Evolutionary insights into the origin of innate and adaptive immune systems: different shades of grey

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Summary

To struggle for survival, all living organisms, from protists to humans, must defend themselves from attack by predators. From the time when life began around 3,500 million years ago, all living cells have evolved mechanisms and strategies to optimally defend themselves, while the invaders also need to survive by evading these immune defenses. The end results would be healthy co-evolution of both parties. Classically, immune host defense is divided into two main categories, namely, innate and adaptive systems. It is well documented that while vertebrates possess both systems, invertebrates and prokaryotes like bacteria and archaea depend almost exclusively on the innate immune functions. Although the adaptive immune system like antibodies and cellular immunity or their equivalents are believed to have evolved at the time when the vertebrates first appeared about 550 million years ago, more recent information from molecular and genomic studies suggest that different forms of adaptive immune system may also be present in the invertebrates as well. These forms of "adaptive" immune system exhibit, for instance, limited degrees of memory, diversity and similarities of their immune receptors with the immunoglobulin domains of the conventional adaptive immune system of vertebrates. Organized lymphoid tissues have been identified in all vertebrates. Very recent molecular and genetic data further suggest that a special type of adaptive system functioning like RNAi of vertebrates is also present in the very ancient form of life like the bacteria and archaea. In this review, I provide some insights, based on recent information gathering from evolutionary data of innate and adaptive immune receptors of invertebrate and vertebrate animals that should convince the readers that our current view on the innate and adaptive immunity may need to be modified. The distinction between the two systems should not be thought of in terms of a "black and white" phenomenon anymore, as recent molecular and genomic information points to the fact that a line of distinction is not as sharp as it was once thought to be, but it is blurred by different shades of grey. *(Asian Pac J Allergy Immunol 2014;32:3-15)*

Key words: *evolution of immune system, adaptive immune system, innate immune system, immune receptor, diversified immune receptor, vertebrate, invertebrate, jawless fish, jawed fish*

Abbreviations

AID-APOBEC	=	activation-induced deaminase of APOBEC family
APOBEC	=	apolipoprotein B mRNA-editing catalytic polypeptide
APAR	=	agnathan-paired receptors resembling antigen receptor
BCR	=	B cell receptor
C	=	constant
CDA	=	cytidine deaminase
CTX	=	cortical thymocyte marker
DSCAM	=	Down syndrome cell adhesion molecule
CYT	=	cytoplasmic domain
FBG	=	fibrinogen-like domain
FREP	=	fibrinogen-related protein
GP1bα	=	glycoprotein 1b α
I	=	intermediate
Ig	=	immunoglobulin
IgSF	=	immunoglobulin superfamily
ITIM	=	immunoreceptor tyrosine-based inhibitory motive
JAM	=	junctional adhesion molecule

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kDa	=	kilodalton
LRR	=	leucine-rich repeat
MHC	=	major histocompatibility complex
MW	=	molecular weight
NAR	=	new antigen receptor
NK	=	natural killer
NICIR	=	novel immunoreceptor tyrosine-based inhibitory motif-containing IgSF receptor
NITR	=	novel immune-type receptor
PBR	=	peptide-binding region
PPO	=	prophenoloxidase
PRR	=	pattern recognition receptor
RAG	=	recombination-activating gene
RSS	=	recombination signal sequence
S	=	Svedberg unit
Siglec	=	sialic acid-binding Ig-like lectin
SIRP	=	signal-regulatory protein
SNP	=	single nucleotide polymorphism
TCR	=	T cell receptor
TLR	=	Toll-like receptor
TM	=	transmembrane domain
V	=	variable
VCBP	=	variable domain-containing chitin binding protein

Introduction

The outcome of the ongoing war between host and microbes depends on the properties of both parties. Microbes, with their relatively short generation times, have evolved adaptation and mutation mechanisms that allow them to outpace host defenses. For survival, all living organisms across the animal kingdom, both invertebrates and vertebrates, employ various innate defense components like pattern recognition receptors (PRRs), antimicrobial peptides and complement components to counteract the attack. In some invertebrates, e.g., sea urchin (an echinoderm) and arthropods, and plants, these innate components have greatly expanded and are more complicated compared to their vertebrate counterparts. To be more successful against the predatory attack, the vertebrates with longer lifespan than the invertebrates must have additional strategies to cope with the high rates of mutation in microbes that have much shorter doubling times. As the variation and mutation exhibited by all kinds of microbes are unpredictable, the best option for the host is to find the most flexible ways and means to diversify its defense mechanism. One way to do that is to rapidly and randomly generate and expand the specificity of their adaptive immune capacity. The diversification of adaptive immune recognition of microbes is said to be unlimited. This is successfully accomplished by generation of somatic gene rearrangement and, to a lesser extent, by somatic hypermutation of their immune receptors in jawed vertebrates, i.e., immunoglobulins (Igs), B cell receptors (BCRs) and T cell receptors (TCRs).[1,2] It is estimated that both BCRs and TCRs can detect as many as 10^{14} different ligands. The most ancient extant vertebrates like the jawless fish (hagfish and sea lampreys) use a different approach to achieve the same purpose.[1-3] These lower vertebrates however use diversified leucine-rich repeat (LRR) proteins instead of the vertebrate Ig superfamily (IgSF) receptors to interfere with the microbial attack. The use of different molecular frameworks by the defensive components in phylogenetically distinct groups of animal species to interact with the same ligands is not unusual as, for example, it was previously reported for the natural killer (NK) cell receptors in mouse and man against the same major histocompatibility complex (MHC) ligand. In fact, NK-cell activity has been shown to have a broad phylogenetic distribution extending from mammals to protochordates or even to the more ancient species.[4] The cells with morphological characteristics typical of lymphoid cells have been identified not only in the vertebrates, but also in several members of the prevertebrate deuterostomes as well.[1] However, whether or not similar cells with similar functions are also present in lower invertebrates (protostomes) remains to be determined. It is known that hemocytes in arthropods can synthesize diversified immune receptors in response to microbial attack. It should be remembered that information regarding the presence of soluble immune components and immune cells of the adaptive system in early vertebrates is difficult to obtain because, to the present day, no other living representatives from the early jawed fish preceding the sharks are available for study. Therefore, the evolutionary events leading to the present day antigen receptor must remain highly speculative. Structurally, the NITRs (novel immune-type receptors) with activating and inhibitory activities found in cytotoxic cells of some

