, the phagocyte oxidase gp91phox/Nox2 was present before the divergence of Choanoflagellata and the metazoans. Thus, it is likely that Nox2 is the most ancient Nox in animals. During the evolution of protostomes, Nox2 was lost in the exuviating animals (Ecdysozoa), as it is missing in both arthropods (*Drosophila*) and nematodes (*Caenorhabditis*) but present in all other metazoan phyla. Thus, the ROS produced by arthropod hematocytes must originate from an enzyme other than Nox2 (Ha *et al.,* 2009). The phagocytic NADPH oxidase of mammals is a multicomponent complex comprising proteins of different subcellular location, both membrane-bound (p22phox and gp91phox forming the flavocytochrome b_{558}) and cytosolic (p40phox, p47phox, p67phox), and a small GTP-binding protein Rac. Upon cell activation, the cytosolic subunits are phosphorylated and migrate to the membranes where they bind to the membrane subunits to assemble the active oxidase (Babior, 1999). The gene encoding p22phox is present in the Choanoflagellata, Cnidaria, and Mollusca, but is absent from Annelida and Echinodermata as well as Ecdysozoa, the latter observation consistent with the absence of the Nox1–4 subfamily in this group. It is interesting that the three residues (Pro152, Pro156 and Arg158) in human p22phox indispensable for binding to p47phox are invariant in all animal p22phox proteins (Sumimoto, 2008), although an identifiable p47phox subunit is only found in chordates. The acrasiid "amoeba" *Dictyostelium* has a protein similar to p67phox, but none homologous to p47phox, p40phox or p22phox (Lardy *et al.,* 2005). This protein contains a Rac-binding tetratricopeptide repeat (TPR) domain, but does not contain any identifiable activation domain. The domain architecture of p47phox and p67phox is conserved from fishes to mammals. The genome of the chordate *Amphioxus* contains both genes (in p47phox the whole domain structure is duplicated). This indicates that this gene emerged early in chordate evolution. On the other hand, p67phox seems to be absent in the urochordates suggesting its loss in this lineage. A urochordate protein that contains solely the Rac-binding TPR domain, but not the other domains of p67phox, is interpreted as a functional p67phox homologue (Inoue *et al.,* 2005). The p67phox-homologous proteins known from molluscs (Kawahara & Lambeth, 2007) and echinoderms (Genebank accession number XP 781982) may participate in oxidase activation without cooperating with p47phox (which is absent there). The gene encoding p40phox occurs only in chordates (Sumimoto, 2008).

Nitric oxide synthase (NOS) activity has been detected in almost all organisms (ref. in Palumbo, 2005). The primary function of nitric oxide is signalling role through activation of guanylate cyclase and cGMP formation. In sponges, NO is a cellular signal for environmental stress (Giovine *et al.,* 2001), which may represent the most primitive NO-based sensory network in the animal kingdom. In invertebrates, NO is a messenger in a variety of tissues, including the nervous system, excretion organs (ink gland, salivary gland), light organs, haemocytes, and endocrine cells. In the ink gland cells of the cephalopod *Sepia*, NO signalling activates tyrosinase leading to the melanin formation responsible for the black color of its ink. The NO/cGMP signalling pathway is also involved in secretion of ink constituents from gland cells. In insects, NOS expression is associated with neuronal signalling (Elphick *et al.,* 1996) and production of NO-loaded salivary gland proteins that facilitate blood-feeding by haematophagous insects (Ribeiro *et al.,* 1993). In *Drosophila* NO induces expression of a gene encoding the antimicrobial peptide diptericin (Nappi *et al.,* 2000). A defense function of nitric oxide molecule was observed in crustacean (Yeh *et al.,* 2006) and mollusc haemocytes (ref. in Palumbo, 2005). In haemocytes of the mollusc *Mytilus,* free radical production has been found to depend mainly on the activity of PI 3-kinase (Garcia-Garcia *et al.,* 2008). In mammals, nitric oxide is a major messenger molecule involved in vascular regulation, immune function and neurotransmission in the brain and peripheral nervous system.

No wonder that nitric oxide synthase shows high similarity across the Metazoa. The enzymes cloned from the cnidarian *Discosoma*, mollusc *Aplysia*, and chordate *Ciona* have putative cofactor recognition sites for haeme, tetrahydrobiopterin, calmodulin, FMN, FAD, and NADPH; all conserved with respect to mammalian NOS (ref. in Palumbo, 2005). The nitric oxide synthase gene of the mosquito *Anopheles* shows the highest homology to the vertebrate neuronal NOS (Luckhart & Rosenberg, 1999). Phylogenetic analysis shows that in the sea urchin *Arbacia,* each of two cloned sequences of NOSs cluster with the mammalian constitutive and inducible isoforms, respectively (Cox *et al.,* 2001). They may represent early homologues of these two isoenzymes. The signals that trigger the inducible synthesis of nitric oxide in invertebrates are unknown (Rivero, 2006), although an LPS- and inflammatory cytokine-responsive transcription binding site has been found (Luckhart & Rosenberg, 1999).

Antimicrobial peptides

The killing of pathogens in invertebrates involves also oxygen-independent mechanisms that include the synthesis of lysozyme and other hydrolytic enzymes, and antimicrobial peptides. Antimicrobial peptides (about 900 are known) are produced by both protozoa and metazoa (Danilova, 2006). Although these peptides are quite different, they all contain clusters of hydrophobic and cationic amino acids conserved during evolution (Zasloff, 2002). They are essential for the attachment to the negatively charged bacterial membranes and integration with membrane phospholipids in pore-forming or membrane destruction (Brogden, 2005). The cytoplasmic granular vesicles of the parasitic *Entamoeba* contain a family of peptides (amoebapore A, B, and C) that display antibacterial activity primarily towards Gram-positive bacteria (Leippe *et al.,* 1994). They resemble defensins of mammals and insects by the presence of three sta-

bilizing disulfide bonds and pore formation in bacterial membranes, although they differ in positions of cysteins and secondary structure (ref. in Leippe, 1999). The 78-residue peptide named NK-lysin of porcine natural killer (NK) and T-cells is a structural and functional analog of the protozoan peptides (Leippe, 1995). Both amoebapores and NK-lysin occur in granules of their cells and are antibacterial as well as cytolytic, but do not lyse red blood cells. A human homologue of NK-lysin termed granulysin was purified from cytotoxic T lymphocytes (Peña *et al.,* 1997). Amoebapores, NK-lysin and granulysin belong to a distinct family of saposin-like proteins which includes saposins (cofactors of lysosomal sphingolipid hydrolases) and lung surfactant associated protein B (Munford *et al.,* 1995). In phylogenetically distant organisms, the architecture of such lytic polypeptides may have developed independently under selection pressure for functionally important units (amphipathic α-helices, stabilizing intramolecular crosslinks). Support for their common ancestry is provided by the cysteine pattern typical for amoebapores and other saposin-like proteins found in putative gene products of flatworms (*Fasciola*, *Schistosoma*), nematodes (*Caenorhabditis*), and arthropods (*Bombyx*) (Leippe, 1999).

Antimicrobial peptides are produced in the phagocytic cells of annelids (Salzet *et al.,* 2006), molluscs, arthropods, and urochordates (ref. in Hancock *et al.,* 2006). A great body of knowledge comes from investigation of insect antimicrobial peptides. They are synthesized primarily by the fat body (a functional equivalent of the mammalian liver) and to a lesser extent by haemocytes, cuticular cells, midgut and salivary glands, and reproductive structures. More than 150 antibacterial and antifungal peptides/polypeptides have been described. The immune peptides of insects may serve as cell adhesion molecules, in regulating the activity of cell surface receptors or signal transduction pathways leading to altered gene expression. *Drosophila* naturally infected by entomopathogenic fungi is able to differentially induce only those antimicrobial peptides with antifungal activities. In *Drosophila* and other insects a NF-κB homologue has been demonstrated to regulate gene transcription of antimicrobial peptides (Hoffmann *et al.,* 1999). Following infection, the gene encoding the cytokine Spätzle was uniquely upregulated in haemocytes but not the fat body (Irving *et al.,* 2005).

In animals, lysozymes constitute key components of the antibacterial defense due to the cationic nature of the protein and, to a lesser degree, its muramidase activity. These enzymes are known from numerous phylogenetically diverse organisms such as bacteria, bacteriophages, fungi, plants, and animals. The phylogeny of lysozymes shows conservation of their function since the last common ancestor of arthropods and vertebrates (Hughes, 1998). Cytoplasmic granules of *Entamoeba* contain two isoforms of lysozyme both of 23 kDa (Leippe, 1999). Multiple lysozyme forms can be recognized in cases when it has also been recruited as a digestive enzyme (Prager, 1996). Amoeba lysozyme II having acidic pI is in this respect similar to one of eight isoforms from *Drosophila*. It is expressed exclusively in the digestive tract and hence is presumably a digestive enzyme (Daffre *et al*., 1994). Like in other phagocytes, amoebic lysozymes prevent microbial growth within the digestive vacuoles and, in addition, fulfill a digestive function to exploit bacteria for nutrition. A striking sequence identity of both amoeba lysozymes with putative gene products of unknown function from the bacteria-feeding nematode *Caenorhabditis* was found (Leippe, 1999).

