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Evolution of Alternative Adaptive Immune Systems in Vertebrates

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Abstract

Adaptive immunity in jawless fishes is based on antigen recognition by three types of variable lymphocyte receptors (VLRs) composed of variable leucine-rich repeats, which are differentially expressed by two T-like lymphocyte lineages and one B-like lymphocyte lineage. The T-like cells express either VLRAs or VLRCs of yet undefined antigen specificity, whereas the VLRB antibodies secreted by B-like cells bind proteinaceous and carbohydrate antigens. The incomplete VLR germline genes are assembled into functional units by a gene conversion–like mechanism that employs flanking variable leucine-rich repeat sequences as templates in association with lineage-specific expression of cytidine deaminases. B-like cells develop in the hematopoietic typhlosole and kidneys, whereas T-like cells develop in the thymoid, a thymus-equivalent region at the gill fold tips. Thus, the dichotomy between T-like and B-like cells and the presence of dedicated lymphopoietic tissues emerge as ancestral vertebrate features, whereas the somatic diversification of structurally distinct antigen receptor genes evolved independently in jawless and jawed vertebrates.

1. INTRODUCTION

The unusual life cycle and anatomical characteristics of jawless fishes have long fascinated evolutionary biologists, developmental biologists, and immunologists alike. The jawless fishes, lampreys and hagfishes, emerged as a sister lineage of jawed vertebrates more than 500 million years ago and are considered living representatives of several extinct early vertebrate clades (1). Several decades ago, it was shown that lampreys (2–6) and hagfishes (7) respond to immunization with bacteria and heterologous erythrocytes by producing specific agglutinins. Moreover, lampreys (2, 8) and hagfishes (9) rejected allogeneic skin grafts, with second-set transplants being rejected at an accelerated rate. Although these early findings clearly suggested an adaptive immunological capacity of jawless fishes, they were not well accepted because of the ambiguous definition of the molecules and cell types underpinning these immune phenomena. In subsequent studies using more advanced procedures, lymphocyte-like cells from hematopoietic tissues of the sea lampreys were isolated by flow cytometry (10) and their transcriptional profiles were characterized; despite the presence of a large number of homologs of genes involved in immunologically relevant activities of mammalian lymphocytes (11; reviewed in 12), the expression profiles of these cells did not reveal genes with significant similarity to MHC, T cell receptor, or immunoglobulin genes (10).

The uncertainty surrounding the nature of agglutinins in jawless fishes was resolved with the discovery of a somatically generated family of diverse leucine-rich repeat (LRR) genes, whose expression was upregulated after immunization in lamprey lymphocyte-like cells (13). The diversity of these unusual antibody-like molecules suggested that they might explain the antigen-specific agglutinins demonstrated decades earlier. Since the initial description of these variable lymphocyte receptors (VLRs) (13), major progress has been made with respect to the cellular and molecular underpinnings of immunity in jawless vertebrates.

2. THE *VLR* GENE FAMILY IN LAMPREYS

2.1. Discovery of VLRs

Multiple attempts were made to identify antibody-like genes by transcriptome analysis of lymphocyte-like cells isolated by using their flow cytometric characteristics. The annotation of these transcripts failed to offer insight into the genetic basis for the previously reported antigen-specific agglutinins. As a last resort, lampreys were immune-stimulated with a mixture of antigens and plant mitogens to induce lymphoblastoid cells, which were used for a more selective transcriptome analysis. This effort yielded a plethora of LRR sequences, each of which proved to be different (13). These VLR sequences provided the essential clue to the lamprey antibody dilemma.

One of the striking characteristics of these VLR antibody-analogs is their composition by variable numbers of leucine-rich repeats of highly diverse sequences (13). This feature is the result of a somatic diversification process; indeed, it was found that the lamprey genome contains an incomplete *VLR* gene and that the relevant donor cassettes required to complete the entire structure in developing lymphocytes are situated in close proximity in the genome (13). This unique genomic arrangement also explained the invariant nature of N-terminal and C-terminal regions flanking the variable middle parts of the predicted VLR proteins.

The finding that individual lymphocytes express only one *VLR* gene (13) indicated that lampreys possess a somatically diversifying repertoire of clonally expressed antigen receptors, akin to the situation in lymphocytes of jawed vertebrates. The originally discovered *VLR* gene is the founding member of a larger family and is now known to encode the so-called VLRB isotype, which is expressed on B-like cells of lampreys (14). Two other VLR isotypes have since been identified:

VLRA (15, 16) and VLRC (17, 18). The presence of the three VLR isotypes in lampreys and hagfishes suggests that this anticipatory receptor system evolved in a common ancestor of the two cyclostome lineages.