bony fish have a framework domain that can fit with being an ancestral immune receptor.[5] Molecular and genetic evidence now available suggest that members of a multigene family found in jawless fishes known as APARs (agnathan paired receptors resembling antigen receptors) appear to be one of the most likely candidates being close relatives of the ancestor of the adaptive immune receptors.[6]

During the last decade, there is evidence showing that some invertebrate animals use different approaches and strategies to diversify their immune recognition receptors.[7] Some of these mechanisms include for example gene polymorphism, genomic instability, gene conversion, RNA editing, etc. In the present review, some evolutionary evidence to be presented should provide convincing insight information that would support the conclusion that the distinction between innate and adaptive immune defense should not be considered to be a "black-and-white" phenomenon anymore because the line of distinction across the animal kingdom is blurred by various shades of grey.

Adaptive immune system in vertebrates

Among the agnathans (cyclostomes) or jawless vertebrates, hagfish and sea lampreys are the most ancient extant animals that have evolved around 500-600 million years ago, i.e., approximately 50 million years before the cartilaginous jawed fish (gnathostomes) first appeared.[8] During this 50-60 million year interval, no other living jawed animals with the exception of, for example, sharks, skates and rays, survived. Jawless and jawed vertebrates possess strikingly different strategies to develop somatic diversity of their adaptive immune receptors. Besides using different reactive domain frameworks, the jawless fish employ RAG (recombination-activating genes)-independent mechanism to increase the diversity of their antigen-binding repertoire.[1-3] All jawed vertebrates, from shark onward employ RAG-dependent mechanism.[9] The term "Immunological Big Bang" sometime mentioned in the literature on the evolution of immune system refers to this abrupt appearance of the typical adaptive immune components, e.g., Ig, BCR, TCR and MHC in the jawed vertebrates during this transitional period.[8,9]

Adaptive immune system in jawed vertebrates (gnathostomes)

Although Ig, BCR and TCR are rather uniform and well conserved throughout the approximately 500 million years of the evolution of jawed vertebrates, some interesting differences are worth mentioning. The adaptive immune system existing from the cartilaginous fish onward has been hypothesized to be related to 2 evolutionary events, namely, the invasion of RAG transposons (RAG1 and RAG2) at the site in the chromosome that would subsequently develop into variable Ig domain and 2 or 3 rounds of whole genome duplication.[1-3] RAGs play a role in generating diversity by creating rearranged genes from the Ig variable regions. It is surprising however that although the *RAG 1 and RAG2* genes could not be detected in the jawless vertebrates, yet a linked *RAG 1 and RAG2* genes were reported to be present in the genome of the echinoderm sea urchin. Similarly, while the MHC could not be found in the jawless vertebrates, some evidence suggesting the presence of gene(s) encoding the primitive complex "proto-MHC" has been reported in some invertebrates.[4]

With regard to the evolution of adaptive immune system in jawed vertebrates, the characteristics of TCRs, BCRs and Ig isotypes and functions remain relatively conserved over the 500 million years of evolution time. This is particularly the case when one considers the TCRs across the evolutionary period of jawed vertebrate.[10] With regard to the types of T cell receptors, the αβTCRs seem to be more evolutionally conserved than the γδTCRs, most probably because the former are constrained by MHC restriction.[10] In marked contrast to the more conserved TCRs, the evolution of BCRs and immunoglobulin isotypes appear to be more labile and more plastic. The primary difference of Ig between humans and lower vertebrates like the amphibians is the number and nature of C region of the H chain isotypes.[10] This difference may depend in part on the nature of their immunoglobulin gene organization (for example, translocon configuration *vs.* cluster configuration) in different groups of animals. It is known that the Ig of tetrapods is encoded by 2-4 gene loci whereas the sharks and possibly other lower vertebrates possess as many as 100-200 loci. The difference observed may have an impact not only on the diversity of their variable Ig rearrangement, but also on various isotypes and class switching. In addition to the variation of V-D-J rearrangement that is triggered by RAGs and RSSs (recombination-signal sequences), other mechanisms of diversification include junctional diversity, combinatorial rearrangement or even gene conversion using pseudogene (e.g., in birds and rabbits) have been identified. Somatic class switch

rearrangement, triggered by the activation-induced cytidine deaminase (CDA) of the AID-APOBEC family expressed by all vertebrate lymphocytes including those of the lampreys, has been reported to be present from the amphibians onward. The latter is consistent with the appearance of lymph nodes and germinal centers in these animals.[1] It is interesting to note that although similar enzyme is present also in the bony fishes (teleosts), no somatic hypermutation has been reported with certainty in these species.