Lectins

Lectins are proteins that recognize and bind carbohydrates. Intracellular lectins recognizing core-type structures and mediating intracellular glycoprotein trafficking are present in plants, yeasts, invertebrates, and vertebrates. Lectins that recognize more complex structures at the cell surface, such as C-type lectins and galectins, are found both in invertebrates and vertebrates, but their functions have evolved independently in different animal lineages (Dodd & Drickamer, 2001). Lectins are present on the surface of invertebrate haemocytes as primitive recognition molecules. Their ability to opsonize and agglutinate foreign cells helps subsequent phagocytosis or encapsulation of microbial pathogens and metazoan parasites (Dodd & Drickamer, 2001). An ancestral S-type lectin has been found in sponges (Pfeifer *et al.,* 1993). A 32-kDa S-type lectin from the nematode *Caenorhabditis* shares 25–30% sequence identity with the vertebrate members of that group (Hirabayashi, *et al.,* 1992). Intermediate forms between S- and C-type lectins have been found in the venom of the rattle snake *Crotalus* (Hirabayashi *et al.,* 1991). Convergent evolution seems responsible for the similarities in structure and/or function of some lectins (Vasta *et al.,* 1994). Individual functional domains of these molecules are related to collagen, growth factors, calmodulin, and complement regulatory proteins. Common to all lectins are the carbohydrate-binding domains covering a set of highly conserved residues (Vasta *et al.,* 1994). Although distinct and probably unrelated groups of proteins are included under the term "lectins" there are essential similarities between invertebrate and vertebrate lectins. One group of invertebrate lectins comprises molecules that show significant homology to membrane-integrated or soluble vertebrate C-type lectins. The second includes β-galactosyl-specific lectins homologous to the S-type vertebrate lectins. The third group are lectins that show homology to vertebrate pentraxins that exhibit lectin-like properties, such as C-reactive protein and serum amyloid. Moreover, there are lectins that do not exhibit similarities to any of the aforementioned categories. Although lectins do not express recombinatorial diversity, they have more than one binding site specific for different carbohydrates in a single molecule and also show certain flexibility of the binding sites allowing recognition of a range of structurally related carbohydrates (Vasta *et al.,* 1994).

Invertebrate cytokine-like molecules

Functional analogues of mammalian inflammatory cytokines, for instance IL-1 and TNF-like molecules, have been found in annelids, molluscs, insects, echinoderms, and urochordates (ref. in Beschin *et al*., 2001). In fact, the functional similarities between cytokines and their invertebrate counterparts need not reflect homology but rather can result from molecular convergence based on the structural similarity of the lectin-like recognition domain. Various vertebrate cytokines possess lectin-like properties that may be involved in regulation of innate immune responses independent of the binding to their cytokine-specific receptor (Lucas *et al.,* 1994; Zanetta & Vergoten, 2003). The carbohydrate-binding domains of cytokines are spatially distinct from the cytokine-receptor binding sites. The action of urochordate IL-1-like molecule may be related to its galactosyl-specific lectin-like property rather than reflecting an evolutionary relation (Raftos, 1996; Beck *et al*., 1993). Coelomic cytolytic fac-

tor (CCF) from the earthworm *Eisenia* triggers activation of the prophenoloxidase (proPo) cascade (involved in biosynthesis of melanin) upon interaction with cell wall components of Gram-negative bacteria or yeast. Despite the functional analogies between CCF and TNF (lytic abilities, lectin-like activities, secretion from coelomocytes or macrophages, respectively, after LPS stimulation) they are not homologous (Beschin *et al.,* 1999).

The TGF-β superfamily is a group of multifunctional cytokines that includes bone morphogenetic proteins (BMPs), TGF-βs and others. The BMP subfamily members have a critical role in embryogenic patterning and in maintaining tissue homeostasis in adult life, whereas TGF-β subfamily members mainly modulate the immune response (Kingsley, 1994; Hogan, 1996; Letterio & Roberts, 1998). TGF-β-like molecules resembling the BMP subfamily members have been identified in molluscs, nematodes, insects, echinoderms, and urochordates (ref. in Beschin *et al*., 2001). Since the identified invertebrate TGF-β and TGF-β-receptor-like proteins are closer to the mammalian BMP subfamily than to the TGF-β one, they may not be involved in immune responses in invertebrates. The molecules involved in the development of invertebrates may have diversified and been recruited by the immune system with the emergence of vertebrates. The example of invertebrate cytokines raises the question whether the immune system arised convergently from different ancestral systems, by diversification from a common ancestor, or by a combination of both routes.

Serine proteinase cascades

Serine proteinases appeared early in evolution (trypsin-coding genes have been found in bacteria) (Rypniewski *et al.,* 1994) and occur in the digestive system of all animals. Proteinases evolved towards fulfilling various advanced physiological functions in the immune and other systems. In the activation of the prophenoloxidase system that leads to melanization in insect haemocytes, a serine proteinase cascade is involved showing similarities to the blood clotting system and the complement system of vertebrates. Proteases organized into cascades mediate dorsal-ventral differentiation, arthropod haemolymph clotting, vertebrate blood clotting, and complement activation. Krem and Di Cera (2002) showed (Fig. 1) that each cascade has a functional core consisting of three serine proteases, the downstream one cleaving the terminal substrate. It seems that the two others, middle and upstream proteases, were added later in evolution. The middle (factor B) and downstream (proclotting enzyme) proteases of the horseshoe crab haemolymph cascade share sequence homology with the Snake (middle protease) and Easter (downstream protease) agents of the *Drosophila* dorsal-ventral polarity controlling cascade. Factor C (the upstream protease) of the horseshoe crab haemolymph cascade (Muta *et al.,* 1991) has homology to vertebrate complement factors C1r and C1s (upstream and middle proteases) (Kusumoto *et al*., 1988). Factors C1r and C1s have functional and sequence similarity to scolexin, a haemolymph serine protease of *Manduca sexta*. This suggests that these proteins had a common ancestor. Thus, the arthropod and vertebrate immune systems use molecules from a pool existing before the protostome-deuterostome split.

The downstream protease of the vertebrate clotting cascade, thrombin, belongs to the same lineage as the complement factors C1r and C1s. The upstream and middle proteases of the clotting cascade (factors VII, IX,

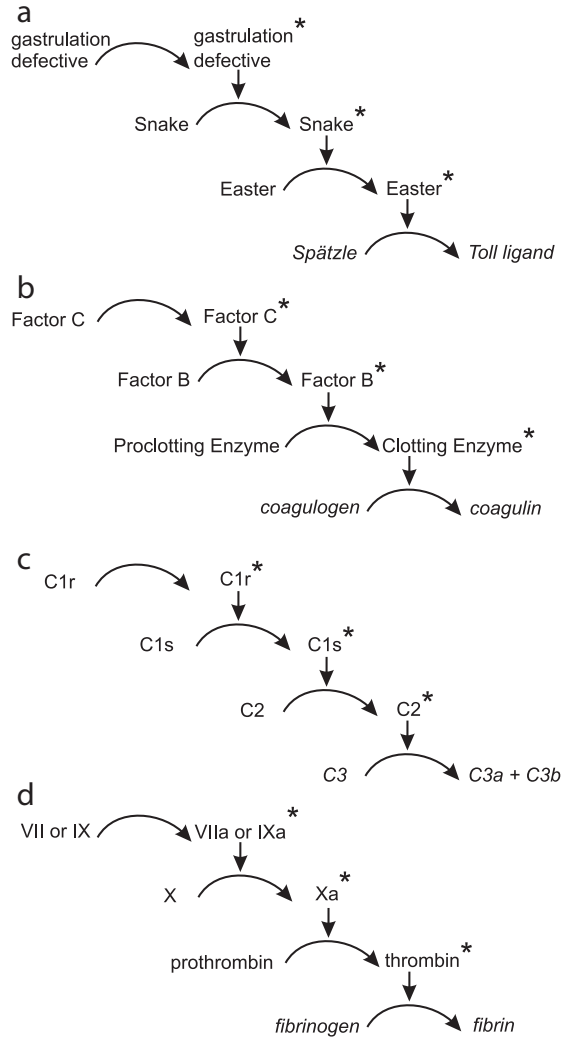

Figure 1. Serine protease cascades of different functions. Shown are cascades governing dorsal-ventral differentiation during *Drosophila* embryogenesis **(a)**, horseshoe crab haemolymph clotting **(b)**, classical pathway of complement activation **(c)**, vertebrate blood clotting **(d)**. In evolution, these cascades expanded by adding homologous proteins at the beginning of the cascade. Activated proteases are marked with asterisks. Substrates are indicated with italics. Based on Krem and di Cera (2002).