2.2. VLR Protein Composition

The VLRs are membrane-bound proteins of conserved general structure composed of an N-terminal cap (LRRNT), LRR1, multiple LRRV modules, a connecting peptide (CP), a C-terminal cap (LRRCT), and an invariant stalk region rich in threonine and proline residues, as revealed by the prototypic VLRB receptor (13) (**Figure 1*a***). The LRRV modules possess a characteristic consensus sequence (XLXXLXXLXLXXNXLXXLPXXXFX, where X represents any amino acid); the LRR module designated LRR1 is composed of 18 residues and thus is shorter than other LRR modules. Sequence diversity among individual VLRs is primarily found in the central portion of the VLR molecules, beginning with the 3′ part of LRRNT and ending with the 5′ part of LRRCT (13, 16–35). In the case of VLRBs, the stalk region has a glycosyl-phosphatidylinositol (GPI) cleavage site and is anchored to the cell membrane by GPI linkage, but the VLRBs can also be secreted after lymphocyte activation and differentiation (13, 14, 19, 24, 30, 35–37); in contrast, VLRAs and VLRCs are transmembrane proteins that are not secreted (14, 30). Crystallographic studies (21, 22, 26, 27, 29, 31, 32, 38, 39) have confirmed the initial model of a curved solenoid structure (13) for all three VLR isotypes; as for other typical LRR proteins, such as the Toll-like receptors (e.g., 38, 40), LRRNT and LRRCT cap the ends of the solenoid to protect the hydrophobic core. Interestingly, most, but not all, of the variable residues in VLRs are located in the concave surface that is formed by parallel β sheets.

Despite the overall similarities of the three-dimensional structures of VLR isotypes, there are several characteristic isotype-specific differences. With respect to the number of LRRVs, *VLRB* assemblies are typically much shorter than those of *VLRA* and *VLRC* (13, 15–20, 28, 32–34). For both *VLRA* and *VLRC* assemblies, a particular preference for four LRRVs has been described (32, 38), and this length preference is considered (32) to be the result of postassembly selection (discussed below). Another notable difference between VLR structures of lampreys concerns an insert of eight amino acid residues in the LRRNT region of VLRC and an LRRCT region insert encoding a loop of variable length that is typically seen in VLRA and VLRB proteins. The LRRCT insert appears to be involved in antigen binding by VLRBs (21, 22, 29, 31, 39) and possibly also by VLRAs (26), but the role of the LRRNT insert in VLRC structures (32, 38) is currently unknown (**Figure 1*b***).

2.3. *VLR* Gene Assembly

The cardinal features of all *VLR* genes are similar. *VLR* genes are incomplete in their germline configuration (**Figure 2*a***) and thus incapable of encoding any functional proteins before their completion. More specifically, the germline versions lack sequences coding for the C-terminal part of LRR1, any LRRV and LRRVe elements, and the N-terminal parts of the CP region (reviewed in 41–44). To generate functional *VLR* genes, the partial germline genes must be completed by introduction of different types of genomic cassettes encoding the other elements found in fully assembled *VLR* genes. The potential donor elements are located in both 5′ and 3′ regions flanking the incomplete germline *VLRB* locus (**Figure 2*b***) (13, 17), and the same is true for the *VLRA* and *VLRC* loci (17, 28, 33). Functional *VLR* gene assemblies are generated by serial stepwise replacement of the intervening sequences of the germline *VLR* genes by *LRR* sequences (15, 19, 20, 28); this gene conversion–like mechanism utilizes short stretches of nucleotide homology

