Evolution of Adaptive Immune Recognition in Jawless Vertebrates

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Abstract

All extant vertebrates possess an adaptive immune system wherein diverse immune receptors are created and deployed in specialized blood cell lineages. Recent advances in DNA sequencing and developmental resources for basal vertebrates have facilitated numerous comparative analyses that have shed new light on the molecular and cellular bases of immune defense and the mechanisms of immune receptor diversification in the "jawless" vertebrates. With data from these key species in hand, it is becoming possible to infer some general aspects of the early evolution of vertebrate adaptive immunity. All jawed vertebrates assemble their antigen-receptor genes through combinatorial recombination of different "diversity" segments into immunoglobulin or T-cell receptor genes. However, the jawless vertebrates employ an analogous, but independently-derived set of immune receptors in order to recognize and bind antigens: the variable lymphocyte receptors (VLRs). The means by which this locus generates receptor diversity and achieves antigen specificity is of considerable interest because these mechanisms represent a completely independent strategy for building a large immune repertoire. Therefore, studies of the VLR system are providing insight into the fundamental principles and evolutionary potential of adaptive immune recognition systems. Here we review and synthesize the wealth of data that have been generated towards understanding the evolution of the adaptive immune system in the jawless vertebrates.

Keywords

Adaptive immune system; Evolution; Jawless vertebrates

1. Introduction

The elimination and neutralization of structurally diverse viruses, bacteria and parasites by metazoan immune systems requires a corresponding diversity of immune receptor molecules. In general, adaptive immune systems must provide clonally diverse anticipatory and self-tolerant repertoires that can be deployed by lymphocyte cell lineages. Each lymphocyte bears a unique cell surface receptor in order to recognize and respond to antigenic determinants of diverse and evolving pathogens in the ongoing struggle for survival. Until recently, the molecular details of adaptive immunity only had been described for the jawed vertebrates (gnathostomes). All jawed vertebrates, including the most phylogenetically divergent extant representatives, such as sharks, are equipped with similar adaptive immune systems that employ VDJ recombination to achieve combinatorial diversity of immunoglobulins (Igs) and T cell receptors (TCRs) [1]. In developing lymphocytes, the recombination activating genes

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(*RAG1* and *RAG2*) mediate recombination of variable region segments by random selection of each of V, D and J segments into the maturing immune receptor loci. In addition, the Activation-Induced Deaminase gene (*AID*) encodes an enzyme that facilitates gene conversion and somatic hypermutation of immunoglobulin loci in higher vertebrates, and also is required for class switch recombination. Recent findings in the cyclostomes (lamprey and hagfish) have revealed that jawless vertebrates possess an alternative form of immune receptor system that evolved independently of the Igs and utilizes a *RAG*-independent strategy to generate receptor variants that recognize and facilitate elimination of pathogens [2;3]. This system undergoes somatic rearrangements of leucine-rich repeat cassettes in the variable lymphocyte receptor (VLR) locus in order to generate immune receptor diversity that is presumably comparable to that of Igs in jawed vertebrates. Two similar VLR genes (VLRA and VLRB) have been identified in jawless vertebrates. Both VLR genes undergo somatic diversification and probably contribute to immune defense system, but the nature of somatic rearrangement and immune functions of the VLRB gene have been studied in greater detail. Two AID-APOBEC family member genes (*CDA1* and *CDA2*) were identified in lamprey and have been implicated as being facilitators of these gene conversion-type rearrangements of the VLR loci in lymphocytes [4]. Lymphocytes expressing rearranged VLR molecules can bind bacterial or erythrocyte antigens and subsequently differentiate into VLR antibody-secreting cells that morphologically resemble plasma cells [5]. The binding affinities of VLR to its cognate antigens have been empirically determined to be similar to that of mammalian Igs [6;7]. Thus, it is now clear that two radically different systems evolved in cyclostomes and gnathostomes in which either LRR (leucine rich repeat) or Ig (and TCR) gene fragments are randomly assembled to generate diverse repertoires of lymphocyte receptors and respond to antigenic challenges. This evolutionary scenario raises intriguing questions as to whether these two adaptive immune strategies represent truly convergent evolution or if one was ancestral to and replaced by the other in either the cyclostome or gnathostome stem lineage.

2. Evolution of Adaptive Immune System in Gnathostomes (brief overview)

Although this review primarily focuses on the adaptive immune system in cyclostomes, it is best understood in the context of the gnathostome adaptive immune system, because the gnathostome system is relatively well-characterized and provides an outgroup perspective on the cyclostome condition. The adaptive immune system of gnathostomes was presumably appended to the innate immune system and provided the capability to mount non-self-challenge by inducing diversity in a limited set of specialized molecules and deploying these in lymphocytes. The gnathostome adaptive immune system's vast repertoire of antigen-specific receptor molecules is generated by somatic gene rearrangement, which is mediated by *RAG* genes. In addition to Igs, which recognize unprocessed antigens and neutralize them, these antigen receptors include TCRs, which recognize antigen fragments presented by highly polymorphic major histocompatibility complex (MHC) molecules that display both self and non-self antigens on the surface of T-cells. The emergence of the adaptive immune system is believed to have occurred when an Ig superfamily (IgSf) gene of the "variable" type was invaded by a transposable element containing *RAG1* and *RAG*2 genes [8;9].

The adaptive immune system may have evolved in the context of a pre-existing innate immune system because the randomly-derived and subsequently activated lymphocytes exhibit memory of previous encounters with infectious agents and facilitate a more rapid response when these agents reappear. Several studies have revealed that jawless vertebrates, protochordates and even invertebrates have evolved sophisticated RAG-independent strategies in order to recognize and facilitate elimination of pathogens. The range of such molecules, which include the Down syndrome cell adhesion molecule (DSCAM) in *Drosophila* [10], fibrinogen-related proteins (FREP) in the snail [11], and variable lymphocyte receptors (VLRs) in jawless vertebrates [2], encompass both the immunoglobulin superfamily and leucine-rich repeat

(LRR) proteins. Despite their structural diversity, these molecules participate in various types of host defense programs. The ability to deploy structurally diverse antigen recognition molecules is a general feature of metazoan immune systems, but DNA-based somatic diversification as a means to facilitate receptor diversity is a characteristic that is apparently unique to the vertebrates.