X) belong to the lineage of the horseshoe crab clotting factor C. This suggests that the vertebrate blood clotting emerged as a by-product of innate immunity, because its entire functional core shares ancestry with the complement proteases. According to Krem and Di Cera (2002), the haemolymph clotting cascades evolved in protostomes after dorsal-ventral cascade with introduction of the complement-like clotting horseshoe crab factor C. In the deuterostomes, a primitive complement system increased its complexity and then blood clotting proteases diverged from the serine proteases of advanced complement system.

There are many functional links between development, immunity, and haemostasis in vertebrates. Enzymes of the coagulation cascade participate in immunity, cell growth and embryogenesis. Various serine proteases belonging to the coagulation system are able to activate the complement cascade independently of the established pathways (Amara *et al.,* 2008). On the other hand, MASP2 a (lectin pathway) is capable of promoting fibrinogen turnover by cleavage of prothrombin generating thrombin (Krarup *et al*., 2007). Thrombin and

prothrombin are known to influence the immune reactions. Thrombin induces, for instance, chemotaxis of monocytes and neutrophils. Prothrombin promotes cell migration through the extracellular matrix (ref. in Krem & Di Cera, 2002). In protostomes, a relation between the clotting cascade and the primitive complement system also exists. Factor C (the horseshoe crab haemolymph cascade) acts as an LPS-responsive C3 convertase in the initial phase of horseshoe crab complement activation (Ariki *et al.*, 2008). Also, substrates of these four protease cascades show evolutionary relations. Spätzle is structurally homologous to coagulogen. The C-terminal domains of Spätzle and coagulogen share structural homology with the nerve growth factor (NGF) indicating that substrates involved in clotting, immunity and development share common ancestry.

THE BEGINNING OF THE COMPLEMENT SYSTEM

The vertebrate proteins that bind pathogen or foreign molecules (antigens) and initiate a cascade of events eventually leading to opsonization or lysis of the invader are called complements. Their ancestry is rooted within the invertebrates. In mammals, the complement consists of about thirty soluble proteins directly involved in immune reactions and their regulation. Most are normally inactive and their activation may be triggered directly by an invading organism or indirectly by an immune response. Components C1 to C4 are proenzymes activated sequentially by limited proteolytic cleavage. Eventually, the components C5–C9 assembly into a large protein **m**embrane **a**ttack **c**omplex (MAC) that mediates microbial cell lysis. Thus the activation of complement is focused on microbial cell membrane, where it is triggered either by the antibody bound to the microorganism (classical pathway), by microbial envelope polysaccharides (alternative pathway), or by host proteins binding microbial surface (lectin pathway). Each of these pathways has been studied in detail and they are now part of the textbook knowledge of immunology (Fig. 2).

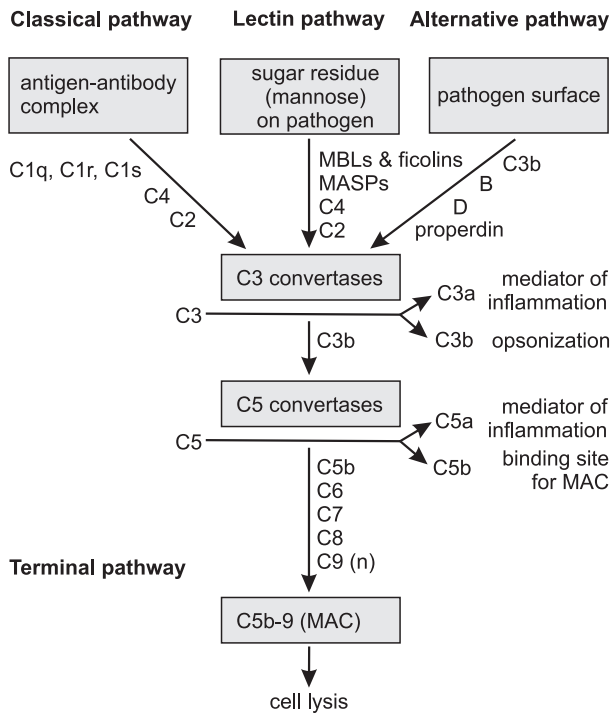

Figure 2. Complement activation pathways
Classical pathway is initiated when C1 component binds to antigen–antibody complexes. C1 consists of C1q and two copies of C1r and C1s each. As a result of this binding, activated C1q r_2s_2 (C1s and C1r are serine protease enzymes) cleaves C4 and C2. Alternative pathway is initiated by binding of spontaneously generated C3b to activating surfaces such as microbial cell walls and involves factor B, factor D and properdin. Lectin pathway is initiated by binding of the serum protein **m**annose-**b**inding **l**ectin, MBL (member of collectin family) to the surface of pathogen. After MBL binds carbohydrate residues on pathogen surface, MBL-associated serine proteases (MASP-1 and MASP-2, structurally similar to C1r and C1s) bind to MBL. Resulting active complex causes cleavage and activation of C4 and C2. All three pathways generate C3 and C5 convertases and produce C5b which is converted into membrane attack complex by a common sequence of terminal reactions (assembly of components C5b through C9).

Ancestry of the complement system

A large family of thioester-containing proteins characterized by the possession of an internal thiol ester bond within the conserved GC**G**EQ motif whose covalent bonding to external targets is essential for the function of the protein (Armstrong, 2006). The protease inhibitor α_2-macroglobulin, the thioester proteins (TEPs), and components C3, C4, and C5 of the complement system belong to this family.

α_2-Macroglobulin is an opsonin that promotes binding and endocytosis of diverse enopeptidases to cell surface receptors such as the low density lipoprotein-related protein (LRP) (Strickland *et al.,* 1990). In this process, the target protease is enclosed in a molecular cage of the α_2-macroglobulin polypeptide chain (Borth, 1992; Armstrong, 2006). The bound proteases are degraded in secondary lysosomes (Van Leuven *et al.,* 1978). A variety of mammalian cell types, including monocytes/macrophages, bind the protease – α_2-macroglobulin complex. Members of the α_2-macroglobulin protein family have been found within metazoans (ref. in Armstrong, 2006). Initially, it was believed that C3 and α_2-macroglobulin diverged from an α_2-macroglobulin-like ancestor. Recently, four genes of thioester-containing proteins (TEPs) have been identified in the cnidarian *Haliplanella lineata* (Fujito *et al.*, 2010). Two of them are clasified to the α_2-macroglobulin subfamily and the other two genes to the C3 subfamily. The *C3* and *a_2-macroglobulin* genes are expressed in different parts of the the anemone body. Some characteristics which are found in the human C3, but not in human α_2-macroglobulin, were present in the common ancestor of the TEP genes. It concerns, for instance, possible cleavage site for C3 convertase, or the catalytic His residue. Their acquisition was believed to be an achievement of C3 subfamily evolution, as it greatly increases the rate of reaction of the thioester with hydroxyl groups and with water (Dodds & Law, 1998). It is still not clear whether the common ancestor had a C3-like function or an α_2-macroglobulin-like function. To resolve this problem, animals presenting the state before the C3/α_2-macroglobulin gene duplication should be found (Fujito *et al.*, 2010). No TEP gene has been identified in sponges and choanoflagellates (Kimura *et al.*, 2009) suggesting that TEPs arose in eumetazoan lineage. Thus, the creation of the TEP gene and subsequent gene duplication and functional differentiation into C3 and α_2-macroglobulin had to occur after the divergence of sponges and before the divergence of cnidaria from the bilateralia lineage.

Insect TEPs (Blandin & Levashina, 2004) show functional and structural similarity to members of the C3 subfamily. Some insect TEPs bind bacteria and promote phagocytosis (Levashina *et al.,* 2001) and therefore are reffered to as complment-like proteins. But according to

Fujito *et al.* (2010) they belong to the α_2-macroglobulin subfamily, which suggests that the functional similarity of the arthropod TEPs and mammalian C3 results from functional convergence.

In view of the foregoing, the complement system has a more ancient evolutionary origin than the acquired immunity. Genes encoding proteins with domains typical for complement components, the complement control protein (CCP), von Willebrand factor type A (vWA), serine protease (SP), CUB domain, epidermal growth factor-like (EGF-like), thrombospondin type 1 repeats (TSP), low-density lipoprotein receptor domain class A (LDLa), and SRCR are present in the cnidarian genome (Kimura *et al.,* 2009). As mentioned earlier, the simplest metazoans, sponges, contain proteins with SCR/CCP domains that show high homology to members of the mammalian regulators of complement activation (RCA family) (Pahler *et al.,* 1998). Also, in marine chelicerates the N-terminal region of factor C (serine protease zymogen involved in haemolymph clotting) contains five SCR repeats and is classified into the branch of the family consisting of complement factors initiating complement activation (MASP/C1r/C1s) (Iwanga & Kawabata, 1998).

The multi-component complement system comprising at least the central molecule of the complement system (C3), Bf, and MASP occurs in the cnidarians (Kimura *et al.,* 2009). While retaining several ancestral features, the cnidarian C3 and Bf genes show close similarity to those of chordates (amphioxus) rather than to those of other invertebrates. This suggests that the ancestral amino-acid sequences of these complement components are well conserved in the cnidarians and chordates. A recurrent loss of complement genes seems to have occurred in other group of cnidaria, *Hydra*, *Caenorhabditis*, *Drosophila*, and the snail *Euphaedusa*.