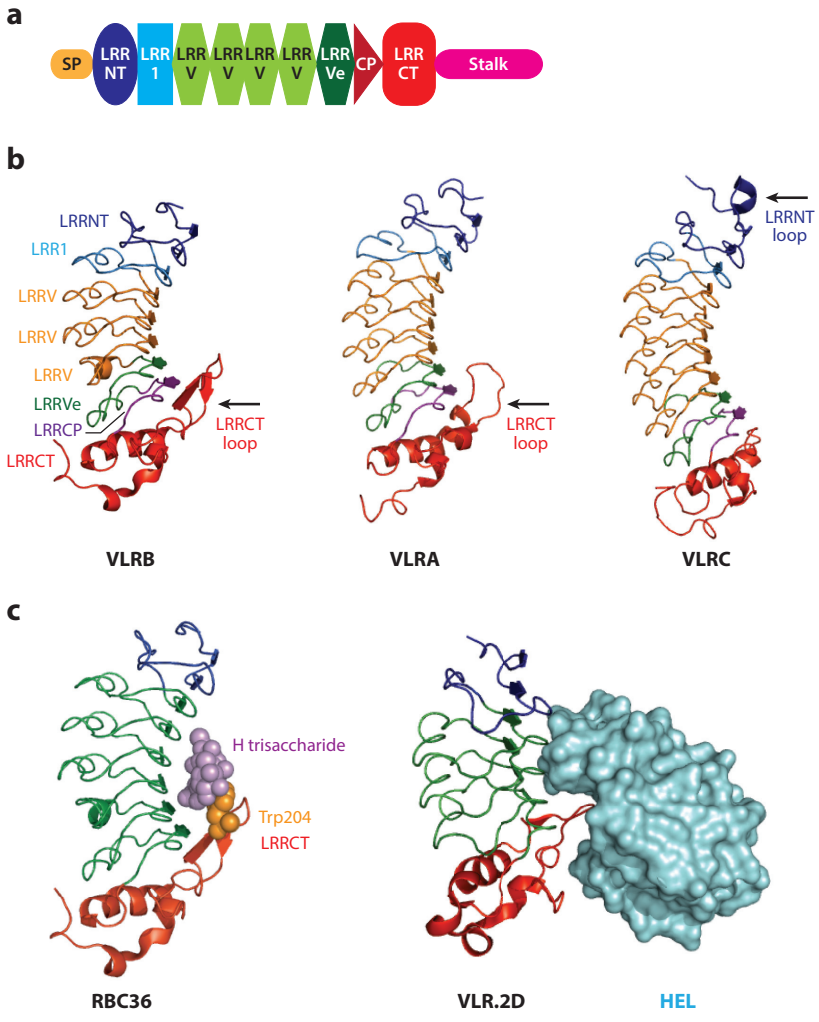


Figure 1

Structural features of VLR isotypes. (*a*) Schematic of a generic VLR protein. VLRs are membrane-bound proteins of conserved general structure composed of a signal peptide (SP), an N-terminal cap (LRRNT), LRR1, multiple LRRV modules, a connecting peptide (CP), a C-terminal cap (LRRCT), and an invariant stalk region rich in threonine and proline residues, as revealed by the prototypic VLRB receptor. Sequence diversity among individual VLRs occurs primarily in the center, beginning with the 3′ part of LRRNT and ending with the 5′ part of LRRCT. (*b*) Structural features of the three VLR isotypes. Arrows point to the positions of the loop inserts in the LRRCT modules of VLRB (21) and VLRA (26) and the position of the loop insert in the LRRNT module of VLRC (27). (*c*) Structure of two VLRB-antigen complexes. (*Left*) The structure of a VLRB antibody (RBC36) in complex with the type II H antigen trisaccharide (21) illustrates that the specificity of the binding is mediated by four hydrogen bonds on the inner concave surface, with additional van der Waals contacts stabilizing the interaction; moreover, a tryptophan residue situated at the tip of the β hairpin that characterizes the highly variable insert of the LRRCT domain makes contact with the galactose moiety in the H-trisaccharide (21). (*Right*) Structure VLR.2D (22) illustrates that a VLRB antibody in complex with a proteinaceous antigen retains a nearly identical conformation, with the exception that the LRRCT insert is adaptable. In this example, it undergoes a hinge movement to accommodate the HEL antigen, compatible with pre-equilibrium conformational diversity of the protruding loop structure (22). Figure modified from Reference 119.

between donor and acceptor sequences and can begin from either the 5′ or the 3′ end of the incomplete germline gene, eventually resulting in a completely assembled *VLR* gene (**Figure 2*c***).

The genomic regions containing the three different *VLR* genes and their associated donor cassettes are still incompletely defined, but several lines of evidence suggest that the lamprey *VLRA* and *VLRC* genes are situated in close proximity to each other, within ∼400 kb (33), and are not close neighbors with the *VLRB* gene locus (**Figure 2*b***). In hagfishes, the *VLRC* and *VLRB* loci are also far apart, at the opposite ends of one chromosome (45). Of note, the structurally variable components of VLRAs and VLRCs often possess partially identical sequences but rarely, if ever, share donor cassette sequences with the structurally analogous VLRB isotype. Analysis of partially assembled genomic sequences (and the results of Southern filter hybridizations) further indicates the interspersed arrangement of isotype-specific and shared genomic donor cassettes of *VLRA* and *VLRC* genes, features that may facilitate the shared cassette use (33). Overall, the genomic structure of the *VLRA/C* locus in lampreys is reminiscent of the interspersed nature of the *TCRA/D* locus in jawed vertebrates, which is also characterized by the shared use of variable gene segments during the formation of complete *TCR* genes (46).