3. Description of the Adaptive Immune System in Cyclostomes

3.1. Presence of immune cells

Mononuclear cells that are morphologically similar to vertebrate lymphocytes have been identified in various tissues and in the peripheral blood of sea lamprey and hagfish. Lamprey lymphocytes possess a very electron dense nucleus and relatively little cytoplasm, which contains numerous ribosomes but a paucity of membranous organelles [12]. The lamprey possesses several organs that may contribute to the maturation of developing lymphocytes. One of the primary immunological organs of the adult lamprey is the supraneural body (also known as the fat body or pro-vertebral arch; Fig. 1A). This structure lies dorsal to spinal column and becomes heavily populated with lymphocytes following after immune challenge [13–15]. The supraneural body from hematopoietically stimulated lampreys appears to be histologically-similar to 'bone marrow' in higher vertebrates and contains all blood cell lineages and their precursors, including lymphocytes at all stages of maturity [16]. Lymphocytes are also abundant in the lamprey kidney, where large populations are intermingled around the renal tubules (Fig. 1B), a situation very similar to that seen in the kidney of teleosts [17]. A third primary immunological organ is the typhlosole, which lies within an invagination of the gut proper (Fig. 1C). This organ contains a wide range of lympho-hematopoietic cells interspersed with stromal-like tissue and blood sinusoids. The general histological organization and hematopoietic cell composition of the typhlosole are similar to that found in the hematopoietic nets occurring in the intestinal submucosa of the plexiform veins of hagfishes [18] and in numerous organs in cartilaginous and bony fishes that are considered to be morphological and functional equivalents of the bone marrow [19].

3.2. Observation of antibody-like agglutinating activities

As is the case in the gnathostomes, lampreys have the capacity to mount considerable agglutination responses following exposure to heterologous antigens. Serum from the arctic lamprey *Lampetra reissneri* had been shown to contain natural agglutinins which react, to varying degrees, with the erythrocytes of different species. Upon repeated immunizations with sheep red blood cells, arctic lampreys showed a dramatic increase in specific hemagglutination titers [20]. The induced agglutinins were heat-stable and displayed a high degree of specificity to sheep erythrocytes. Similarly, the sea lamprey, *Petromyzon marinus*, possesses low titers of agglutinins to the H surface antigen of human "O" cells but subsequent immunization gives rise to a substantial increase in titer of these agglutinins [21]. Furthermore, these agglutinins have been shown to be antigen-specific for the O-type erythrocytes [13;21;22]. In adult *P. marinus*, the agglutinin-producing cells were found to be abundant within the supraneural body [16], whereas in ammocoetes (larvae) of the arctic lamprey the specific agglutinin producing cells were present in the typhlosole and corresponded morphologically to plasma cells [18]. Thus, the typhlosole and kidney, and supraneural organ are the most likely sites for definitive lymphopoiesis in the larval and adult lamprey, respectively. Alternatively, the general mode of lymphocyte development, differentiation and maturation in lampreys (and cyclostomes, in general) may be different than what is observed in gnathostomes [23].

3.3. Expression of immune related transcription factors in developing lymphocytes

A number of transcription factors that are known to be important in lymphocyte development and early fate specification have presumptive lamprey homologs that also are important in

lymphogenesis. The transcription factor Ets is a primary regulator of hematopoiesis in gnathostomes [24] and is expressed in embryonic blood islands and in developing lymphocytes [25;26]. The transcription factor, Bcl11a, which may function as a leukemogenic factor in gnathostomes, also is expressed in lamprey lymphocytes [27]. Other transcription factor genes found as transcripts in lamprey and/or hagfish include: *ikaros* (zinc finger lymphocyte differentiation factor) [28], *blimp-1* (B-cell induced maturation protein, PR domain containing) [29], ubiquitin conjugated enzyme E2A [30], the B-cell homeodomain-containing gene *Pax5* [31], and *Gata2/3* genes that regulate gene expression in hematopoietic cells. Altogether, expression of numerous transcription factor homologs in both gnathostome and lamprey lymphogenesis suggest that their role in early development of the lymphocyte *per se* may have been established prior to the divergence of jawless and jawed vertebrates [23].

3.4. Discovery of adaptive immune related genes

Mature cyclostome lymphocytes express many genes that are homologous to those expressed in the mature lymphocytes of higher vertebrates; including genes that have an essential role in lymphocyte proliferation and activation. Sea lamprey lymphocytes express: (1) CD45, which is involved in the regulation of T and B cell stimulation and proliferation [32]; (2) CD9/CD81, which stabilizes the molecular complexes involved in lymphocyte activation, adhesion, migration, and differentiation [33]; (3) BCAP, which serves an adaptor function in signaling and B lymphocyte activation [5;30;33]; (4) CAST, which serves a similar role as BCAP but in T-cell activation [30;33]; (5) CD98, which transports amino acids associated with lymphocyte activation[33]; and (6) SYK protein tyrosine kinase, Src family members, and HS-1 adaptor molecule [30;33], which are lymphocyte activators. Similarly, CD45, a receptor-type protein tyrosine phosphatase that regulates lymphocyte development and activation, has been identified in the lymphocyte population of the hagfish [34]. Other critical genes necessary for the mammalian adaptive immune system have been identified within the lamprey genome, including: (1) CXCR4, a chemokine homing receptor known for mammalian hematopoietic progenitors [35] as well as four additional chemokine receptors [23;33]; (2) two CC chemokines [23]; (3) CXCL12, a chemokine receptor that pairs with CXCR4 and is involved in regulating the precise spatial distribution of lymphoid progenitors in hematopoietic tissues [23]; (4) the chemotactic inflammatory cytokine IL-8 [36]; (5) the IL-17 receptor [5]; (6) CD4-like molecules [37] and (7) Toll-like receptor (TLR) orthologs TLR2a, TLR2b, TLR2c, TLR7 and TLR10 [5], which function in B-cell activation. Cyclostomes also express genes that are similar to those encoding gnathostome adaptive immune receptors, but which lack the diversity of these receptors. A single copy of a TCR-like gene having divergent V and J type sequences and a VpreB-like gene is expressed in lymphocyte-like cells of sea lamprey [38] and a family of paired Ig-like receptor genes encoding transmembrane proteins with activating and inhibitory potential has been isolated from hagfish lymphocytes [37]. Surveys for homologs of genes that are expressed in vertebrate lymphocytes provide evidence for shared regulatory and signaling functions in cyclostome and gnathostome immunity.

3.5. Identification of jawed vertebrate thymus-associated orthologous structures in lamprey

3.5.1. Histological investigation—The search for a thymus in the lamprey was based on the presumption that all higher vertebrates possess and require this structural innovation for immune function. Classical light microscopic studies represented the first attempts to identify a thymus analog in lamprey. These studies revealed numerous lymphocytes and macrophages within structures that are located under the pharyngeal epithelium of ammocoetes [17]. These structures (known as pharyngea lamina propria) appear to be involved in the uptake of foreign materials (particulates) from the pharyngeal cavity [39], but on the basis of more recent observations these clearly were neither a thymus nor a primordial precursor of such an organ [40]. Zurbrigg and Beamish [41] showed that the presumptive lymphocytes of sea lamprey stained positive with Thy-1 (CD90), which is typically associated with the thymus and T-cell

reactivity in gnathostomes. They found Thy-1 reactivity in different tissues including typhlosole, opisthonephros, liver, external gill openings in larval lamprey, but were unable to locate a thymus-like structure. Based on all available evidence, the lamprey lacks an identifiable thymus, implying that the thymus, an extremely specialized structure, probably arose as an innovation within the lineage that led to the jawed vertebrates [42].