The cnidarian complement genes show endodermal cell-specific expression, thus a connection with the gut. The unexpectedly ancient origin of the multi-component complement system can thus be traced back to before the divergence of the cnidarian and bilaterian lineages, perhaps more than 600 million years ago (Ayala *et al.*, 1998). Thus, it is likely that the complement system arose in their common ancestor by the *de novo* creation of the C3/TEP gene and the establishment of the domain composition of Bf and MASP genes by shuffling of pre-existing domains. The finding of the MASP gene in non-chordates is particularly important, indicating that the lectin pathway is equally old as the alternative pathway.

In echinoderms, the C3 and C2/factor B-like components are present (Smith *et al.,* 2001). In the urochordates, occupying a position between the invertebrates and vertebrates, proteins involved in the lectin and alternative activation pathways, such as glucose-binding lectin (GBL) homologous to mannose-binding lectin (MBL) (Sekine *et al.,* 2001), ficolins (Kenjo *et al.,* 2001), MBL-associated serine proteases (MASPs) (Ji *et al.*, 1997), C3 (Nonaka *et al.,* 1999), and C2/factor B (Nonaka & Miyazawa, 2002) have been identified. Mannose-binding lectin (MBL) and ficolin are lectins composed of a lectin domain attached to a collagen-like region. Their structure is similar to that of complement C1q owing to the collagen-like stalk. However, they use a different lectin domain for carbohydrate recognition: a carbohydrate recognition domain (CRD) is found in MBL and a fibrinogen-like domain in ficolin. Glucose-binding lectin (GBL) contains a carbohydrate recognition domain that is homologous to C-type lectin. The collagen-like domain is replaced in this molecule by another sequence that has a helical structure similar to the configuration of the Gly-X-Y repeats of collagens. Ficolins as well as GBL probably act as recognition molecules of the primitive urochordate complement system in a similar manner to the mammalian lectin pathway. Complement-mediated phagocytosis is a central part of the physiological function of the urochordate complement system.

The jawless fishes of the class Agnatha (lamprey and, even more primitive, hagfish) are the least derived of all extant vertebrates. C3 from the hagfish *Eptatretus* originally identified as hagfish Ig (Varner *et al.,* 1991), apparently has a two-chain structure containing a thioester site in the α chain. It acts as an opsonin (Hanley *et al.,* 1992). A protein from the lamprey homologous to mammalian C3/C4 (Nonaka & Takahashi, 1992) has been shown to have opsonic activity as well. The sequence of factor B (alternative activation) from lamprey is equally similar to those of mouse factor B and C2, suggesting that it represents a stage before the Bf/C2 gene duplication (Nonaka *et al.*, 1994). Two lectins from lamprey serum (Endo *et al.,* 2006) have been identified: an MBL and a homologue of C1q. Both lectins are associated with a serine protease of the MASP family (MASP-A) which exhibits a proteolytic activity against lamprey C3. The single MASP molecule found in the lamprey (Matsushita *et al.,* 2004) appears to be more related to the MASP-2 isoform found in other animal species. The lamprey C1q consists of a collagen-like domain and an antibody recognition domain, gC1q, found in a variety of proteins including mammalian C1q. In a phylogenetic tree of the gC1q domains lamprey C1q and mammalian C1q form a cluster. These observations strongly suggest that C1q may have emerged as a lectin and functioned as an initial recognition molecule of the complement system before the appearance of acquired immunity components, such as immunoglobulins, in the cartilagineous fishes. Therefore, the lamprey complement system consists of at least the lectin MASP complex and C3. However, identification of lamprey C1q clearly indicates that the classical pathway originates at the agnathan stage.

There is no evidence for the presence of the membrane attack complex (MAC) in lamprey (Fujii *et al.,* 1992), although a single component with lytic activity has been described in lamprey serum. However, a gene with sequence similarity to the mammalian complement membrane attack regulatory molecule CD59 (protectin) has been reported in the hagfish (dos Remedios *et al.,* 1999). The presence of this gene suggests that the terminal lytic complement pathway (C5b-9) could operate in these primitive vertebrates. Thus, the agnathan complement represents an early intermediate stage in the complement phylogeny. It is worth noting that in the anthozoan cnidarians, several proteins with membrane attack complex/perforin domain have been found (Miller *et al.*, 2007).

EVOLUTION OF THE COMPLEMENT SYSTEM

There is empirical evidence that the complement system increased in complexity while evolving from the fish to mammalian grade.

Cartilagineous fishes

Sharks are the most primitive vertebrates having partial molecular machinery required to mount an adaptive

immune response, i.e., antibodies (Schluter *et al.,* 1997), TCR (Rast & Litman, 1994), and MHC class I and II molecules (Salter-Cid & Flajnik, 1995). In addition, they possess the simplest form of the classical pathway of complement activation, not present in jawless fishes. The nurse shark *Ginglymostoma* (Jensen *et al.,* 1981) has six complement-like proteins that interact sequentially and form functional cascades corresponding to the mammalian classical and lytic pathways. Shark C1q is composed of at least two chain types with 50% identity to human C1q A and B chains. MASP genes (lectin pathway) known from two shark species show greater homology to mammalian MASP-2/C1r/C1s lineage than to MASP-2 (Smith, 1998). Moreover, the presence of a functional alternative pathway as well as of proteins resembling C3, factor B, and a putative factor H, a regulator of complement activation in the alternative pathway (Dodds *et al.,* 1998; Shin *et al.,* 2007), was assessed. The presence of a lytic pathway in sharks is postulated on the basis of the isolation of C8 and C9 in the shark, although the molecular mass of those proteins (185 and 200 kDa, respectively) is higher than those of their mammalian counterparts (Smith, 1998).

Bony fishes

The complement system of the teleosts covers all three pathways of complement activation and involves C1r/C3/C4/C5/C8/C9/fB/fD/fH/MASP/MBL-like molecules (Zarkadis *et al*., 2001). However, the complement function in these fishes differs in a number of important aspects from that in mammals. As could be expected, the optimal activation temperature for fish complement is much lower (20–25 °C) than in the mammals (37 °C). Furthermore, in contrast to the human complement, complements from a variety of fishes can lyse erythrocytes of tetrapodes and humans with high efficiency through activation of the alternative pathway. Because the antibody response in fishes is quite rudimentary, it is postulated that the complement in these species may act against invading pathogens. Some of the components (C3 and factor B) of the complement system of bony fishes are present in multiple isoforms encoded by different genes. The most important feature of these C3 isoforms is that they differ in their binding specificities for a number of complement-activating surfaces. All the purified C3 isoforms were found to contain an α and β chain and to have an internal thioester bond in the α chain (ref. in Zarkadis *et al*., 2001).

In the trout *Salmo* factor Bf-2 acts in both the classical and alternative pathways of complement activation. They may represent primordial molecules that in warm-blooded vertebrates have evolved to function exclusively in either the alternative or classical pathways. This suggests that before the divergence of C2 and factor B from the common ancestor, a molecule existed that was able to function in both the alternative and classical pathways. The presence of factor B-like molecules in urochordates, agnatha, sharks and teleosts, as well as the absence of C2-like molecules from those species, provide evidence that the fB/C2 duplication and the appearance of C2 took place after the divergence of teleosts (Zarkadis *et al*., 2001). In teleost fishes, two MASP-3 and two C1r/C1s/MASP-2-like isotypes have been found (Nakao *et al.,* 2003b). They are structurally similar to each other, belonging to the MASP/C1r/C1s family comprising MASP-1-like and MASP-2/C1r/C1s-like proteins (Thiel *et al.,* 1997; Matsushita *et al.,* 1998). The exact point of divergence of MASP-1 and MASP-2/C1r/C1s within the lower vertebrates remains unclear. As suggested by studies on urochordates, MASP-1 may have emerged prior to MASP-2/C1r/C1s (Nonaka, 2001). Thanks to the cloning of cnidarian complement genes (Kimura *et al.,* 2009) it is known that the cnidarian MASP shares with invertebrate MASPs and vertebrate MASP-1 some canonical features of the serine protease (SP) domain which were lost in vertebrate MASP-2, MASP-3, C1r and C1s, indicating that the ancestral MASP was MASP-1 type molecule. The C1r and C1s components of C1 are clearly derived from the MASP lineage. C1q is closely related to MBL or ficolins with the substitution of antibody recognition domains for the CRDs in MBL or fibrinogen-like domain in ficolins. Two C1 genes from the carp *Cyprinus* with 36% and 34% homology to human C1r and C1s, respectively, may be close to ancestors of the mammalian C1r/C1s (Nakao *et al.,* 2001). From the evolutionary point of view, the primitive lectin pathway in innate immunity appears to have developed into the more sophisticated, multifunctional complement system of the classical pathway through gene duplication, to serve as an effector system of acquired immunity.