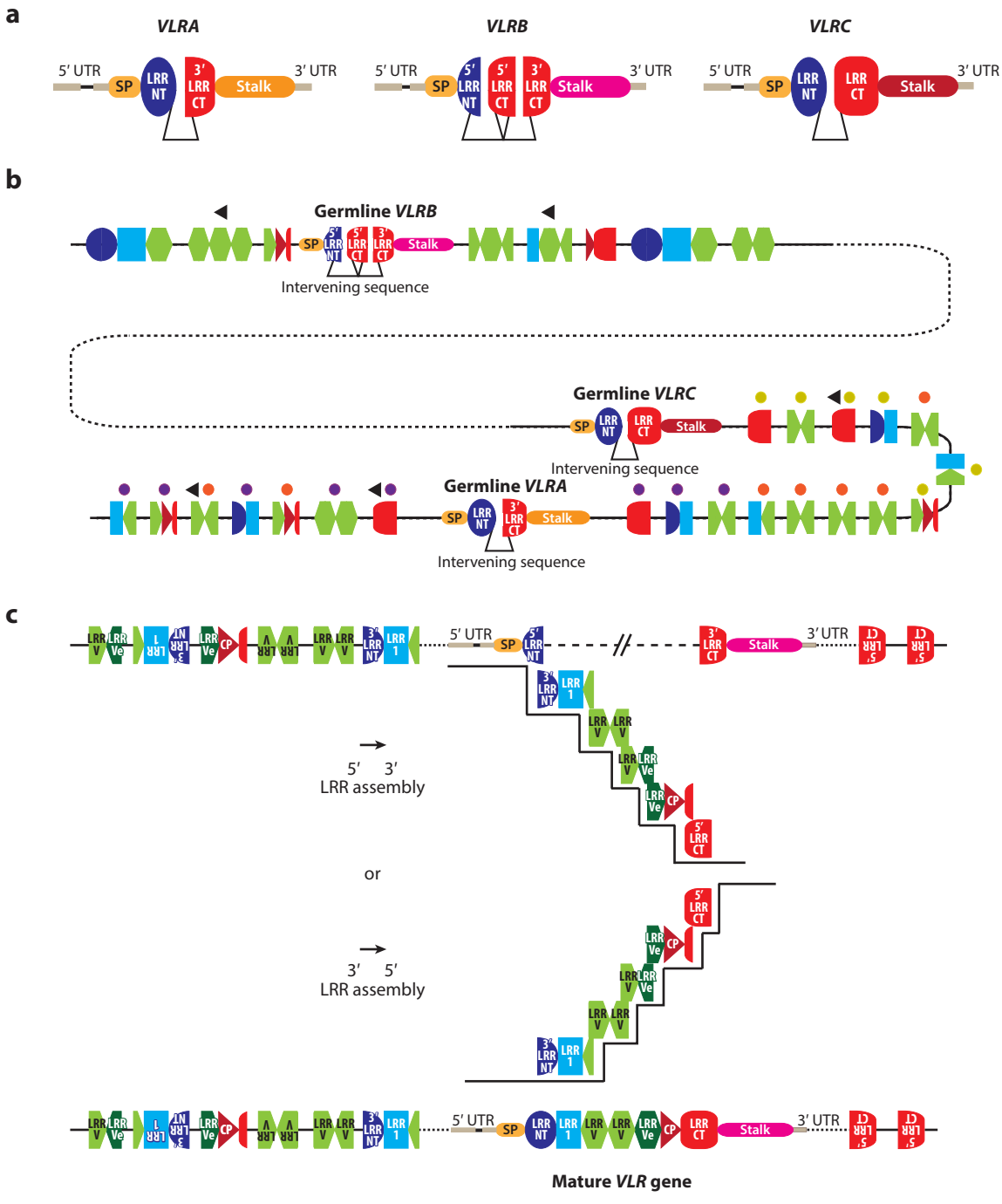
2.4. Lineage-Restricted *VLR* Gene Assembly

VLRB gene assembly typically occurs on only one *VLRB* allele, thus ensuring the expression of a single receptor specificity for each VLRB-bearing lymphocyte (13, 20); this is analogous to the phenomenon of allelic exclusion observed for immunoglobulin genes (47). When biallelic assemblies occur in a cell, one of them typically is nonfunctional (13, 20, 23). Notably, neither *VLRA* nor *VLRC* assemblies are observed in lymphocytes expressing the VLRB receptor on the cell surface, and vice versa (14, 30), indicating the lineage-specific execution of the assembly process.