3.5.2. Molecular investigation—In mammals, T lymphocytes develop from a common lymphocyte progenitor in the bone marrow. These progenitor cells leave the bone marrow and home to the thymus where they undergo development, differentiation and thymic selection. The developing thymocytes are embedded in the interstices of an extensive network of epithelial cells mainly in the cortex of the thymus. The gnathostome transcription factor, Foxn1, occupies a central position in the thymic genetic network and has a role in establishing a functional thymic rudiment. Foxn1 is expressed in the thymus in cartilaginous fishes and all other jawed vertebrates and is considered a key factor in the evolutionary emergence of the vertebrate thymus. A presumed ortholog of the *Foxn1* gene (*Foxn4L*) has been discovered in lamprey, and found to be expressed in epithelial lining of the gill basket in lamprey ammocoetes [23]. In the gnathostome thymus, the differentiation of lymphocyte progenitor cells into the T cell lineage is dependent on the Notch ligand Delta-like 4 (Dll4) [43;44]. Analysis of whole genome sequence data revealed that the lamprey genome contains three distinct *Dll4* genes, whereas the teleost fishes possess orthologs of five such genes. One of the lamprey delta-like genes is expressed in a circumscribed region of the epithelium lining the gill basket of ammocoetes, precisely overlapping with expression of the *Foxn4L* domain, indicating an evolutionarily conserved pattern of co-expression of *Foxn4/Foxn4L* and *Delta*-like genes. Moreover, genes for two Notch orthologs (NotchA and NotchB), which transmit Delta-like signals to developing hematopoietic cells in vertebrates [45] have been identified in the lamprey genome and shown to be highly expressed in leukocytes and lymphocytes. However, neither *VLRB* (a marker of mature lymphocytes) nor *CXCR4* (chemokine receptor which is expressed in virtually all leukocytes) were found within the same epithelial structures in histological sections of ammocoetes of *Lampetra planeri* [30]. The cells expressing *VLRB* are located largely in the vasculature of gills and gill-associated structures. Importantly, no large aggregations of lymphocytes are localized in any particular site within those tissues. In addition, no *VLRB*-positive cells were detected within the epithelial structures, corroborating the overall differences between these tissues and thymus [23]. In summary, molecular surveys for thymus-related genes in lamprey provide evidence that the thymic and gill basket epithelium likely share a common developmental origin and that the evolutionary emergence of the thymus may have occurred in the common ancestor of gnathostomes through modification of this epithelium. However, the structures in lamprey are clearly not histologically or functionally equivalent to the gnathostome thymus.

4. Discovery of the Adaptive Immune Receptor of Cyclostomes (VLR)

The first evidence for the existence of the VLR receptors came from a subtractive EST survey of lymphoid cells of immunized ammocoetes, which resulted in the identification of a large number of sequences encoding a particularly complex set of leucine-rich repeat (LRR)-containing molecules [2]. These proteins were named variable lymphocyte receptors because the transcripts were expressed predominantly or exclusively by lymphocytes and each transcript encoded an ectodomain that differed among all other VLR transcripts. The basic structure of the variable lymphocyte receptor is similar to many other LRR-containing proteins and consists of: (1) conserved signal peptide; (2) N-terminal LRR (LRRNT); (3) variable number of diverse LRRs ("diversity region"); (4) connecting peptide followed by C-terminal LRR (LRRCT); (5) conserved C-terminus composed of a threonine/proline-rich stalk (analogous to Ig "constant region"); (6) glycosyl-phosphatidyl-inositol (GPI)-anchor site; and (7) hydrophobic tail. There is no detectable signaling tail. BAC clone analysis revealed that

the germline VLR is invariant, lacks the LRR modules that impart the enormous diversity of this molecule, and that the missing LRR modules reside 5' and 3' of the transcription start and stop sites. Diversity of the transcribed receptors is thought to occur by subsequent recruitment of LRR cassettes into the diversity region through a novel recombination mechanism that accesses the large banks of flanking LRR cassettes.

4.1. Genomic Structure of VLR Loci in cyclostomes

The germline VLR locus (gVLR) lacks the capacity to encode a functional immune receptor and must undergo rearrangement in order to achieve this functionality [2]. The gVLR core contains the presumptive promoter, transcription initiation and termination sites and the functional start and stop codons, all arranged in the same relative orientation as the mature VLR locus (mVLR) (Fig. 2). However, the region between the start and stop codons does not encode an open reading frame and does not possess cassettes of variable leucine-rich repeats (LRRs) that build the mature locus. Rather, the region that is ultimately populated by LRRs consists of two long (~6kb) intervening regions that are interrupted by a partial LRR-CT module. The LRR minigene cassettes are inserted [46] randomly from elsewhere in the genome between invariant N- terminal and C-terminal LRR cassettes during lymphocyte ontogeny.

Many, if not all, of the LRRs that are incorporated into the mVLR are found at the upstream and downstream regions that flank the presumptive promoter and core region. These belong to several defined classes. The so-called N-terminal repeats (LRRNT) and C-terminal (LRRCT) repeats are respectively invariant to the 5' and 3' regions of the VLR locus that immediately flank the intervening (non-coding) sequence. Other classes of repeats include: LRR1, which always abuts LRRNT, LRRV (variable), LRRVe (a distinct subclass and basically the last LRRV), and CP ("connecting peptide" a truncated LRR), which always abuts LRRCT. Sequencing surveys and reverse transcriptase assays have revealed substantial variation in the length of mVLR molecules, which derives from the incorporation of variable numbers of LRRV cassettes. Thus, a single basic structure can be defined for the mVLR locus: SP-LRRNT-LRR1-LRRV$_{1~9}$-CP-LRRCT-C terminus.

Rogozin *et al.* [4] performed a survey of all available sequences from the ~5X lamprey genome sequencing project in order to identify genomic intervals that contribute to the mVLR. This survey searched for matches (>30bp) to a larger number of functional (transcribed) mVLRs and identified 2.2 Mb of sequence fragments (genomic contigs) that contained exact matches to mVLRA molecules and 2.1 Mb that correspond to mVLRB molecules. Understanding how these repeats are distributed in the genome will be the key to understanding the cellular basis of VLR rearrangement/maturation. Specifically this will reveal whether all incorporated cassettes lie in the regions that immediately flank the VLR locus, or if some are incorporated from other chromosomes or distant genomic regions.