All jawed vertebrates possess IgM and at least one other type of immunoglobulin isotypes or Ig-like-H chain isotype.[10] Among the known Ig isotypes, IgM is most likely representing primordial Ig isotype. In the cartilaginous fish (elasmobranch), this isotype is a predominant immunoglobulin secreted in the animal plasma. However, unlike in higher vertebrates, the cartilaginous fish IgM is found to be secreted in 2 forms, namely, multimeric (19S) and the more abundant monomeric (7S).[10,11] Ontogenically, the 19S also appears first and to be replaced later by the higher affinity 7S form. This phenomenon is homology to the IgM to IgG switching in higher vertebrates.[10,11] In addition to these 2 forms, other variations also exist, for example, IgM_{1gj}. Another unique primordial Ig isotype found in cartilaginous fish and in some bony fish is IgW.[10,11] The structure of the IgW in shark in particular has large variation. For example, the number of C domain varies from as few as 2 to as high as 6. The IgW isotype appears to be phylogenetically related to the IgD found also in the bony fish and higher vertebrates.[10] However, in the fish it is found to be more abundant in the secreted form and is often associated with granulocytes and inflammation. It should be reminded that the IgD isotype is predominantly a membrane-associated isotype in mammals and man. Another unique Ig isotype in the cartilaginous fish is the IgNAR (new antigen receptor) which is secreted as a dimer of covalently bonded H chains with no associated L chain.[10] It is interested to note that this structural characteristic and function has been previously reported for IgG in a phylogenetically more remote species, a camel. Another Ig isotype found in teleost fish is IgT (also called IgZ).[12-15] It is present in monomeric form in plasma, but is present in secretory component-containing polymeric form in mucosa. In fact, it is probably the most ancient reported Ig isotype specialized in mucosal immunity. In the amphibians, the mucosally associated Ig isotype is known as IgX[14,14a]. IgA, probably an IgX equivalent, is known to have evolved first in the reptiles and also plays a role in mucosal immunity in higher vertebrates.[12,13] The "IgY" isotype reported to be present in amphibians, birds and reptiles appear to be a forerunner of mammalian IgG and IgE. The expression of IgG subclasses on the other hand appears to be unique for mammals, as it has not been reported in vertebrates more ancient than the mouse. In addition to the variation in isotypes mentioned above, many of these isotypes, particularly obvious in cartilaginous and some bony fishes, are present in several other forms, i.e., difference in nature and number of constant IgH domains.

Adaptive immune system in jawless vertebrates (cyclostomes)

Until the last decade, it was believed that, unlike the jawed vertebrates, these evolutionally more ancient jawless animals had no conventional adaptive immune system to counteract against invading microbes. However, it was subsequently observed that hagfish and lampreys could mount an antibody-like high molecular weight agglutinin that is distinct from immunoglobulin M following stimulation by some particulate antigens.[16-20] Such a response could be readily enhanced following a booster injection with the same antigen. Similarly, these animals could mount accelerated allograft rejection even though in the absence of MHC and they also display delayed type responses characteristics of cellular immunity in vertebrate animals.[12] These observations [17,18,20] suggest that the jawless vertebrates may have a form of adaptive immunity that is distinct from that is known in jawed vertebrates (Figure 1). This unconventional system, which from now on will be referred to as "anticipatory" or "alternative" type of adaptive immune system, diversifies its antigen receptors by a special type of somatic gene rearrangement that is RAG independent.[20] It does not use the Ig domains but instead uses leucine-rich-repeat (LRR) domain that is rearranged by a gene conversion-like mechanism to generate its diversity (Figure 1).[1,2,21] Organs and tissues containing large number of lymphocyte-like cells have been identified (e.g., adaptive immune-related gene like *CD45* and lymphocyte-specific transcription factor genes like *Ikaros*, *Gata2/3* and *Spi-B*). The receptors on these cells are known as variable lymphocyte receptors (VLRs).[1,22,23] Up until a few years ago, this immune system was thought to have 2 distinct lymphocyte

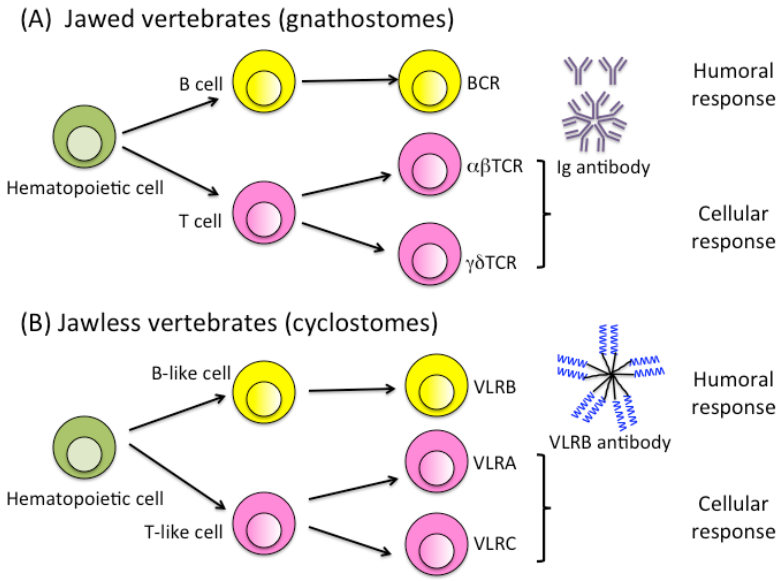

Figure 1. Compartmentalization of adaptive immune systems in jawed and jawless vertebrates.

lineages, VLRA and VLRB, that function respectively similar to the jawed vertebrate T and B cells. Both lineages show typical clonal expansion following immunization. However, very recently a third lineage with characteristics of T cells has been identified (VLRC).[1,24] Whether or not these two T cell lineages are analogous to the vertebrate αβT and γδT cells remained to be determined.