Terminal complement components — lytic pathway

Molecules homologous to mammalian C5 have been described in several species of teleost fishes (ref. in Zarkadis *et al.,* 2001). Although C5 is closely related to C3 and C4, it seems that the other components of the lytic pathway (C6, C7, C8a, C8b, and C9) are related and homologous to perforin, the lytic protein of natural killer cells and cytotoxic lymphocytes (Podack *et al.,* 1989). All these molecules share common structural motifs, i.e., thrombospondin (TS), low-density lipoprotein receptor (LDL-R), and epidermal growth factor precursor (EGFP) domains. In addition, C6 and C7 possess short consensus repeats (SCRs) and FIM modules in the C-terminal domain. The cloning of a C6-like gene from the most primitive of present-day chordates, the amphioxus *Branchiostoma*, suggests an ancient origin of the C6/C7/C8/C9/perforin gene family. Sequence analysis studies suggest that C6 and C7 emerged first, followed by the appearance of C8 and C9 (Mondragon-Palomino *et al.,* 1999). It seems reasonable that the duplication of an ancestral gene proceeded through two pathways. One pathway presumably led to the simple form of perforin, while the second produced the ancestor of C6–C7 with its complex modular structure. Further duplication and loss of modules may have led to the creation of C8 and C9 molecules. Following the emergence of cyclostomes, a primitive lytic pathway appeared. The membrane attack complex present in teleost fish closely resembles the mammalian complex.

Complement regulatory proteins

The survival of hosT-cells requires their protection from autologous complement attack. Various regulatory molecules belong to a human gene family of **r**egulators of **c**omplement **a**ctivation (RCA) (Hourcade *et al.,* 1989). Each of these proteins is composed exclusively of short consensus repeats (SCRs) (also known as complement control protein modules CCPs), and their binding to the active complement fragments C4b/C3b inhibits activation of the complement pathways. Complement regulatory-like molecules with SCR domains, probably close to ancestors of the RCA gene superfamily, have been found in urochordates (Li *et al.,* 2000). Also, identification of soluble regulatory proteins of the complement

system such as lamprey C4-bp (Kimura *et al.,* 2004) and factor H (Endo *et al.,* 2006) has led to the suggestion that the lamprey complement system may be more sophisticated than the urochordate system. Two isotypes of complement regulatory factor I (Nakao *et al.,* 2003a) and factor H have been cloned and characterized in teleost fish (Krushkal *et al.,* 1998). A GPI-anchored membrane protein (CD59, also called protectin) is the main regulatory molecule protecting hosT-cells from the lytic attack of terminal complement complexes (MAC) (Morgan, 1999). A CD59-like gene from the trout (*Salmo*) has high sequence similarity to mammalian CD59 (Lee & Goetz, 1998). A gene encoding a CD59-like molecule has also been cloned from the hagfish. The presence of CD59 in the hagfish, together with the observation of a C5a-like activity in hagfish plasma, suggests that an ancient form of the lytic pathway is present in these primitive vertebrates (dos Remedios *et al.,* 1999).

According to Nonaka and Kimura (2006) the evolution of the complement can be summarized as follows: A primitive complement system most likely composed of C3 and Bf and thus similar to the mammalian alternative pathway emerged in the common ancestor of cnidaria and bilaterians. The structural features of these cnidarian genes suggests that the ancestral C3 was proteolytically activated by Bf, and that it formed a covalent bond with non-self molecules using its intramolecular thioester bond. Whereas the C3 and Bf genes were retained by deuterostomes, they were lost many times independently in the protostome lineages. The recent finding of a MASP gene in cnidarians (Kimura *et al.,* 2009) suggest that the primitive lectin pathway could operate besides the alternative pathway in those animals. MASP, MBL, and ficolin constitute the urochordate lectin pathway similar to the mammalian one. Finally, vertebrate-specific complement gene duplications, such as those leading to C3/C4/C5, Bf/C2 and MASP/C1r/s, occurred before the emergence of cartilagineous fish, most probably contributing to the establishment of the third activation pathway, the classical pathway. The classical and lytic pathways of the complement system seem to have emerged at the cartilagineous fish stage, coincident with the emergence of adaptive immunity. Genes for the ancestral terminal complement components appear to have been recruited by the complement system and duplicated to C6/C7/C8a/C8b/C9 before the appearance of the jawed vertebrates, although the timing of those events still needs to be clarified in detail.

ALLORECOGNITION

Although only jawed vertebrates possess major histocompatibility complex MHC, allorecognition is well documented in urochordates and non-chordate invertebrates. All urochordates seem to have natural killer (NK) cells involved in allorecognition (Kasahara *et al.,* 2004). One of the urochordate *Botryllus* genes whose expression is down-regulated after allogenic contact is a homologue of CD94/NKR-P1 (natural killer cell receptor, P1); this gene is expressed in the blood cells of *Botryllus* (Khalturin *et al.,* 2003). Natural killer cell receptors are, however, unrelated to the MHC, use also other ligands than MHC, are not involved directly in AIS, and are not an evolutionarily homogenous group. De Tomaso *et al.* (2005) have found that in *Botryllus* allorecognition is determined by a single genetic locus, *FuHC* (for fusibility/histocompatibility). Fusion occurs when two colonies share at least one *FuHC* gene variant, or allele; rejection occurs when no *FuHC* alleles are shared. A gene encoding an immunoglobulin superfamily member was isolated that, by itself, determines the outcome of histocompatibility reactions (De Tomaso *et al.,* 2005). This gene is believed to encode epidermal growth factor repeats and also immunoglobulin domains. However, cFuHC is not homologous to any proteins of the vertebrate MHC-based histocompatibility system (Klein, 2006); for example, the immunoglobulin domains do not correspond to the C1 type found in MHC. cFuHC has high homology with human IGSF4, the more ancient immunoglobulin domains, which may be descendents of the first antigen receptors (Du Pasquier *et al.,* 2004; Cannon *et al.,* 2004). According to Laird *et al.* (2005), the function of FuHC may be to protect against parasitism by conspecific stem cells. The genome of the related urochordate *Ciona* harbours a number of genes highly homologous to those involved in natural killing in vertebrates (Azumi *et al.,* 2003) but not homologues of the *cFuHC* gene. A transmembrane protein that has three short consensus repeats (SCR) domains termed variable complement receptor-like protein 1 (vCRL1) is expressed in follicle cells and haemocytes of *Ciona*. This protein is strikingly variable between *Ciona* individuals. In the urochordate *Halocynthia*, the putative self sterility protein is a transmembrane receptor (HrVC120) expressed on oocytes. It has 12 EGF-like domains and one zona pellucida domain. These EGF-like repeats also show genetic polymorphism between *Halocynthia* individuals. One may hypothesize that the self-recognition required during fertilization co-opted a subset of complement molecules from a defense pathway. Apparently, each of the three urochordates studied (*Ciona*, *Botryllus*, *Halocynthia*) employs completely different molecules to distinguish self from non-self (Khalturin & Bosch, 2007).

MAJOR HISTOCOMPATIBILITY COMPLEX

In vertebrates, allorecognition depends on proteins encoded by **m**ajor **h**istocompatibility **c**omplex (MHC) genes. In higher vertebrates MHC is represented by two distinct classes, MHC I and MHC II. A candidate for the primordial MHC gene is not known. Molecular phylogenetic analysis supports a relationship between the class II MHC α chain and class I β_2-microglobulin and between the class II MHC β chain and class I α chain. The most likely hypothesis is that the ancestral MHC molecule had a class II-like structure and later gave rise to a class I molecule (Hughes & Nei, 1993; Klein & O'hUigin, 1993), although now class I is more widely distributed, being expressed on most nucleated cells, whereas class II is restricted to B-cells, macrophages and dendritic cells. The plasma membrane cell adhesion proteins (N-CAM) involved in ontogenic organogenesis is probably ancestral to the adaptive immune system. N-CAM of chicken neuronal organogenesis possesses four β_2-microglobulin-like domains, and it is this domain from which the adaptive immune system possibly originated (Ohno, 1987). An MHC-like region is certainly very ancient and is believed to be present in the common ancestor of proto- and deuterostomes (Danchin *et al.,* 2003). The human genome contains at least three MHC-like paralogous regions (Flajnik & Kasahara, 2001). It has been proposed that the MHC region arose as a result of chromosomal duplications. Phylogenetic analysis shows that all duplication events in those regions occurred after the split of