The genomic structures of the distinct VLRA and VLRB loci have been characterized for both lamprey and hagfish [2;47] and all loci share the same general modular structure. Notably, this structural conservation contrasts with extensive divergence at the nucleotide sequence level, as might be expected for two lineages that have been evolving independently for >500MY. The most obvious difference among these is between the between gVLRBs. Hagfish gVLRB lacks the 5' end of the invariant LRRNT module and has only one LRRCT module in contrast to two that have been identified in lamprey. The only notable structural difference in the VLRAs is the presence of an intron in the 5' UTR of the locus in hagfish that is not seen in lamprey [47] .

4.2. Cellular Basis of Rearrangement

Studies of mVLR molecules, rearrangement intermediates and the lamprey genome have provided several insights into the cellular basis of VLR rearrangements. Notably, these rearrangements are certainly not dependent on the same RAG-mediated mechanism that generates diversity in the immune receptors of jawed vertebrates as no *RAG* genes have yet been identified in the lamprey genome and patterns of VLR recombination are not consistent with homologous recombination at defined consensus sites [4;48]. The VLR assembly also lacks recombination signal sequences (RSS) that characterize the immunoglobulin variable, diversity and joining segments [30]. Rearrangement intermediates have been isolated from the sea lamprey (*Petromyzon marinus*) and Japanese lamprey (*Lethenteron japonicum*) by targeted sequencing of lymphocyte DNAs. These intermediates reveal that LRRs are incorporated onto one and/or both ends (5' and/or 3') of the maturing molecule. Incorporated modules show evidence of multiple switches between distinct LRRs that presumably are located in non-continuous segments of the VLR flanking region. The distinct segments that are incorporated into the maturing VLR exhibit short stretches of sequence homology at their boundaries (6–30 nucleotides) [4;48]. In mature lymphocytes, regions of the intervening region that are replaced by LRRs are not observed elsewhere in the genome, arguing against an equal exchange (i.e., cross-over) mechanism. In conjunction with the apparent directionality of LRR incorporation, LRR insertion events likely are mediated by replication, rather than exchange.

This pattern of replication-mediated recombination has been likened to 'copy-choice' gene conversion that occurs during mating-type switching in fission yeast [4;48;49]. Copy-choice recombination is, in essence, a targeted version of replication-recombination events that are collectively termed synthesis-dependant strand annealing [50]. Synthesis-dependant strand annealing occurs broadly during DNA repair in yeast [50] and likely, in vertebrates [51]. These recombination mechanisms require invasion of a free (i.e., broken) 3' end into a non-allelic (donor) region with sequence homology. The homologous sequence acts as a template from which continued synthesis can proceed. Reinvasion and homologous pairing with the parent strand permits resolution of the recombination event without disruption of the donor region. There are, however, distinct differences between VLR recombination and known replication-recombination mechanisms. The most obvious difference between the two is the need for multiple strand invasions, extensions, and terminations that are targeted to different genomic sites (Fig. 2). Moreover, the events are programmed to occur upon maturation of lymphocyte cell populations. Hence, VLR recombination events are seemingly more tightly-regulated (in a developmental sense) and recombinationally-complex than other known forms of replication-mediated exchange.

If, as all evidence seems to indicate, the mVLR is synthesized by a replication-recombination mechanism, then a fuller understanding of the mechanisms underlying VLR diversification will require identification of the molecular players that mediate strand breakage, invasion, synthesis initiation/termination and juxtaposition of the core VLR and imported LRRs. Recently, we have gained an appreciation for the fact that VLR rearrangements are superimposed on a genome that itself undergoes more broad-scale rearrangements much earlier in development [52]. Given that VLR diversification and broad-scale rearrangement result in highly regulated somatic recombinations that are not typical of other vertebrate species, it seems likely that their underlying mechanisms may share some common molecular players.

4.3. Crystal-structure and antigen-binding properties of VLRs

The molecular basis of antigen recognition by antibodies in the vertebrate immune system is well studied and has revealed how the immunoglobulin folds with their CDR (complementarity determining region) loops can form a high-affinity binding site for virtually any antigen it encounters, whether natural or synthetic [53]. How VLR proteins can assess and recognize

antigen has also been revealed [54;55], though not as broadly. High sequence variability in the Ig fold is concentrated in CDRs H1, H2, H3, L1, L2, and L3, whereas that of VLRs is confined to the concave surface of each LRR module. The crystal structures of the cyclostome VLRs adopt a solenoid-shaped structure common to LRR family proteins and the backbone structure of the LRR modules between VLRA and VLRB are highly similar [54;56;57] and the sequence variation is concentrated on the concave surface of the protein, which presumably is the site of antigen contact. In general, the length of LRRCT insert is known to differ depending on the size of antigens [7]. Variation in the numbers and sequence of LRRs affects the structure at the concave surface, and modularity that is inherent in the LRR scaffold permits changes in the residues within the this surface without affecting the overall stability of the protein [58]. Recent findings on a VLR-HEL (hen egg lysozyme) protein complex reveal that although lamprey VLR antibodies are fundamentally and structurally different from the antibodies of jawed vertebrates, the soluble monomeric VLRB binds to HEL with similar affinity as that of IgM antibodies, which exist as multimers in the plasma of higher vertebrates[7]. The crystal structure of VLR-HEL protein-antigen complexes reveal that nearly the entire concave surface of lamprey is accessible for antigen binding. The binding surface area by VLR to HEL is similar to the surface buried by Ig-antigen complexes [7]. The VLR binds over the catalytic site of HEL with a loop of LRRCT that penetrates into the carbohydrate-binding cleft. This form of binding is completely distinct from that of mouse $V_L H_H$ antibodies, which bind to the flatter surface of HEL, but is similar to the heavy chain V_H antibodies of camelid and IgNAR of sharks [6;7;59–61]. *In vitro* mutagenesis of VLRB can increase the binding affinity by altering the electrostatic potential surface, a mechanism which is reminiscent of the effects of somatic hypermutation of Ig antibodies which normally occur in the maturing lymphocytes of jawed vertebrates.

It has recently been shown that VLRA antibodies also are capable of very high-affinity interactions with antigens [6]. The binding ability of VLRB to heterologous RBC and HEL and VLRA to HEL provide further evidence that both VLRs act as adaptive immune receptors in sea lamprey. The extreme conservation of key residues suggests that the few resolved structures are representative of the structures of the entire repertoire of jawless fish VLRs and that these highly stable, modular and relatively small (15–25 kDa) single-chain peptides can bind a broad range of antigenic determinants with high affinity and specificity. Moreover, binding sites can be readily engineered *in vitro*, and perhaps *in vivo* [6] for improved binding properties. These findings indicate a functional parallelism between VLR-based and Ig-TCR-based antibodies and support the notion that both classes of antigen receptors were optimized over hundreds of millions of years and have evolved in the context of similar underlying lymphocyte gene networks.