Assembly of *VLRA* and *VLRC* antigen receptor genes is more complex. It has been shown that a certain fraction of lamprey VLRA$^+$ cells contain complete *VLRC* gene assemblies. When they are examined at the single-cell level, it appears that only 11% of VLRC$^+$ cells contain additional *VLRA* gene assemblies, whereas 43% of VLRA$^+$ cells display *VLRC* gene assemblies (30). These findings support the notion of a close developmental relationship of the VLRA- and VLRC-expressing lymphocytes and suggest that, during development of the VLRA/C-type lymphocyte lineages, *VLRC* assembly begins before *VLRA* assembly. Some VLRA$^+$ and VLRC$^+$ cells carry biallelic *VLR* gene assemblies, but in these situations, one allele is generally rendered nonfunctional by frameshift mutations and/or in frame-stop codons, presumably as a consequence of errors during the assembly process (30).

2.5. Diversity of VLR Repertoires

In contrast to the situation in mammals and some teleost fishes, for which the results of comprehensive analyses of the T cell receptor (TCR) and B cell receptor (BCR) repertoires are available, the sequence space represented in VLR repertoires of lamprey peripheral lymphocytes is not yet precisely known. For instance, in addition to the incomplete knowledge about the structure of the respective genomic regions, we lack definitive information on how many of the identifiable genomic donor cassettes are used in completely assembled VLRs. Such data are important to assess the relationship between potential and actual diversity in VLR repertoires, and whether the position of a cassette in the genomic locus influences the frequency of its utilization in the assembly process. Despite this caveat, a number of important conclusions can already be drawn from the available datasets. For instance, evidence for limited clonal amplification of particular VLRB$^+$ lymphocytes has been obtained (13, 48), but the generality of this phenomenon remains



uncertain. Repetitive *VLRC* sequences are abundant in the skin but are rarely found in kidney and blood samples; the clonal restriction is specific to *VLRC*, as it was not observed for *VLRA* sequences isolated from the same skin samples (30). The finding of several examples of identical or almost identical *VLRC* sequences in skin samples from different animals indicates the presence of public clones and offers further evidence that the VLRC repertoire is less diverse and more stereotypic in the skin than elsewhere (30), reminiscent of the situation observed with some epithelial lymphocyte types in mammals (49, 50). The functional significance of such lineage-specific asymmetries in VLR repertoires remains unclear.

2.6. Selection of Primary Repertoires of VLRAs and VLRCs

Estimates of the sequence complexities of *VLRB* assemblies in lamprey larvae (13, 19) indicate that these are comparable in magnitude to those observed in BCRs and TCRs of jawed vertebrates, which are known to be purged of self-reactivities during the development of B and T cells (51, 52). It is conceivable that the primary VLR repertoires are also selected for self-compatibility to avoid self-damage. Indeed, lampreys have been shown to reject skin allografts but maintain autografts (2); a similar phenomenon occurs in hagfishes (9). However, although the mechanisms governing selection of antigen receptors expressed by lymphocytes in jawed vertebrates are well understood, no such information exists for the jawless vertebrates.

The most compelling evidence for selection operating on primary repertoires has been obtained for *VLRC* assemblies (32). Strikingly, more than 70% of all functionally assembled *VLRC* genes found in peripheral lymphocytes exhibit a preference for four LRRV modules (28, 32, 38). By contrast, the size distributions of *VLRC* assemblies found in VLRA$^+$ cells and those in the thymoid [the presumptive site of selection processes (53)] are much broader, providing evidence for preferred admission of assemblies with four LRRV elements to the peripheral repertoire of VLRC$^+$ cells. In addition to an apparent preferential size selection, evidence for protein sequence–related selection was found for VLRCs (32), specifically implicating the LRR1 module of VLRC assemblies as a primary target of sequence-specific selection.

Unfortunately, no information is yet available as to the mechanism of selection. It has been speculated that the size selection of VLRCs might be the result of interactions with one or more proteins acting either as simple molecular rulers, forming productive interactions only with VLRs of a certain length, or as functional equivalents of MHC molecules, requiring both length and sequence compatibility for productive interactions (32).