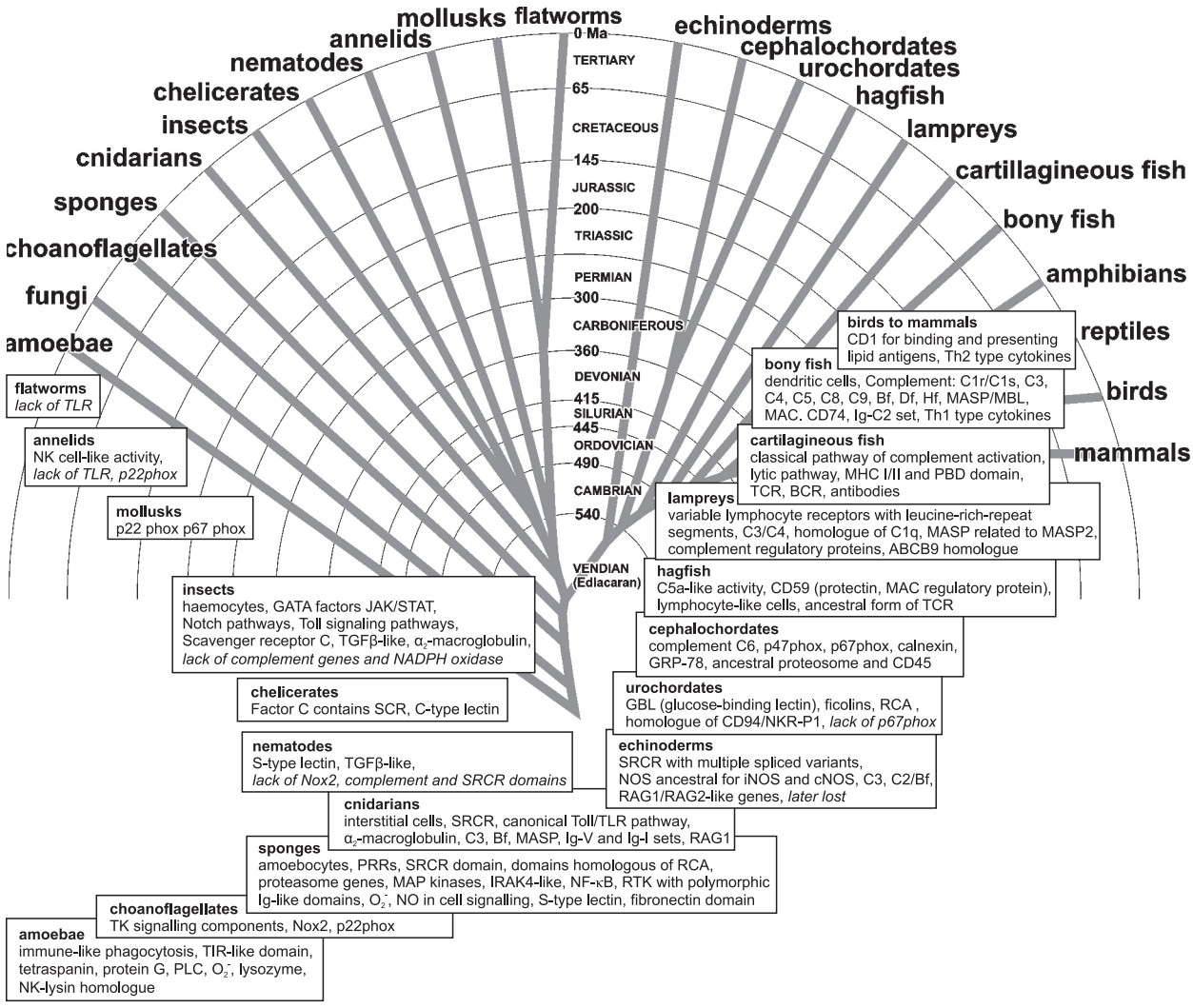

Figure 3. Cumulative pattern of introduction of immunity mechanisms in the evolution of animals.
First appearance of molecules or processes is superimposed on phylogenetic relationships among major taxa referred to in the text, with special emphasis on most ancient events.
Abbreviations: MAC, membrane attack complex; NK-lysin, natural killer cell-lysin; NOS, nitric oxide synthase (**i**nducible or **c**onstitutive isoforms); Nox, phagocyte NADPH oxidase; PLC, phospholipase C; PRRs, pattern recognition receptors; RAG, recombination activating genes; RCA, regulators of complement activation; RTK, receptor tyrosine kinase; SCR, short consensus repeats; SRCR domain, scavenger receptor cysteine-rich domain; TIR, **T**oll, **i**nterleukin-1 receptor, and plant disease **r**esistance genes; TK, tyrosine kinase.

the agnathans but before the origin of gnathostomes (Klein, 1986; Kasahara *et al.,* 1997). Perhaps the duplication allowed one copy of the genes to preserve their housekeeping function and the other copy to diversify.

Vertebrate MHC-based histocompatibility is thought to be a by-product of peptide presentation and polymorphism. The function of MHC is to present peptides (antigens in general) to T-cell receptors. The immune system uses different pathways to eliminate intracellular and extracellular antigens. As a general rule, endogenous antigens (those generated within the cell) are processed in the cytosolic pathway, transported with class I MHC molecules to the plasma membrane of most nucleated cells, and presented on its surface. Exogenous antigens (those taken up by phagocytosis) are processed in the endocytic pathway, transported with class II MHC molecules and presented on the membrane of antigen presenting cells (macrophages, dendritic cells, T-cells).

The **p**eptide-**b**inding **d**omain (PBD) is responsible for the association of the peptide and the MHC molecule. The peptide-binding cleft is formed by the membrane-distal domains in both class I and class II MHC. No clear homologue of the PBD module has been found in any non-vertebrate genome (Klein & Nikolaidis, 2005 and references there). Possibly, it originated by an inter-domain exchange. One of the two domains involved in the putative recombination could have been an **I**g-**l**ike **d**omain (ILD), which contributed a part of its β-sheet, whereas the *a*-helix could have derived from another domain (e.g., from the phage MS2 coat protein by a transposon-mediated transfer). Not only the β-sheets of the Ig-like domains resemble the β-sheet of the peptide-binding domain, but also ILDs constitute the remainder of the extracellular part in the MHC polypeptide, and genes encoding other ILD-bearing molecules are abundantly represented in the MHC region.

The cytosolic pathway

The pathway by which endogenous peptides are degraded for presentation with class I MHC molecules (expressed on most nucleated cells) utilizes mechanisms similar to those involved in the normal turnover of intracellular proteins, but how particular peptides are selected remains unclear. The resulting peptides are translocated by the transporter (TAP) to the rough endoplasmic reticulum, which is the site of MHC biosynthesis. In the reticulum, they bind the MHC molecules and the resulting peptide complexes are then transported to the cell surface for recognition by T-cells (Kindt *et al.,* 2007). The assembly of these components into a stable molecular complex involves several steps and includes the participation of molecular chaperones calnexin, calreticulin, and tapasin that facilitate folding of the polypeptides (Watts & Powis, 1999). An additional protein with enzymatic activity, glucose-regulated protein (GRP58, also called ERP57), forms a disulfide bond to tapasin, and noncovalently associates with calreticulin. This association promotes binding of an antigenic peptide which stabilizes the class I molecule – peptide complex, allowing its release from the rough endoplasmic reticulum (Van Endert, 1999). Calnexin is a transmembrane endoplasmic reticulum chaperone with a lectin activity. Calreticulin is a soluble homologue (paralog) of calnexin; GRP58 is a member of the thioredoxin enzyme family which includes protein disulfide isomerase (PDI) as well. These enzymes catalyze the isomerization of disulfide bonds of proteins undergoing folding and assembly. Phylogenetic analysis shows that calreticulin and GRP58 have homologues in all eukaryotes (Danchin *et al.,* 2003). Vertebrate calnexin and calmegin are orthologues of non-vertebrate calnexin. In vertebrates, calnexin has retained the ancestral function and has directly been co-opted for a new process, while the other duplicate, calmegin, has evolved toward a specific function in sperm fertility (Ikawa *et al.,* 1997).

The transporter associated with antigen processing protein (TAP) is a membrane-spanning heterodimer. TAP belongs to the drug peptide and lipid export (DPL) subgroup of the adenosine triphosphate-binding cassette (ABC) family and is optimized to transport peptides that would interact with class I MHC molecules (Bouige *et al.,* 2002). Within this group TAP1, TAP2, ABCB9 and Mdl1 form a monophyletic group. Mdl1 in yeast, like TAP1 and TAP2, is involved in peptide transport (Young *et al.,* 2001). Phylogenetic analysis shows that TAP1 and TAP2 arose from a duplication of the ancestor of ABCB9/TAP1/TAP2 and that an ABCB9 homologue is present in the lamprey (Uinuk-Ool *et al.,* 2003a). ABCB9 is associated with lysosomes (Zhang *et al.,* 2000) and it is therefore possible that ABCB9 is involved in peptide transport in this organelle. Tapasin is found in all bony vertebrates and probably in all jawed vertebrates, and is a transmembrane protein. Proximal to the transmembrane portion, an IgC1 domain is found, phylogenetically related to class I and class II β chains, implying that tapasin was derived from an MHC class II/class I β-like gene.