Somatic diversity of immune receptors

Following the failure to detect immunoglobulin antibody in immunized jawless fish, it was then speculated that these jawless animals might have a different form of adaptive immune system. Furthermore no BCR, TCR or RAG could be identified in the lymphocyte-like cells of immunized animals. Instead, large quantities of uniquely diverse leucine-rich repeat (LRR)-containing transcripts were found in the proliferating large lymphocyte-like cells of these immunized animals.[16,17] These receptors, encoded by a single *VLR* gene, were subsequently named variable lymphocyte receptors (VLRs). The immature germline *VLR* genes are flanked by more than 100 LRR cassettes.[18,19] Each lymphocyte expresses a unique specific *VLR* gene in monoallelic fashion. After this initial discovery, two *VLR* genes (*VLRA and VLRB*) were identified. These jawless animals were found to diversify their antigen receptors by somatic gene rearrangement that is RAG-independent, but instead the process of diversification is mediated by a gene conversion-like mechanism involving CDA enzyme. The maturation of VLRA and VLRB lymphocytes depends on the enzymes CDA1 and CDA2 respectively. These enzymes function in a lineage-specific manner in that the CDA1 is associated with VLRA cell and CDA2 with VLRB cell. This association is unlike that found in the lymphocytes of jawed vertebrates whereby the use of RAG1 and RAG2 is lineage independent.[17,18] The LRR molecules on VLRA lymphocytes are membrane associated and do not appear to bind free antigen, acting just like the TCRs of vertebrate T lymphocytes. The LRR molecules on VLRB lymphocytes are also membrane associated, but are also secreted as multivalent proteins (VLRB antibody) just like the BCRs of jawed vertebrate B lymphocytes (Figure 1B). Very recently, a third lymphocyte lineage VLRC with characteristics similar to the VLRA has been identified.[24] It has been suggested that these T-like cells, VLRA and VLRC, may be comparable to αβT and γδT cells in jawed animals respectively.

Structures of VLR and VLRB antibody

The basic structure of VLR is similar to several other LRR molecules.[19] The membrane-associated VLR is made up of a single peptide consisting of variable number of central LRR cassettes (diversity region) inserted between N-terminal LRR module

and C-terminal LRR modules that is attached to cell membrane by an invariant stalk and GPI-anchor site and relatively short hydrophobic cytoplasmic tail.[19,20] Each LRR module is made up of 8-10 LRR cassettes consisting of approximately 24 variable amino acids, folded in an anti-parallel β-sheet configuration.[20] Each VLR protein appears to have a curve solenoid structure similar to other LRR containing proteins, e.g., TLRs. The secreted multimeric protein (i.e., VLRB antibody) with MW of 320-400 kDa from VLRB cells is made up of 4 or 5 pairs of identical LRR peptides (Figure 1). It has been calculated that, by using gene conversion-like mechanism to generate diversity, the magnitude of diversification of LRR proteins with affinity comparable to that of IgG antibody of mammals can approach that of the Ig antibody, i.e., between 10^{14} and $10.^{17}$ It is of interest to observe that the VLR-like sequences have also recently been identified in some bony fishes whose adaptive immune system has already been well developed.[25,26] However, the role of the *VLR*-like gene sequences in host defense in these bony fish remains to be determined.

Evidence for the presence of common vertebrate ancestor

It is clear from the above discussion that both jawed and jawless vertebrates use structurally unrelated immune receptors to recognize and respond to structurally similar ligands. A common ancestor of jawed and jawless vertebrates is hypothesized to have possessed primordial version of TCR/BCR and VLR[1,4] (Figure 2). For the TCR/BCR, this was suggested to be V-type Ig-like domain adjoining J sequence that could later evolve into those of TCR/BCR.[4] An ancestor of VLR was suggested to have emerged from a glycoprotein GP1bα-like protein, a component of the platelet glycoprotein of receptor complex known to be conserved in all vertebrates.[2] Similarly, the CDAs, known for their role in the diversification of *VLR* genes, are encoded by genes that are believed to be descendants of the ancient *Aid-Apobec* genes.[2] These genes are not only present in the common vertebrate ancestor and jawless vertebrates, but they also continue to be present in the genomes of jawed vertebrates as well (Figure 2). In the latter, they

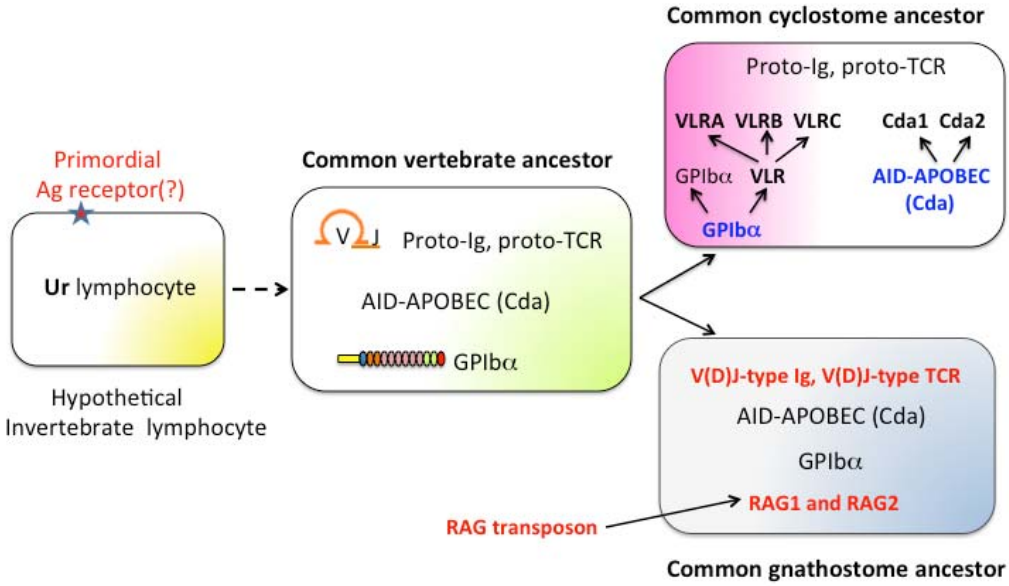

Figure 2. Genetic elements of adaptive immune systems in common jawed and jawless vertebrate lymphocytes. Genome of common vertebrate ancestor is predicted to contain ancestral version of all genes present in common jawed (gnathostome) and jawless (cyclostome) lymphocytes. The common jawed vertebrate lymphocytes are speculated to have been invaded by RAG transposon needed for V-D-J gene rearrangement. Ur-lymphocyte represents a hypothetical invertebrate lymphocyte speculated to be a predecessor of lymphocytes in vertebrate animals (based on references 1 and 2).