5. Phylogeny of the VLR system

Empirical and *in silico* experiments have shown that an analogous VLR system capable of recombination is probably not present in urochordates (*Ciona*), arthropods (*Drosophila*), amphioxus (*Branchiostoma*) or mammals. In the teleost fishes (zebrafish, pufferfish), we have identified two adjacent VLR-like sequences that have the same modules as found in cyclostome VLRs (Fig. 3); however, their genomic structures indicate that they cannot undergo rearrangement (i.e., they are fixed in organization and resemble cyclostome mVLR loci). The expression of these genes (named zVLR1 and zVLR2) in zebrafish has been detected by RT-PCR in hematopoietic tissues in adults. Expressed sequence tags (ESTs) have been identified from several tissues for zVLR1 and *in situ* hybridization has suggested that it is expressed in a number of developing structures, including the blood stem cell compartment (unpublished). The zVLR2 gene also has been identified in zebrafish EST databases but at lower frequencies. It is known that zebrafish possess a well-developed Ig- and TCR-based adaptive immune system [62], but the role of these VLR-like genes in immunity is unknown. On the basis of

gene organization and sequence identity, these zebrafish genes appear to be distant homologs of cyclostome VLRs. A more generic inference was put forward by Rogozin *et al.* [4], who, on the basis of genomic organization and amino acid conservation profiles, suggested that the VLRs share an evolutionary history with the platelet receptor glycoproteins, a class of genes found only in vertebrates. The evolutionary relationship between the cyclostome VLRs and vertebrate LRR-containing loci is not obvious, although remnants of shared ancestry of VLR-like genes have been identified.

6. Distributions and functions of VLR-expressing cells

6.1. Distribution of VLRA+ and VLRB+ lymphocytes

In gnathostomes, B-cells are generated and develop in the bone marrow (or its equivalent), whereas T-cells migrate from distal sites (bone marrow) and undergo development and maturation in the thymus. Recent studies with two-channel FACS-sorted cells by VLRA-and VLRB-specific monoclonal antibodies from four major tissues have shown that VLRA+ and VLRB+ cells belong to discrete lymphocyte populations and exhibit characteristic tissue distribution patterns in lamprey [5]. Although both lymphocyte populations are distributed in the primary lymphoid tissues of lamprey larvae such as blood, kidneys, typhlosole and gill region, the VLRB+ lymphocytes outnumber VLRA+ lymphocytes in all tissues, except the gill region. The VLRB+ lymphocytes are most abundant in the blood and kidneys; whereas, VLRA+ lymphocytes are found primarily in the gills. The distribution pattern is similar in the tissues obtained from both adults and larvae. These results were validated by qPCR with cDNA from flow-sorted cells from all of those tissues from both adult and larvae. The VLRA+ lymphocytes express only VLRA transcripts, whereas the VLRB+ lymphocytes exclusively express VLRB transcripts. The double-negative population did not express VLR transcripts of either type. The relatively high concentration of VLRA+ lymphocytes in the gill region may reflect a developmental origin of these cells or a preferred site of secondary residence [5].

6.2. Differential gene expression by VLRA+ AND VLRB+ cell populations

Conventional monoclonal antibody reagents that specifically detect VLRA and VLRB have allowed the isolation of respective lymphocyte populations and subsequent analysis of differential gene expression by respective populations [5]. Lymphocytes from immunized lamprey express homologs of mammalian cytokines and cytokine receptors. The most notable finding is the expression of *IL-8R* and *IL-17* by the VLRA+ lymphocytes but *IL-8* and *IL-17R* by the VLRB+ lymphocytes [5] (Fig. 4). The VLRA+ cells expressing *IL-17* may attract IL-17R-bearing VLRB+ cells and conversely, VLRB+ cells may utilize IL-8 to attract and engage IL-8R-bearing VLRA+ lymphocytes. This *ad hoc* hypothesis of cell-cell interaction is based on knowledge of the functions of interleukins and interleukin receptors in gnathostomes, and requires experimental validation. Lamprey lymphocytes also express many chemokines and their receptors. An ortholog for CCR9, which facilitates the first wave of embryonic thymus colonization in mouse, also has been identified in the lamprey genome; however, its expression patterns have not been resolved. CXCR4, another chemokine receptor, which acts as a homing receptor for hematopoietic progenitors, is expressed by VLRB+ cells [5] and is localized in the vasculature of gills [23].

TLRs regulate the development and persistence of T- and B-cell responses and memory in mammals [63]. Studies have shown that the murine splenic naïve B cells express a variety of TLRs with the exception of TLR5 and TLR8. Induction of robust antibody production and responses to antigenic challenge appears to require direct stimulation of B-cells by TLRs [64][65]. In lamprey, at least three different *TLR* genes (*TLR2*, *TLR7* and *TLR10*) have been identified and are expressed by VLRB+ lymphocytes [5]. The expression of TLR ligands by the VLRB lymphocytes suggests that TLRs may facilitate the activation of this population in

a manner similar to their role in mammalian B lymphocyte activation. In addition, CD4-like and TCR-like molecules have been reported to be differentially expressed in lamprey lymphocytes [37]. These data suggest that all of these differentially expressed lymphocyte factors probably existed in lymphocyte-like cells prior to the divergence of cyclostomes and gnathostomes. However, it still remains to be seen what role these factors play in VLR function and in lamprey immunity.

6.3. VLRs in humoral and cellular immunity

Experimental evidence suggests compartmentalized roles in humoral (VLRB) and cellular (VLRA) immunity [5]. Immunization of sea lamprey larvae with a single dose of either mouse or human erythrocytes increases VLRB antibody responses, which peaks at around 19 days post-inoculation. However, the hemagglutinin response to erythrocytes was highly variable and depended on antigen dose and individual mouse or human donor. A booster immunization on day-14 results a dramatically increased the specific hemagglutinin response, with a 20-fold amplification. Importantly, when plasma samples were depleted of VLRB by treatment with anti-VLRB coated Sepharose beads, the hemagglutinin activity was almost completely abrogated [66]. This result provides strong evidence that the erythrocytes were indeed agglutinated by VLRB that was produced by the immunized lampreys. The VLRB-bearing lymphocytes are presumed to bind bacterial or erythrocyte antigens and respond by proliferation and differentiation into plasmacytes that secrete multimeric VLRB antibodies specific for protein or carbohydrate epitopes (humoral immunity). Comparative and experimental evidence suggests that the VLRAs remain tethered to the cell surface [5]. This, in combination with experimental evidence that directly supports the idea that VLRA antibodies also are capable of very high-affinity interactions with antigens [6] and the molecular similarity between VLRA lymphocytes and T cells, strongly implies a primary role of VLRA-expressing cells in cellular immunity. These findings provide ample evidence for functional parallelism between VLR-based and Ig-based antibodies and provide further evidence that the origin of adaptive immune system likely predates the common ancestor of all extant vertebrate lineages.