The immune system of vertebrates appears to have recruited the proteasome for the proteolytic generation of MHC class I epitopes. These ubiquitous multisubunit endoproteases are phylogenetically ancient, as they are found both in bacteria and eukaryotes. Peptides generated by the proteasome in *Drosophila* and yeast, which lack MHC function and genes, have a size and composition suitable for loading onto MHC molecules (Niedermann *et al.,* 1997), and therefore it is likely that the product of the proteasome was re-routed towards a new biochemical pathway *via* the peptide transporters. Agnathans lack the ability to produce immunoproteasomes; like in other eukaryotes, their proteasomes contain 7 isoforms of α subunit and 7 of β subunit. In vertebrates, following the *in vitro* action of interferon-γ, PSMB5, PSMB6, and PSMB7 (the three proteolytically active β subunits), are replaced by their paralogues, immunosubunits, PSMB8, PSMB9, and PSMB10, respectively. After the gene duplication events, it seems that PSMB5, PSMB6, and PSMB7 kept their ancestral molecular behavior (protein degradation), while PSMB8, PSMB9, and PSMB10 evolved toward the production of specific peptides for MHC presentation (Danchin *et al.,* 2003). Genes for PSMB and TAP proteins are still evolving divergently under selective pressure in different vertebrate lineages (Nonaka *et al.,* 2000; Powis *et al.*, 1992).

The endocytic pathway

The second location for proteolysis involves the endosomal/lysosomal compartments, where proteases digest any external or internal proteins that find their way into these organelles acquired through immune surveillance of exogenous pathogens. Similarly to class I, class II MHC molecules are assembled within the rough endoplasmic reticulum, where they associate with a transmembrane glycoprotein called invariant chain (Ii, CD74). The bound invariant chain prevents premature binding of any endogenously derived peptides, while the class II molecule is within the reticulum and helps to direct the complex to endocytic compartments containing peptides derived from exogenous antigens. Inside the loading compartments, CD74 is digested by cathepsins S and L, which leaves the binding fragment (CLIP). The removal of CLIP and peptide loading require an endosome-resident accessory molecule HLA-DM. In mammalian B-cells, peptide loading is further modulated by another molecule, HLA-DO. These molecules belong to the non-classical MHC class II family (Hiltbold & Roche, 2002).

The invariant chain glycoprotein CD74 is found only in the gnathostome vertebrates (Dijkstra *et al.,* 2003); several cathepsins seem to have been co-opted for MHC class II peptide presentation several times during evolution (Uinuk-Ool *et al.,* 2003b), at the level of exogenous peptide processing and processing of CD74. As for the cytosolic pathway system, the housekeeping chaperones such as calnexin (remains bound to incomplete complexes) are found in all eukaryotes; therefore, they probably have been recruited directly by the MHC class II system. Some of the proteins involved come from an ancestral duplication that gave rise to classical class II genes and in the other part to HLA-DM (Kasahara *et al.,* 1995). Therefore, classical class II MHC and the chaperone HLA-DM probably come from the major tinkering that happened in the proto-MHC region in the early vertebrate evolution (Abi-Rached *et al.,* 1999).

ANTIGEN PRESENTING

The function of sampling the environment for pathogens is provided in vertebrates by the lymphoid and myeloid dendritic cells, present in small quantities in various tissues in peripheral regions of the body. Those from the skin are referred to as Langerhans

cells. Like any other phagocyting cells they constantly sample the environment for viruses, bacteria, or parasites using their pattern recognition receptors to recognize specific chemical signatures of the pathogens. Immature dendritic cells capture them and then migrate to lymph nodes, where they present the antigen to T-cells. Dendritic cells mature after being contacted with antigens, which means that they shift from an antigen-capturing phenotype to one supporting antigen presentation. The chemotactic receptor CCR7 induces the dendritic cell to travel through the blood stream to the spleen or through the lymphatic system to a lymph node (Penna *et al.,* 2002). In the node, the cell-surface receptors of mature dendritic cells act as co-receptors in activation of T-cells; that is they perform their function as antigen-presenting cells to initiate the immune response.

Although originally identified in mammals, dendritic cells are more ancient than tetrapods, as they occur in the epidermis of the teleost bony fish (Wölfle *et al.,* 2009). Birds appear to already possess genes for the family of CD1 proteins (MHC-like genes) (Miller *et al.,* 2005; Salomonsen *et al.,* 2005) with the property of binding and presenting lipid rather than peptide antigens, earlier known only from mammals. In the brown rat (but not mouse), a subset of dendritic cells still exist that display pronounced killer cell-like activity, which seems to reflect conservation of a rather primitive state in their evolution (Trinité *et al.,* 2000).

THYMUS

The thymus is the only organ of the adaptive immunity system organ present in the gnathostomes and absent in the agnathans. In the thymus, the differentiation of T-cell is regulated by *SPI-B, GATA3* and genes of the IKAROS family (ref. in Klein & Nicolaidis; Suzuki *et al.,* 2004). Among the blood cells of lamprey and hagfish there are lymphocyte-like cells expressing homologues of these genes (Uinuk-Ool *et al.,* 2002 Suzuki *et al.,* 2004). Nevertheless, the agnathan cells do not express the receptor molecules TCR and BCR, thus being not fully equivalent to the gnathostomes lymphocytes (Guo *et al.,* 2009).

The membrane-bound antigen-binding receptor (TCR) of the lymphocytes produced in the vertebrate thymus (T-cells) can recognize only an antigen that is bound to the major histocompatibility complex molecules. The antigen-binding receptors on T-cells are very specific and extremely diverse. This is because in the process of T-cell maturation, a random rearrangement of a series of gene segments that encode the cell's antigen-binding receptor takes place. The mechanism of the rearrangament is similar to that in the reviewed below for B-cells, which is better known. The role of the antigen becomes critical when it interacts with and activates a mature, antigenically committed lymphocyte, bringing about expansion of the population of cells with a given antigenic specificity. In this process of clonal selection, an antigen binds to the particular cell and stimulates it to divide repeatedly into a clone of cells with the same antigenic specificity as the original parental cell. It is a Darwinian mechanism: enormous variation and then selection. The self-non-self discrimination is accomplished by the elimination (during development) of lymphocytes bearing self-reactive receptors or by functional suppression of these cells if they reach maturity. The presence of lymphocytes activated and multiplicated *via* clonal selection provides the basis of immunological memory (Klein & Nikolaidis, 2005).

The antibody-based immune system is defined by the presence of MHC, TCR, BCR and recombination activating genes, the *RAG* genes. It arose after the divergence of the gnathostomes from the agnathans (Takezaki *et al.,* 2003). The origin of the adaptive immunity represents a culmination of a long gradual accumulation of small changes in organs, cells and molecules over hundreds of million years (Klein & Nikolaidis, 2005).

ANTIBODY-BASED IMMUNE SYSTEM

About 90% of animal species have no adaptive immunity, yet they thrive, with many living for decades, in a world of microbes. Presumably the adaptive response provides a competitive advantage to animals equipped with it. They are able to fight pathogens at a much lower cost than is possible with the innate immune system alone.

A membrane-bound antigen-binding receptor (i.e., antibody) on B-cells matured in the birds' *bursa Fabricii* or in the mammalian bone marrow can recognize a free antigen. The antigenic specificity of each B-cell is determined by its membrane-bound antibodies. As in the case of T-cells, this specificity is created by random rearrangements of a series of gene segments that encode the antibody molecule. All antibody molecules on a given B lymphocyte have identical specificity. A selection process in the bone marrow eliminates any B-cells with membrane-bound antibody that recognizes self components.

A substantial diversity of immunoglobulin molecules is generated by the RAG1/RAG2 recombinase which catalyses random gene segment rearrangements during early development of the lymphocyte (the primary repertoire). Following antigen encounter, somatic hypermutation triggered by the **a**ctivation-**i**nduced **d**eaminase (AID; an enzyme that deaminates cytosine residues and thus converts them into uracil) occurs yielding a secondary repertoire. A maturing B-cell starts with dozens to hundreds of three classes of gene segments and, as it develops, the cell excises all but one of each class. The surviving segments then get stitched together into a DNA sequence that encodes the antibody unique to each mature B-cell. The T-cell similarly recombines gene segments to create distincT-cell surface receptors for pathogens (Travis, 2009). In mammalian B lymphocytes, recombination is followed by further diversification of the BCR genes by two or three mechanisms, depending on species: untemplated somatic hypermutation, pseudogene-templated gene conversion, and switch recombination (Kindt *et al.,* 2007). All three mechanisms rely on the participation of the activation-induced deaminase (Honjo *et al.,* 2004).