function in somatic hypermutation, somatic class switching, as well as in somatic gene rearrangement in some species. The genes encoding RAG1 and RAG2 in jawed vertebrates are speculated to be the results of gene transfer and insertion of a transposon, most likely from a bacterial or viral origin.[1,2] Although searching for BCR, TCR, RAG and MHC in jawless vertebrates failed to disclose the presence of these components, more rigorous transcriptome analyses in lampreys identified molecules thought to be related to the evolutionary precursors of TCR.[6,27,28] One of these proteins consists of an extracellular V-C2 domain connected to the cytoplasmic ITIM (immunoreceptor tyrosine-based inhibitory motive). This novel immunoreceptor tyrosine-based inhibitory motif-containing IgSF receptor (NICIR) is sometime referred to as "TCR-like" receptor.[27]Similarly, another molecule structurally similar to VpreB in jawed vertebrates has also been identified in sea lampreys.[28] This soluble single Ig V domain has been suggested to represent a possible forerunner of BCR. Another IgSF-containing protein now known as agnathan-paired receptors resembling antigen receptors (APARs) has been identified in hagfish.[6] Its extracellular domain consists of V-type Ig-like domain with a canonical J segment. Due to the presence of these receptors in the jawless fish, it is hypothesized that a common ancestor of all vertebrate animals must have V-type IgSF domains that could be subsequently evolved into classical BCR and TRC in higher vertebrates (Figure 2). In fact, several other investigators have previously reported that IgSF V-like sequences have existed since the very early evolutionary period, as at the time of emergence of sponges more than 600 million years ago.[7] Moreover, the V sequences fused to recognizable sequences are known to have existed prior to emergence of jawed vertebrates.[4,5]

Evolution of antigen immune receptors

Why and exactly when the adaptive immune systems in both jawless and jawed vertebrates evolved around 500 million years ago remain speculative because the resource specimens required to trace back to ancient times are unavailable, i.e., there are no living representatives from early jawed fishes preceding sharks. It has been generally speculated that the adaptive immune system emerged at the time when an IgSF gene of the "variable" V-type was invaded by a transposon containing *RAG1* and *RAG2* genes (Figure 2).[25,26,29,30] Different lines of evidence are consistent with a hypothesis that RAG plays a central role in creating rearranged genes in jawed vertebrates. On the other hand, *RAG1* and *RAG2* gene cluster and sequence elements similar to *RAG1* have also been reported in echinoderms, and in amphioxi and other invertebrates, respectively.[30] Although their function with regard to evolution is uncertain, it is possible

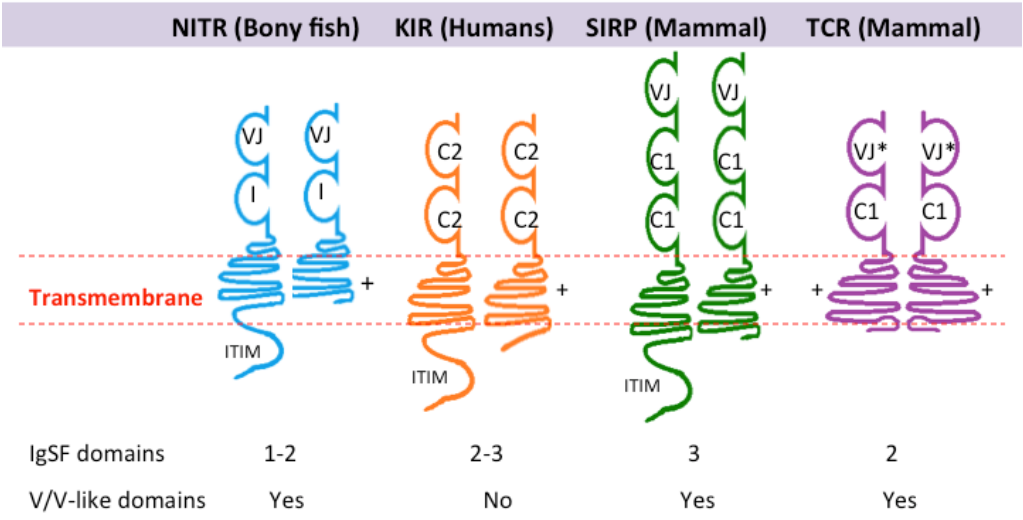

Figure 3. Similar characteristics between adaptive and innate immune receptors in vertebrates. The structural characteristics of the 3 innate (NITR, KIR and SIRP) receptors are compared with a reference adaptive (TCR) receptor. Abbreviations: ITIM = immunoreceptor tyrosine-based inhibitory motif; VJ = V-set immunoglobulin domain containing J-like sequence; VJ* = rearranged VJ domain (based on reference 25).

that in these animals these genes may have other function(s).