6.4. Lamprey VLRA[+] and VLRB[+] lymphocytes functionally resemble gnathostome T- and B-cells, respectively

Both VLRA[+] and VLRB[+] lymphocyte populations responded strongly when larval lampreys were immunized with *Bacillus anthracis* exosporium by intraperitoneal injection. The most notable functional difference between VLRA[+] and VLRB[+] populations is that VLRB^{+} cells secreted their VLRB antibodies in response to immunization [66], whereas the VLRA[+] population did not secrete antibodies [5]. Similar results also were observed when the lampreys were immunized with *E. coli* [5]. Consistent with this observation, 293T human embryonic kidney cells that were transfected with VLRA cDNA produced VLRA proteins that were tethered to the cell surface (i.e., not secreted); whereas transfection of the same cell line with VLRB cDNAs resulted in the secretion of VLRB protein products as multimeric forms [5]. It also is worth noting that a large number of VLRB[+] lymphocytes were detected in the blood of both naive and immunized animals, whereas no VLRA^{+} cells that could bind anthrax spores were detected either before or after immunization. These features raise the possibility that VLRA[+] lymphocytes respond to antigenic stimulation in a manner that strongly resembles the T-cell response and that the VLRA proteins, like TCRs in jawed vertebrates are expressed exclusively as transmembrane-bound molecules [5]. Conversely, VLRB[+] lymphocytes respond to antigenic stimulation in a manner that more closely resembles the B-cell response. The preferential expression of *TLR* orthologs by the VLRB[+] lymphocytes suggests that TLR ligands may facilitate activation of this population of lymphocytes in a manner similar to that described for mammalian B lymphocytes. Enhanced expression of *IL-17* and *MIF* transcripts by VLRA[+] lymphocytes after immune stimulation indicates that VLRA[+] lymphocytes may

use these pro-inflammatory cytokines to engage the IL-17R-bearing VLRB^{+} cells in a manner similar to mammalian T-cells. Conversely, upregulation of IL-8 in activated VLRB^{+} cells suggests that they may use this cytokine to attract IL-8R-bearing VLRA[+] lymphocytes [5]. These observations suggest that VLRA[+] lymphocytes may be analogous to gnathostome T-cells and VLRB[+] lymphocytes analogous to gnathostome B-cells.

7. Conclusions and future prospects

Jawless vertebrates use receptors comprised of variable leucine-rich-repeat modules as counterparts of the immunoglobulin-based receptors (Ig and TCR) that form the basis of the gnathostome antigen recognition system. The discovery that cyclostomes and gnathostomes possess independently-derived immune receptors, which act in the context of very similar underlying lymphocyte genetic programs, suggests that either VLR or Ig, or both evolved in the context of specialized lymphocyte lineages that evolved prior to diversification of the ancestral vertebrate lineage. The question remains open as to whether: (1) VLR evolved in the context of an ancestral Ig expressing system; (2) Ig evolved in the context of an ancestral VLR expressing system; or (3) both systems evolved in parallel, being appended upon the same ancestral system. However the possibility that hagfish are basal to the lamprey-vertebrate split and the observation of VLR-like genes within the zebrafish and fugu genome, suggest the intriguing hypothesis that VLRs represent an ancestral immune receptor molecule.

Major challenges remain in elucidating the evolutionary history of the VLR locus, unraveling the mechanisms underlying its recombinational diversification, and dissecting the mechanisms that regulate its expression and ontogeny. Progress toward sequencing and assembly of an ever-increasing number of genomes from phylogenetically important vertebrate groups (including lamprey and hagfish) should provide broad-scale synteny information that will aid in reconstructing ancestral genomes and may reveal the origin of VLR and the fate of its vertebrate orthologs. Identifying the proteins that interact with the VLR locus at the time of rearrangement will be critical to understanding the precise recombinational mechanisms that give rise to receptor diversity. It is anticipated such studies also will provide insight into broad-scale rearrangements that occur during embryonic development. Dissecting the signaling mechanisms that underlie development and activation of VLRA[+] and VLRB[+] lymphocytes will be important for deducing whether the lymphocyte-type populations that existed in the ancestral vertebrate lineage gave rise to gnathostome T- and B-cell subsets. Finally, a better understanding of the cellular aspects of the VLR signaling cascade and the mechanisms underlying immunological tolerance are imperative, and will greatly aid in revealing general evolutionary strategies that underlie the emergence of the vertebrate adaptive immune system.