VLRAs also exhibit signs of selection, with the highly skewed length distribution being the most obvious feature (32, 34). In contrast to VLRB sequences, distinctive sequence preferences

Figure 2

Schematic illustrations of *VLR* genes and their assembly. (*a*) Incomplete germline configurations of the three known lamprey *VLR* genes. Note that the germline versions lack sequences coding for the C-terminal part of LRR1, any LRRV or LRRVe element, and the N-terminal parts of the CP region. (*b*) Genomic configuration of *VLR* genes and their flanking donor *LRR* cassettes. Although the genomic regions containing the three different *VLR* genes and their associated donor cassettes are still incompletely defined, several lines of evidence suggest that the lamprey *VLRA* and *VLRC* genes are situated in close proximity to each other, within ~400 kb, but are not close neighbors with the *VLRB* gene locus (*dotted line*). (*c*) Sequential stepwise assembly of *VLR* genes. To generate functional *VLR* genes, the partial germline genes must be completed by introduction of different types of genomic cassettes encoding the other elements found in fully assembled *VLR* genes. The assembly process consists of a serial stepwise replacement of the intervening sequences of the germline *VLR* genes by *LRR* sequences via a gene conversion–like mechanism, utilizing short stretches of nucleotide homology between donor and acceptor sequences. Assembly can begin from either the 5′ or the 3′ end of the incomplete germline gene, eventually resulting in a completely assembled *VLR* gene. Figure modified from Reference 120.

were additionally noted for the second LRRV module in VLRA (and VLRC) sequences (34), supporting the notion that primary repertoires of these two receptor isotypes are subject to selection at several levels.

2.7. Secondary Modifications of VLR Repertoires

Somatic hypermutation of BCRs during immune responses is a well-known phenomenon in essentially all gnathostomes (54) and has also been shown for TCRs of cartilaginous fishes (55). Whether somatic hypermutation of VLRs occurs is unclear. An analysis of LRRV sequences of in vitro affinity-selected VLRAs compared with the sequences of the genomic donor cassettes (25) suggested that somatic diversification could occur in lamprey lymphocytes during the immunization process. However, considerable sequence diversity exists among individuals of the same lamprey species/ecotype, precluding firm conclusions about the presence and magnitude of somatic diversification processes. Indeed, a study comparing the germline repertoire of VLR elements with sequences found in high-affinity VLR binders of the same lamprey individual has yet to be conducted.

Other changes in the VLR repertoires might occur apart from potential hypermutation. For instance, it would be interesting to examine the clonal diversity of VLR repertoires over the lifetime of lampreys, the larval phase of which is typically measured in years. It is well known that the diversity of TCR sequences for naive T cells of aged mammals is influenced by homeostatic proliferation, which often differs for individual clones and potentially influences the magnitude of immune responses (56); this phenomenon has been linked to reduced output of naive cells as a result of thymic involution (57). Whether thymoid involution occurs during the many years of the lamprey larval stage is currently unknown.

3. AID-LIKE CYTIDINE DEAMINASE GENES IN LAMPREYS

3.1. Potential Cytidine Deaminase Participation in *VLR* Gene Assembly

A gene conversion–like process has been proposed for the assembly of the incomplete *VLR* germline genes in agnathan lymphocyte progenitors (13, 15, 20). Gene conversion also underlies the generation of antibody diversity in certain gnathostome species (58–61) and depends on the activity of activation-induced cytidine deaminase (AID) (62, 63), a member of the AID-APOBEC family of cytidine deaminases (CDA) (64). Two genes encoding presumptive AID-APOBEC CDAs, designated *CDA1* and *CDA2*, were initially discovered in the genome of the sea lamprey (15), and these genes are conserved in other lamprey species (65). The expression patterns of *CDA1* and *CDA2* differ among lymphocyte populations of the sea lamprey (14). Whereas *CDA1* expression is preferentially observed in lamprey lymphocytes of VLRA and VLRC lineages, *CDA2* expression is associated with the VLRB-expressing lymphocyte lineage (14, 30) (**Figure 3**); this contrasts with the situation in jawed vertebrates, wherein expression of *RAG1* and *RAG2* occurs in both T and B lymphocytes (66). When the expression of *CDA1* and *CDA2* is examined by RNA in situ hybridization, *CDA1* is found to be expressed exclusively by cells in the thymoid, the presumptive thymus equivalent in lamprey larvae (see below), whereas *CDA2* expression is found primarily in cells of the typhlosole and the kidney (53), the major hematopoietic sites of lamprey larvae (67). Of note, information on the ontogeny of *CDA* gene expression is not yet available.