Transition between innate and adaptive immune receptors

In order to obtain information regarding the ancient "variable" type of receptors, it might be useful to compare the basic structure of rearranged adaptive immune receptors like those of BCRs or TCRs with that of non-rearranged innate cells, as innate system has evolved together with adaptive system for over 500 million years.[25,31] NK cell is probably the best candidate for this study as it shares a common progenitor with T and B lymphocytes but does not rearrange its receptor. As shown in Figure 3, the human KIR consists of 2 extracellular C2 IgSF domains, while the TCR chains consist of V and C1 domains. Comparing TCR with NITR which represent a multigene family of NK cell-like in bony fish shows an even more convincing relationship between the conventional adaptive immune receptors with innate immune receptor. The NITR consists of one extracellular V region type domain connecting to a membrane-associated I (intermediate)-type IgSF domain. More than 100 alleles of NITRs have been identified in some bony fishes.[5] Both mammalian KIRs and bony fish NITRs possess similar signaling characteristics. The NITRs in these bony fishes are found associated with cells having cytotoxic activity like the NK cell in mammals.[5] Signaling regulators like those of the family SIRP show an even closer relationship with the TCR (Figure 3). These signal regulating receptors consist of one extracellular V-like domain linked to 2 C1 domains.[31] The identification of C1 domains in SIRP differs from other non-rearranged IgSF members of the innate system including the NITRs mentioned above. The presence of 2 or more C1 domains in SIRP is analogous to the property of vertebrate Ig heavy chains. In fact, the first C1 domain found in non-vertebrate animals is part of the nectin-like sequences in the protochordate *Ciona intestinalis.*[4] Moreover, some SIRP genes are suspected to encode a single soluble V domain (similar to some V domain containing NITRs) (Figure 3 and 4). In general, SIRP members regulate immune function by interacting with "self" ligands. Some SIRPs serve as negative regulators for phagocytic activity in macrophages. There is evidence suggesting that SIRP genes may be expressed in animals phylogenetically more ancient (such as in birds) than mammals. It should be mentioned that the V-like domains of NITR and SIRP members are made up of V domain with joining (J) motive (Figures 3 and 4). Altogether, these structural and functional data suggest that both NITRs and SIRPs may be considered innate immune receptors that are closely related to antigen receptor predecessors.

Putative ancestors of antigen receptors in the prevertebrate protochordates

Prior to being invaded by RAG transposon, the ancestor of antigen immune receptors (e.g., Ig, BCR and TCR) probably had a V domain in which V and J segments were encoded by a single exon (Figure 2). It is speculated that a transposition process mediated by the *RAG* transposon was responsible for the insertion of a recombination-signal sequence (RSS) into the exon of this V domain, resulting in a split of gene organization into V and J segments[4,5] (Figure 2). Thus the insertion of a RAG transposable element into this V domain formed a key event in the development of somatic rearrangement in jawed vertebrates. On the other hand, in the jawless vertebrates a *RAG* insertion did not take place, and in these animals therefore a VLR-like LRR protein was instead chosen as an antigen receptor[1,2,25,26] (Figure 2). Moreover, as certain jawed vertebrates, i.e., cartilaginous fish and camel, possess an immunoglobulin isotype made from only a single pair of heavy chain, it is logical to predict that the least complex ancestral receptor in invertebrates is a single chain structure made from IgSF domains with the extracellular V and C domains connecting to a membrane segment with cytoplasmic tail. Genetic studies[4] of some antigen receptor-like components of the protochordate *Ciona intestinalis* showed that these components possess from 2 to 4 extracellular IgSF domains and the V domain may be encoded by a single exon with VJ sequence (Figure 4). Altogether, different lines of evidence from contemporary genetic studies allow one to conclude that ancestral structural elements related to vertebrate antigen receptors were present in the prevertebrate protochordates.

Diversified immune type receptors in invertebrates

In order to survive in an environment heavily contaminated with microbes, it is not unexpected that in the absence of adaptive system, early metazoans and protochordates would have an innate system that is more diverse and complicated than in the vertebrate animals which possess adaptive immunity. Because the invertebrates encompass a large group of animal species, it is to be expected

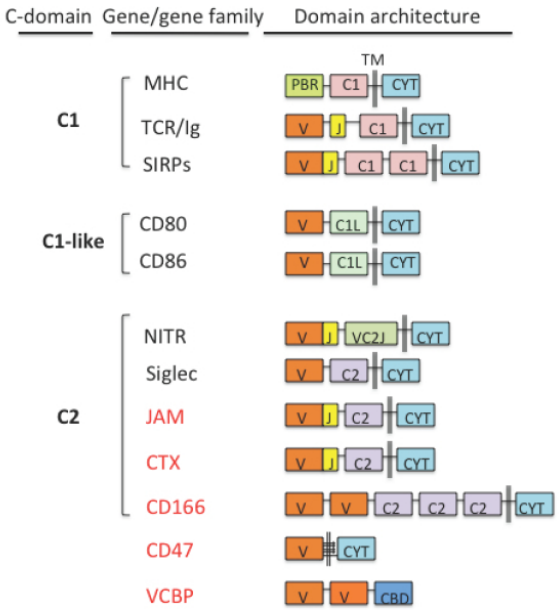

Figure 4. Gene organization and antigen receptor-like immune receptors in protochordates. Genes/gene families in protochordates (red font) include JAM (junctional adhesion molecule), CTX (cortical thymocyte marker), CD166, CD47, VCBP (variable region containing chitin-binding protein). Abbreviations: C1L = C1-like; CBD = chitin-binding domain; CYT = cytoplasmic domain; IgSF = Ig domain; NITR = novel immune type receptor; PBR = peptide-binding region; SIRP = signal-regulatory proteins; TCR = T cell receptor; TM = transmembrane domain; VCBP = V region containing chitin-binding protein (based on references 4 and 5).