Acknowledgments

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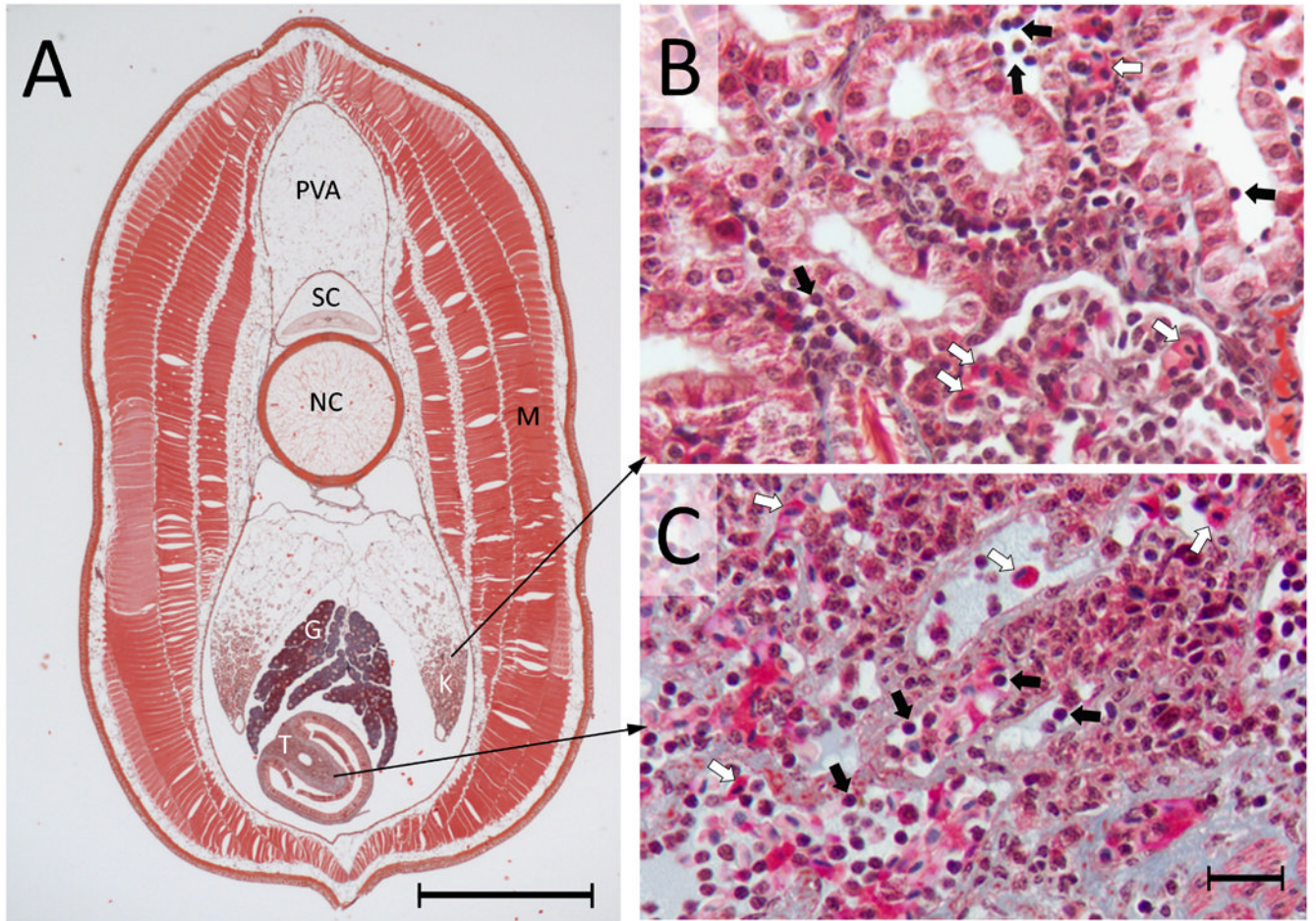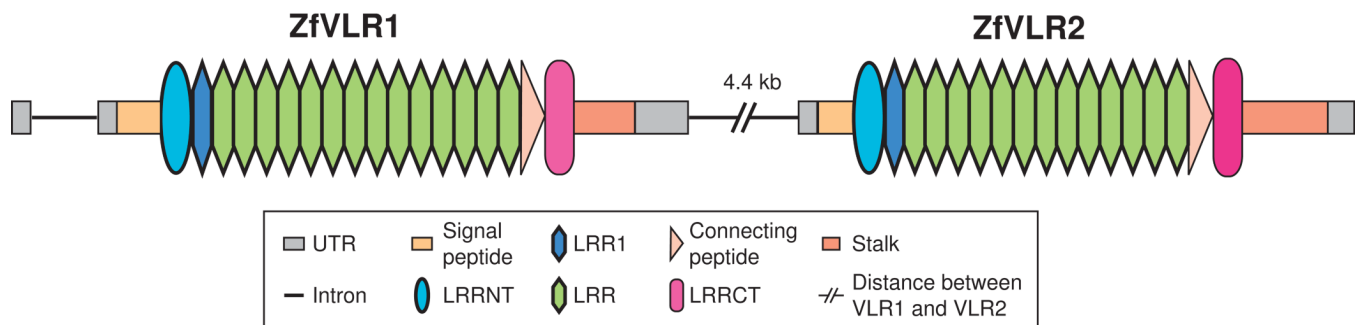
Figure 1.
Distribution of cells in primary hematopoietic tissues in larval lamprey. (A) A transverse section from the mid-body region of an ammocoete lamprey (~ 13 cm in length). The 10 µm processed section stained with Masson Trichrome is showing different major internal organs, which include protovertebral arch (PVA), spinal cord (SC), notochord (NC), gonad (G), kidney (K), typhlosole (T) and muscle (M). Scale bar = 1 mm. (B) A magnified view of the kidney showing the distribution of blood cells, including many lymphocytes and erythrocytes (black and white arrows, respectively). Collections of blood cells are seen in and amongst the renal tubules. (C) A magnified view of the typhlosole showing diverse blood cells (lymphocytes and erythrocytes are indicated by black and white arrows, respectively). Scale bar = 10 µm. The structures that are stained light blue are largely extracellular matrix, which is highly abundant in the typhlosole.
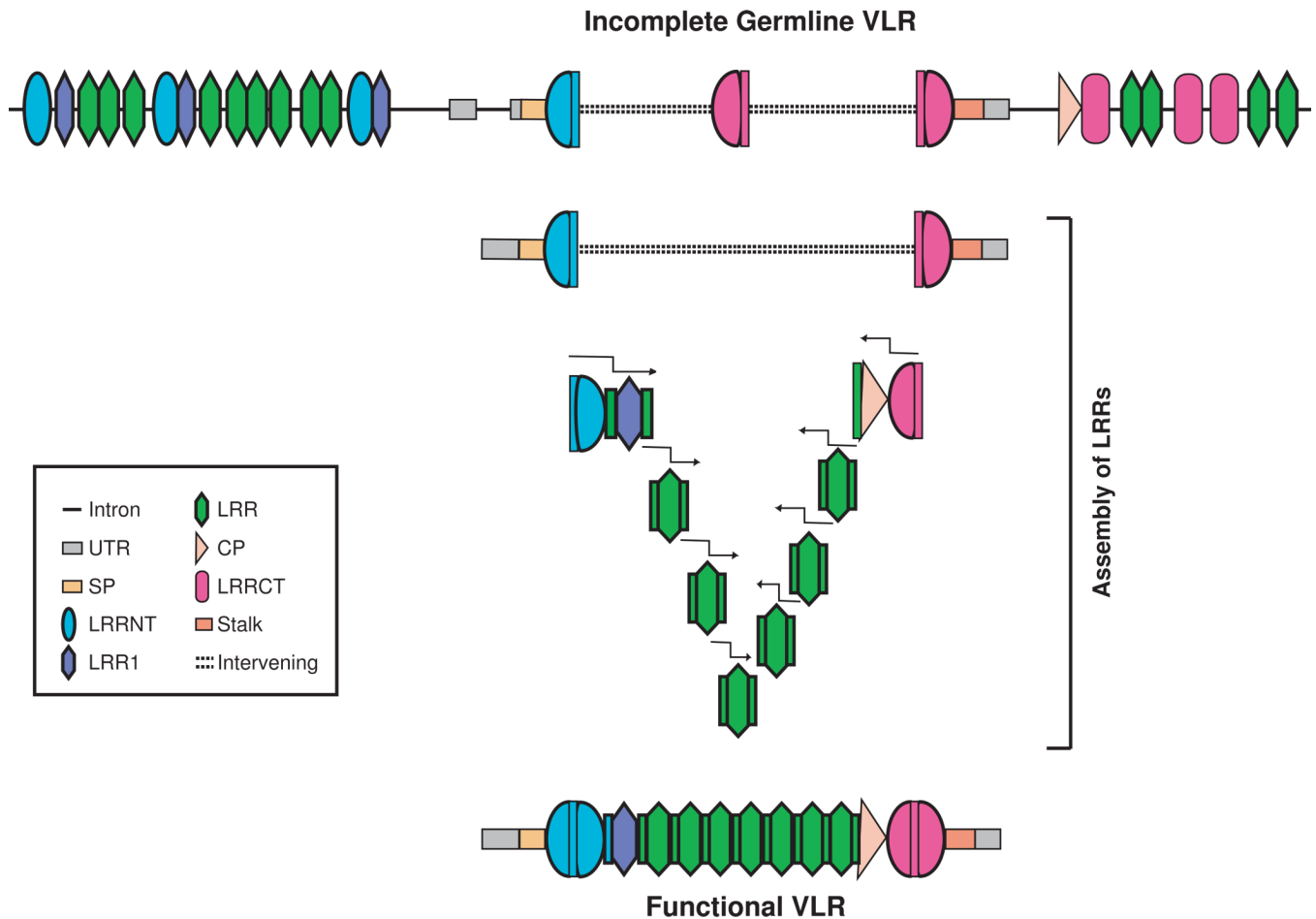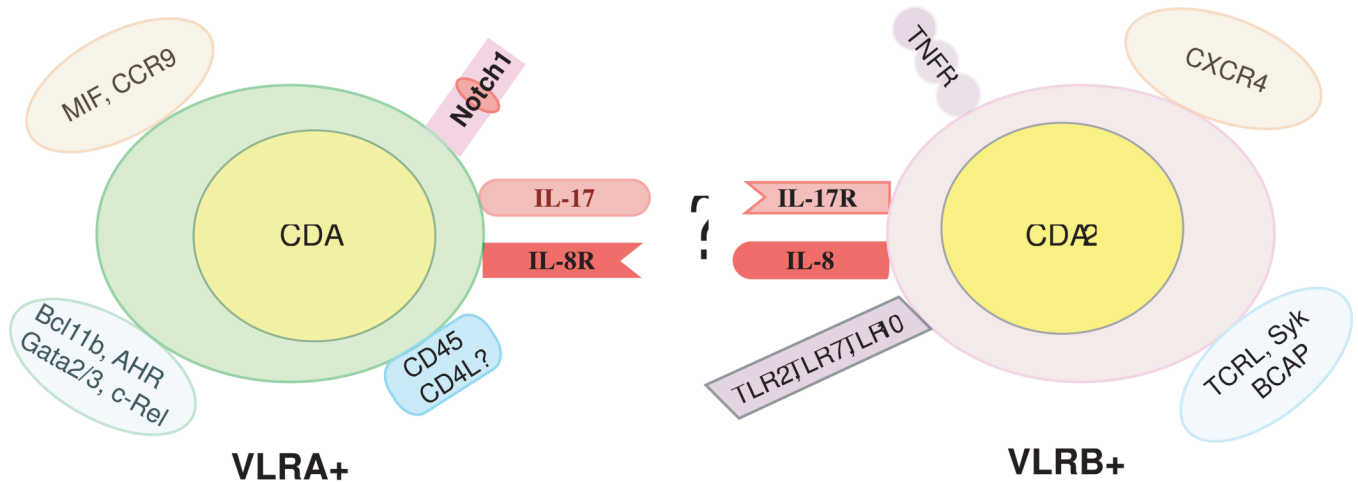
Figure 2.
Genomic organization and rearrangement of a mature VLR gene of sea lamprey. The germline VLR (gVLR) configuration with 5' and 3'-encoding LRR genetic segments (**top**) contains an additional 13.2 kb of non-coding intervening sequence and lacks the key LRR modules, which are essential to the structure of functional VLR genes. The inserted LRR modules lie both 5' and 3' of gVLR. The VLR locus undergoes stepwise assembly (**middle**) via recombination between short stretches of nucleotide homology found at the junctions of various LRR modules. This process may occur on either (or both) strand(s) during replication and gradually replaces the intervening sequence with all the variable LRR segments. The end product of these recombination events (**bottom**) is a mature VLR locus capped with an invariant 5' end of the LRRNT module, an invariant 3' end of the LRRCT module and a variable number of LRR cassettes (each encoding 24 amino acids) that vary in number and sequence. The illustration is not drawn to scale.