Immunoglobulin domains (ILDs), categorized in seven main types (V, C1, C2, C3, C4, I and FNIII), are found in all molecules essential for antibody-based immunity: MHC molecules, TCR, BCR, CD4 and CD8 coreceptors, and others (Kindt *et al.,* 2007). The presence of immunoglobulin domains in many different proteins involved in the immune response and cell adhesion suggests that these domains have been distributed among different proteins mainly by exon shuffling through intronic recombination. These domains share the basic Ig-fold structure (consisting of two β-pleated sheets of a sand-

wich rolled into a cylinder). It is generally accepted that all members of the Ig superfamily derive from a single common ancestor. However, independent origin by convergent evolution of some of them cannot be excluded (Halaby & Mornon, 1998). In the genome of the cnidarian starlet see anemone *Nematostella*, both I type and V type domains have been found. On the basis of this analysis, it is still not possible to distinguish which is the most ancestral set of immunoglobulin domains (Buljan & Bateman, 2009). Presently, only these two domains are found in lower invertebrates. The C2 type that includes domains present in the CD4 and CD8 coreceptors and adhesion molecules is known from arthropods (Buljan & Bateman, 2009). The V-domains of the subtype found in the TCRs and BCRs are known from both agnathans (Pancer *et al.,* 2004) and cephalochordates (Cannon *et al.,* 2002; Sato *et al.,* 2003), although in these they do not rearrange. Thus, the nonrearranging V-type domains arose before the AIS was established. The V-domains of TCRs and BCRs share the rearrangement mechanism, which suggests that they derived from a common ancestral domain, present before the divergence of the extant gnathostome classes (Hood *et al.,* 1985). The species distribution of immunoglobulin domains shows that the C1 type is found only in gnathostome vertebrates and developed late in the metazoan evolution. Many of the immunoglobulin-like domains found in sponges are associated with kinase domains, as it was mentioned earlier, suggesting that the immunoglobulins' ancestral function involved signalling (Buljan & Bateman, 2009).

The RAG1 protein is a large multifunctional recombinase which binds to specific recombination signal sequences flanking the V, D, and J gene segments, cleaves the DNA between these sequences and the coding sequence, opens hairpins formed by the broken ends, joins the broken DNA ends and acts as transposase, at least *in vitro*. The RAG2 protein seems to act primarily as a stabilizing cofactor of RAG1. The RAG proteins are not closely related to any other eukaryotic recombinase (Agrawal *et al.,* 1998). It has been proposed that these RAG enzymes were originally transposons, for instance a Transib family of DNA transposons (Kapitonov & Jurka, 2005), which, however, do not contain sequences related to a RAG2 protein, or arose by insertion of an infectious DNA virus resembling a herpes virus adjacent to a sequence encoding a RAG2 protein (Dreyfus, 2009), but this transposon theory remains controversional (Hughes, 1999). In the genome of the purple see urchin genes that closely resemble *RAG1* and *RAG2* have been found (Rast *et al.*, 2006). Suprisingly, the cnidarian genomes encode a protein related to deuterostome RAG1 (Hemmrich *et al.*, 2007). The occurrence of RAG proteins in echinoderms suggests that the putative transposon encoding these enzymes invaded early deuterostomes to be subsequently lost in most their lineages except for the jawed vertebrates, which adopted them to perform the VDJ recombination (Travis, 2009).

Activation-induced deaminase (AID) is a member of a vertebrate family of related enzymes which perform a variety of functions (Beale *et al.,* 2004). For instance, proteins of this family are related to RNA-editing enzymes in yeast (Xie *et al.,* 2004). Hence the ability to convert cytosine to uracil by deamination was established early in the eukaryote evolution and was then deployed repeatedly to serve specific needs in the various emerging taxa. No homologus protein has been found in the agnathan lymphocyte-like cell transcriptome or the *Ciona* genome, so these subfamilies of deaminating enzymes may represent a gnathostome innovation. Some gnathostome species possess only one of the three AID functions, which suggests that they may have been acquired sequentially in gnathostome evolution. The antigen receptor diversification triggered by AID probably arose earlier in evolution than the RAG-mediated repertoire generation (Neuberger, 2008). Quite recently, a new role of AID in active DNA demethylation and reprogramming towards pluripotency in mammalian somatic cells has been discovered (Bhutani *et al.*, 2010).

CYTOKINE EVOLUTION

The innate and adaptive immune systems do not operate independently of each other. Any interaction between receptors on macrophages and microbial components generates growth factor-like molecules, the cytokines that stimulate and direct the adaptive immune response. Below, only a general overview considering cytokine evolution is presented based on the reviews by Krause and Pestka (2005) and Huising *et al.,* (2006). It is generally believed that our adaptive immune system evolved from a novel mechanism of self *versus* non-self recognition (by virtue of discrimination of peptide sequence rather than pathogen-specific sugars or foreign lipids) to allow an effective way to eliminate viruses and virus-infected cells from an organism. In the *Ciona* genome no homologues of receptors employed by the adaptive immune system have been identified, but distant homologues of type I interferon receptors are present. According to Krause and Pestka (2005), IFNs and IFN receptors evolved during the chordate evolution but prior to the origin of vertebrates. Proteins involved in antiviral activity diverged before those involved in adaptive immunity. Genes encoding IFNs and IFN receptors duplicated multiple times during chordate evolution. The types I and II interferon (IFNγ) and IL-28-like proteins evolved as duplications from a primordial antiviral cytokine to improve or expand the innate response to viral and microbial infection. IL-10-like cytokines were derived from an IFN species as well. This suggests that many types of antiviral cytokines (interferon and interferon-like molecules) developed from the original "antiviral cytokine", and numerous cytokines evolved to assist the emerging adaptive immune system.

Many mammalian helical cytokines have orthologues in fishes; presumably, these cytokines emerged well before the divergence of tetrapods. Then, this cytokine family experienced gene duplication (IL-11, IL-12p40) and somatolactin became specific for the teleost fish lineage, whereas placental lactogens are restricted to the placental mammals. Pro-inflammatory cytokines (IL-1β, TNF-α) and anti-inflammatory ones (TGF-β and IL-10) have been discovered in teleost fishes as well. Also IFNγ and IL-18, which together with IL-12 drive the immune response towards Th1 (inflammatory response), are present in the teleost fishes. The only major group of cytokines for which not a single fish ortholog has been reported to date is the Th2 cluster (secreted in allergic diseases and helminthic infections). These cytokines are not found outside birds and mammals. This shows that Th1 and the cross-regulatory Th2 need not necesssarily have developed simultaneusly. Representatives of several classes of chemokines are also present in fish, although there is compelling evidence that the chemokine repertoires of fish and mammals differ extensively due to lineage-specific gene duplications. A common feature of all fish cytokines discovered to date is the low degree of primary sequence conservation they share with their mammalian orthologs. This relatively poor se-

quence conservation prevents identification of non-mammalian orthologs of cytokine genes.

CONCLUSIONS

In this review the thesis is supported that many defense mechanisms generally believed to be specific to the immunity systems of advanced metazoans have been inherited from unicellular eukaryotic ancestors. Phagocytosis, used as a tool of immunity by specialized cells of invertebrates and vertebrates, serves for both food acquisition and defense in amoebae. Signalling, used in many cell pathways of the metazoans against foreign cells, is based on the TIR domain, which is indispensable for protists. Mammalian lysins are related to lytic factors secreted by amoebae. The immunity of sponges, nowadays the simplest multicellular organisms, is based on the same proteins or domains as that of higher organisms. Sponge molecules involved in self/non-self recognition, sensing of pathogens, cellular signalling and transport are homologous to those of higher animals.

A great body of such proteins survived during metazoan evolution until the origin of vertebrates. Thioester-containing α_2-macroglobulin and C3 subfamilies, as well as I type and V type domains of immunoglobulin family occur in the cnidarians. Although many mechanisms of immunity are common for invertebrates and vertebrates (phagocytosis, cytotoxicity, lectins, proteinases), others are only used in invertebrates (haemolymph clotting system, melanization) although the general plan on which they operate is realized in vertebrates as well. Some of those molecules, for instance Toll-like receptors, complement components or catalytic subunit of the phagocyte NADPH oxidase were lost in various animal groups.

The antibody-based immunity did not arise instantaneously. Individual components of this mechanism existed much earlier and only had to be adapted to a new function. Molecules assisting the synthesis of MHC class I proteins: calnexin, calreticulin, glucose-regulated protein of 78 kDa, and others are examples. Another group of proteins were modified after dupliction of their encoding genes. The modification adjusted the molecules to the requirements of the antibody-based immunity. Activation induced deaminase and three pairs of proteasome subunits of β type are examples of this process. Duplications of the common ancestor of each gene pair encoding the β subunits took place within the gnathostomes. But there is also a group of molecules that did not exist prior to the appearence of antibody-based immunity. It includes the MHC molecules, TCRs, and BCRs, all of fundamental importance for the antibody-based immunity. The MHCs presumably originated by shuffling of gene segments at the genomic level, which resulted in assembling together different domains or modifying preexisting ones. Probably also the new design of the peptide-binding domain (PBD) arose in such a way.

It can be concluded that the immunity of mammals is a result of sequential introduction of immunity mechanisms operating at various stages in the evolution, from the earliest unicellular eukaryotes through worm-like metazoans, fishes, and amphibian to reptile grade tetrapods. All the time those mechanisms were enriched with evolutionary innovations as a result of the Darwinian selection of spontaneously generated genetic variability. Also the role of gene duplication and exon shuffling in the creation of the most effective ways of fighting viruses, bacteria, and parasites cannot be overestimated.

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