therefore that they would have diverse strategies and mechanisms to diversify their immune recognition receptors and effector molecules.[7] These include, for example, gene duplication, deletion and recombination, multigene families, gene copy number variation, genomic instability, allelic diversity, single nucleotide polymorphisms (SNPs) and RNA splicing and editing. As will be seen later, some of these invertebrates are able to synthesize diversified molecules that may have from a few to several thousand different isoforms. It was mentioned earlier in this review that RAG-like proteins have been identified in the animals in *Protochordate* group as well as in lower invertebrate animals like sea urchin in the phylum *Echinoderm*.[25,26,30] However, whether or not this means that some type of somatic gene rearrangement is also present during this early stage of phylogenetic development remains to be determined. The diversified receptors that have been more thoroughly studied[7,32-34] include variable domain-containing chitin binding proteins (VCBPs) in amphioxi, fibrinogen related proteins (FREPs) in molluscs and Down syndrome cell adhesion molecules (Dscams) in arthropods (Figures 5 and 6). These components are complex molecules composed of more than one structural framework, e.g., different IgSF domains and lectin domain (Figure 5). VCBP family members are secreted proteins having 2 V-like IgSF domains at N terminal linked to a chitin-binding domain at C terminal.[32] The V-like domains are encoded by a single exon containing VJ sequence. FREPs are both membrane-anchored hemocyte proteins that are also secreted into hemolymph.[34] The proteins are made up of an IgSF domain at N terminal connecting to the fibrinogen-related protein with lectin activity at the C terminal. The Dscam proteins were reported initially to be present in arthropods, but were subsequently found also in other invertebrates like crustaceans and even in vertebrate animals like humans.[7] They are expressed predominantly in hemocytes and fat body cells, and are also secreted into hemolymph. The structure of this group of proteins has been predicted from sequencing data to have tandemly repeated IgSF domains, fibronectin domains, and a transmembrane region connecting to a cytoplasmic tail. It has been calculated that *Drosophila melanogaster* may have as many as 38,000 or more Dscam isoforms.[7]

Different lines of evidence indicate that these highly diversified groups of molecules function in defense against infections. They exhibit specific anti-microbial activity and demonstrate certain degrees of memory as evidenced by significant increased activity levels following reinfection.[32,33] These components have been shown to act as opsonins and bind specifically to some pathogenic microorganisms, agglutinating and precipitating microbial secretions, facilitating phagocytosis as well as interfering with biofilm formation.

In addition to the potential defensive role of these diversified receptors against infection against various groups of microbes, the animals that evolved during the very early metazoan evolution like those in the phyla *Porifera* (e.g., sponges) and *Cnidaria* (e.g., jellyfish) also possess the ability to diversify their alloreactive receptors against "self" ligands. Diversification and biological specificity of both the receptors and "self" ligands are shown to be related to the IgSF domains.[7,31]

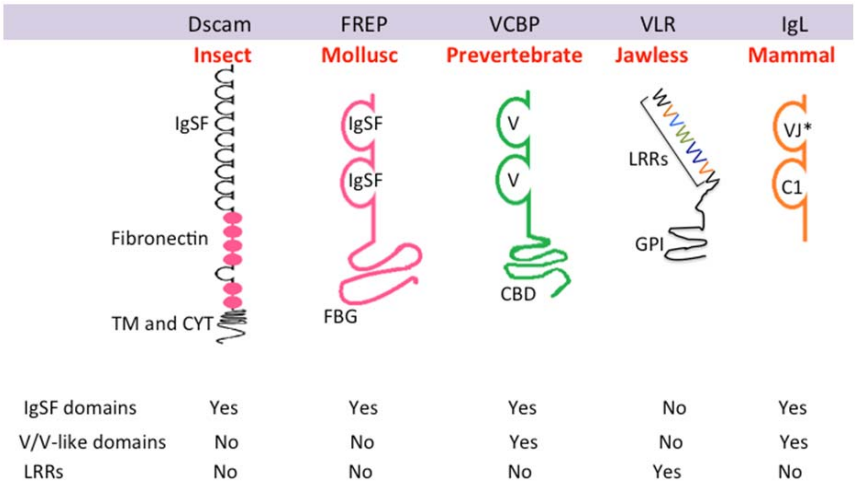

Figure 5. Diversified immune receptors in invertebrates. Main structural characteristics of the various diversified invertebrate immune receptors are compared with vertebrate immunoglobulin light chain (IgL). CBD = chitin-binding domain; GPI = glycosylphosphatidylinositol; Dscam = Down syndrome cell adhesion molecule; FREP = fibrinogen-related protein; FBG = fibrinogen-like domain; VCBP = variable-region-containing chitin-binding protein; LRR = leucine-rich repeat; VLR = variable lymphocyte receptor (modified from reference 25).

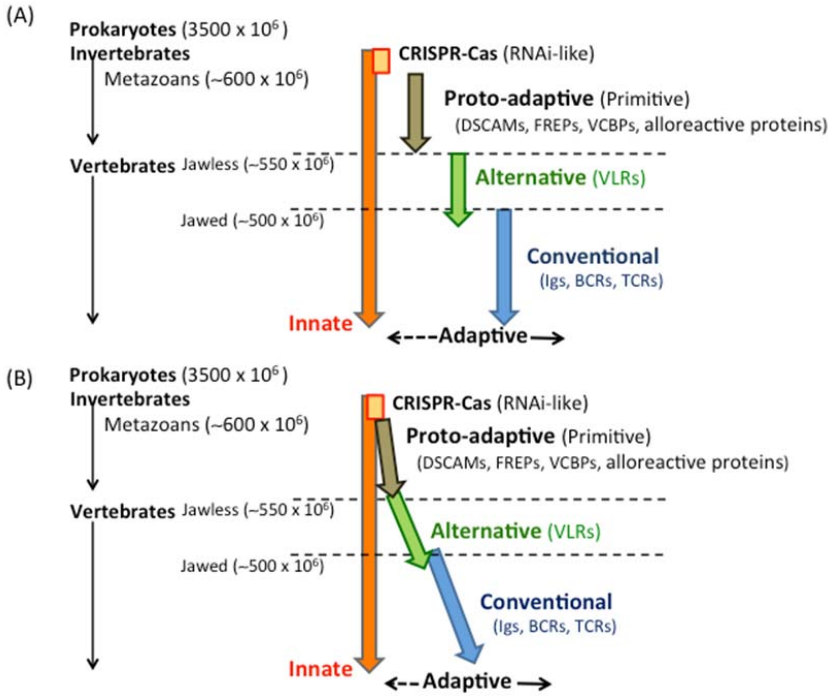

Figure 6. Hypothetical origin of "adaptive-like" immune receptors from innate counterparts. The IgSF domains which are hallmarks for adaptive immune system are detected in innate immune receptors in representative groups of invertebrate animals (A), suggesting a possible common evolutionary origin of immune receptors of the 2 immune systems (B).