The association of CDA activity with *VLR* gene assembly is primarily based on coexpression patterns. Whereas CDA1-like proteins exhibit mutagenic activity in heterologous systems (15, 68),

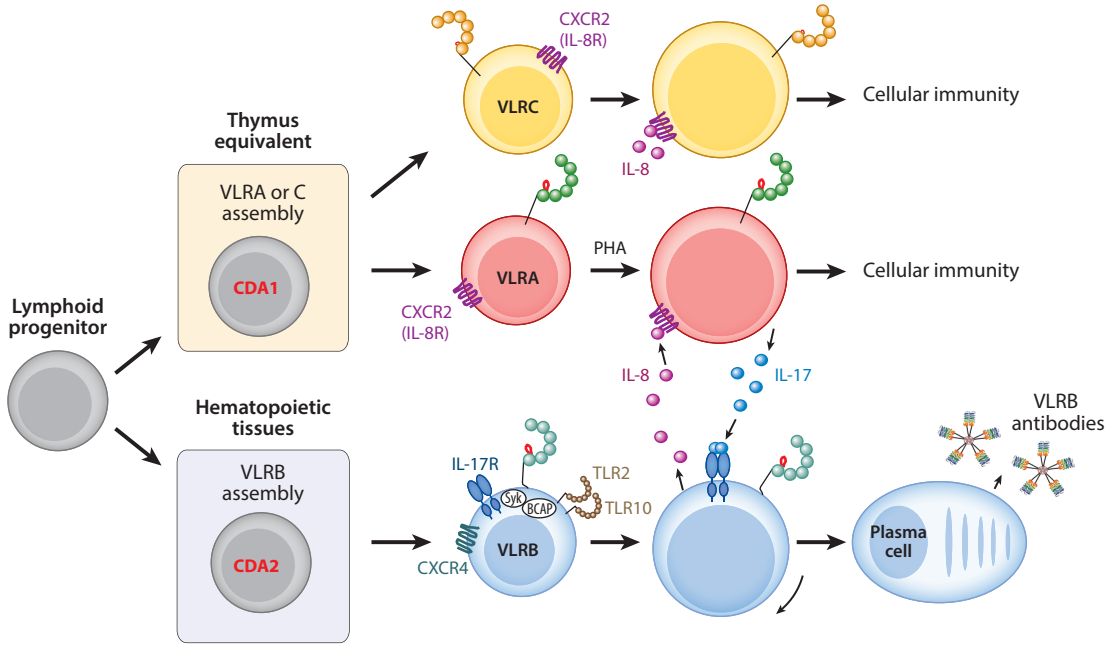


Figure 3

Characteristics of VLR-expressing lymphocyte lineages. In this simplified scheme, a hypothetical lymphoid progenitor gives rise to precursors that differentiate after gene assembly in the thymus equivalent, presumably mediated by CDA1, into VLRC- and VLRA-expressing T-like cells. These cells express, in addition to their antigen receptors, other cell surface molecules, such as CXCR2, that allow them to communicate with other immune cells, in this example those that secrete IL-8. After stimulation, they may themselves secrete effector molecules, such as IL-17, that can activate other cell types, in this example VLRB-expressing B-like cells. *VLRB* gene assembly in hematopoietic tissues is thought to be mediated by CDA2. The VLRB$^+$ cells can express chemokine receptors (for instance, CXCR4), cytokine receptors (such as IL-17R), and pattern recognition receptors (such as Toll-like receptors). Antigenic stimulation of VLRB$^+$ lymphocytes may lead to limited clonal amplification and differentiation into plasma cells that secrete VLRB antibodies. Figure modified from Reference 120.

no such activity has yet been reported for CDA2. Given the complications associated with genetic analyses in lampreys, with their long generation time, genetic interference—for instance, with the *CDA*-like genes using the CRISPR/Cas9 system (69)—might eventually provide the long-sought functional link between CDA activity and *VLR* gene assembly. Alternatively, it may be possible to combine large genomic fragments of *VLR* loci with *CDA* genes in heterologous cells in order to assay for assembly, and/or use an in vitro reconstitution strategy with suitable test templates for this purpose.

3.2. Application of Cytidine Deaminase as a Genome-Editing Tool

Lamprey CDA1 induces C-to-T mutations in *Escherichia coli* and yeast genomes much more efficiently than does human AID (15, 68, 70). The hypermutagenic CDA1 induces clustered mutations in yeast diploid cells that are very similar to those found in human cancers (68, 71, 72). These findings support the idea that AID/APOBEC CDA activity is a prominent source of genome-wide hypermutation in human tumors and that an evolutionary ancestor of this family had already acquired this potential.