Figure 3.
Genomic organization of two VLR-like genes in the zebrafish. The two VLR-like genes separated by 4.4 kb in the zebrafish genome assembly (Accession No. BX569794). Both genes contain all core VLR components identically juxtaposed as in cyclostome mVLRs. However, these genes contain comparatively more LRR cassettes than the average VLR molecule in cyclostomes and lack the ability to undergo genomic diversification. The exact phylogenetic relationship to cyclostome VLRs is unclear, though there is a distinct possibility that they are evolutionarily related at least on the basis of their similar genomic organizations. The functionality of these molecules is largely unknown. Color coding of structural features is presented in the inset and is identical to that in Figure 2. The illustration is not drawn to scale.

Prediction of Signaling Mechanism by VLRA and VLRB

Figure 4.
A conceptual diagram of differential gene expression by VLRA[+] and VLRB[+] lymphocytes. This illustration highlights the various proteins that were empirically shown [5] to be differentially expressed in flow sorted VLRA[+] and VLRB[+] lymphocyte populations [5]. These include possible enzymes for genomic rearrangement, signaling molecules, transcription factors, cell surface receptors, and chemokines/cytokines and their cognate receptor genes. The restriction of CDA1 and to VLRA[+] and CDA2 to VLRB^{+} cells implies the potential for their selective involvement in the assembly of the VLRA and VLRB loci during lymphocyte development. It is speculated that the VLRA[+] lymphocytes expressing IL-17 may attract IL-17R bearing VLRB[+] lymphocytes, and VLRB+ lymphocytes may use IL-8 to attract and engage IL-8R bearing VLRA[+] lymphocytes. Likewise, expression of TLR ligands by VLRB[+] lymphocytes might trigger the activation of this cell population. Molecules are grouped on the basis of functionality and are not necessarily coexpressed (for example, TCRL, Syk and BCAP are B-cell signaling molecules, whereas Bcl11b, AHR, Gata2/3 and c-REL are T-cell transcription factors, etc).