Conclusions

All forms of life from the most primitive form (i.e., protists) to humans have mechanisms and strategies to protect themselves against predatory attack. Bacteria and archaea must protect themselves against bacteriophages, so that they could survive and coexist during the early stage of evolution. It was reported recently that this simplest form of life, in addition to innate immunity, possess also a special form of immunity with some degrees of adaptive characteristics, e.g., memory, diversity and specificity.[35-37] To counteract against the threat from bacteriophage attack, these prokaryotes employ an RNAi-like mechanism known as CRISPR-Cas system to enhance resistance to reinfection by the same type of bacteriophages. This novel mechanism represents a special, and probably the most primitive form of adaptive defense that can be found in cellular life.[38,39] It appears therefore that in addition to the innate system, all forms of life across the animal kingdom may also have other ways and means to fight against invading pathogens. One such mechanism is by diversification of their immune receptors (Figures 6 and 7). Different groups of animals use different mechanisms to achieve the same outcome.[38-40] For example, jawed vertebrates diversify their immune receptors TCRs and BCRs by a RAG-dependent V-D-J rearrangement. This is commonly referred to as conventional adaptive immune system with that of humans representing the prototype for this system[1,2] (Figure 6). Although the adaptive system is highly conserved throughout the 400-500 million years of jawed vertebrate evolution, minor differences in the humoral arm have been observed for example when comparing the most ancient extant jawed vertebrates like the sharks with mammals.[10,30] The extant jawless vertebrates, i.e., hagfish and sea lampreys, employ a RAG-independent mechanism to achieve the same purpose.[17,18] These animals use LRR framework rather than Ig domain and diversify their immune receptors by a gene conversion-like mechanism.[25] This type of adaptive system, referred to as alternative adaptive immune system (Figure 6), is shown to be as effective as the conventional adaptive system in terms of generating immune diversity.[3,17,18] Although initially the classical hallmarks of conventional adaptive system could not be detected in jawless fish, recent studies using more sophisticated and more sensitive technologies identified unusual immune receptors consisting of IgSF framework both in the hagfish and sea lampreys, i.e., APARs, NICIRs and VpreBs.[6,25-30] However, the functions of these receptors remain to be determined.

The invertebrate animals on the other hand diversify their immune receptors by mechanisms other than those employed by the vertebrate animals.[7] The diversified components that have been studied in more detail, e.g., VCBPs, FREPs and Dscams, have a rather complex structure, employing a combination of IgSF and lectin

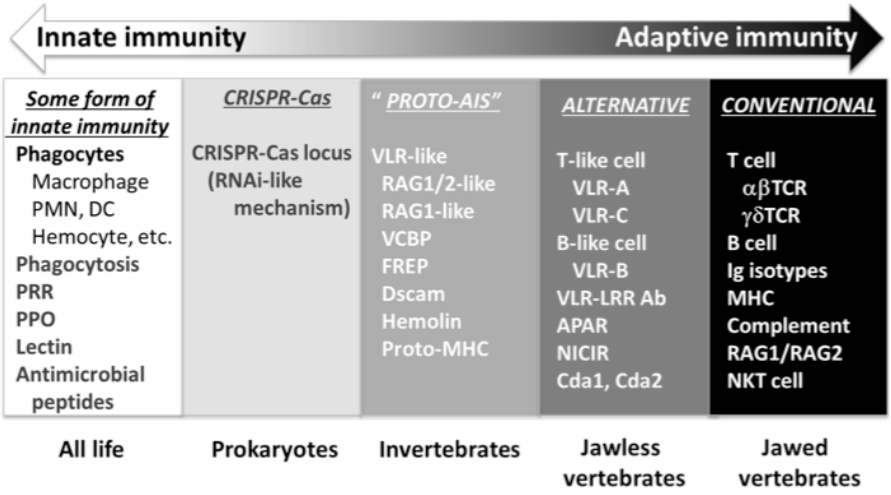

Figure 7. Different shades of adaptive immune defense across the animal kingdom. Although all forms of life are known to possess innate immune mechanisms to defense against pathogen attack, recent data suggest the possible presence of different forms of adaptive immune mechanisms in all living cells (modified from reference 38).

domains to achieve defensive functions. This system is probably the most primitive type of adaptive system and is referred to by some investigators as "Proto-adaptive" system[1] (Figure 6). It should be noted that across the animal kingdom, the organisms use different mechanisms to diversify their immune receptors. This goes all the way down the line of evolution from humans to the most ancient metazoans like the *Porifera*.[4,7,39,40] Considered all together, this makes the adaptive immunity a system that is as old as cellular life itself. Our current concept on a sharp distinction line dividing innate and adaptive immune systems may have to be modified because there are different shades of grey blurring the line separating the 2 systems into black and white areas (Figure 7).[31,38,39] Humans, which are supposed to be the most well developed animals, possess the most advanced and flexible adaptive immune system. The complexity of the system gradually reduces as one moving down the line of evolution, reaching minimal complexity in the protists (e.g., using RNAi-like mechanism in the CRISPR-Cas system in bacteria). On the other hand, there is evidence suggesting that the innate immune system which is known to have existed in all living organisms is more complex and more diverse in lower vertebrates than that found in the most advanced vertebrates.[39-43] Therefore, our current concept on a sharp distinction line dividing innate and adaptive immune system into black and white areas may have to be modified because there are different shades of grey in between that blur the line of separation (Figure 7). It is to be expected that with rapid progression in this area, our concept regarding the evolution of immune systems will be modified further in the near future.

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