In recent studies, an enzymatically inactive form of the Cas9 nuclease was fused with lamprey CDA1; this modified version of the CRISPR/Cas9 system was highly efficient in directed mutagenesis (73, 74), indicating the potential of lamprey CDAs for bioengineering.

4. IMMUNOCHEMISTRY OF VLRs

4.1. Structural Diversity of Known VLRs

The three known VLR isotypes differ in their modes of membrane association and secretion. VLRBs are attached to the lymphocyte membrane by GPI linkage and are secreted by maturing plasma cells following antigenic stimulation (13, 14, 30). The secreted VLRB antibodies are typically composed of five disulfide-linked dimer pairs (24, 36), akin to the multimeric IgMs secreted by plasma cells of mammals. By contrast, VLRAs and VLRCs remain as membrane-integral monomeric proteins after antigenic stimulus (14, 30) and thus resemble the functionally equivalent TCRs of jawed vertebrates.

4.2. Structural Features of VLRBs

In the initial description of VLRB antibodies, it was suggested that VLRBs, akin to Toll-like receptors (75, 76), form a curved solenoid (19). Moreover, their variable concave surfaces were suggested to serve as the binding sites for antigens; this was later confirmed by mutational analysis of a VLRB antibody directed against BclA, a major coat protein of *Bacillus anthracis* spores (24). The first VLRB molecules whose LRR-region structures were solved were two VLRBs of the inshore hagfish *Eptatretus burgeri*, one possessing two LRRV elements and the other three (27). Interestingly, it was found that the difference in one LRRV element does not cause detectable changes in the overall geometry of the horseshoe-like structure; moreover, the structural effect of the characteristic LRRCT loop insertion appears to be local, which has important consequences for the mode of antigen binding by VLRBs. Indeed, in a comparison of an unbound lamprey VLRB antibody and the corresponding VLRB-antigen complex (22), the VLRB antibody retained a nearly identical conformation, with the exception that the LRRCT insert underwent a hinge movement to accommodate the HEL (hen egg white lysozyme) antigen.

Several other structures of VLRB antibodies in complex with their cognate antigens were solved by X-ray crystallography (**Figure 1*c***), with particular emphasis on the interaction of carbohydrates with VLRB antibodies. In the first cocrystal of a VLRB antibody with its cognate antigen, the type II H antigen trisaccharide, specificity of the binding is mediated by four hydrogen bonds on the inner concave surface, with additional van der Waals contacts stabilizing the interaction; interestingly, a tryptophan residue situated at the tip of the β hairpin that characterizes the highly variable insert of the LRRCT domain makes important contacts with the galactose moiety in the H-trisaccharide (21). VLRB antibodies recognizing other biomedically relevant glycans (77) were isolated using yeast surface-display (25). A VLRB antibody recognizing the tumor-associated Thomsen-Friedenreich (TF) antigen Galβ1–3GalNAcα indicated that this VLRB forms a tight hydrophobic cage encasing the disaccharide between the LRRCT insert loop and the concave surface of the solenoid (31). The VLRBs specific for the H-trisaccharide and the TF antigen both consist of LRR1, three LRRV elements, and one LRRVe element. However, since VLRBs exhibit different numbers of LRRV elements (up to six), lamprey antibodies could in principle accommodate longer glycans, albeit associated with bending of the oligosaccharide to maintain contacts with the rigid solenoid framework of the VLR structure. By contrast, the binding mode of lectins (78) and immunoglobulins (79) is different, typically accommodating glycans in long

convex grooves through the stacking of aromatic residues against the sugar ring and hydrogen bonding of the hydroxyl groups of the sugar to the side chains of polar amino acid residues. The importance of the LRRCT loop insert for specific antigen recognition is also highlighted in the interaction of a BclA glycoprotein-binding VLRB antibody (29). The LRRCT insert buries more surface area of this antigen than any other single LRR motif from the concave surface and provides the most hydrogen bonds, suggesting that the LRRCT insert contributes substantially to the binding specificity of this antibody (29).

A recent study (37) addressed whether intrinsic chemical and structural features of antigens would determine the modes of interaction between antigen and immunoglobulins and VLRBs. The immune responses of mice and lampreys to influenza A virus in this study were strikingly similar; in both species, a large majority of the antibodies were directed against the hemagglutinin globular head domain. Both VLRBs and immunoglobulins shared a preference for aromatic residues (tyrosine, tryptophan, asparagine, and aspartic acid), supporting the notion that antigen recognition by immune receptors follows a set of universal rules. Indeed, a VLRB antibody was shown to recognize an epitope on HEL (22) that almost completely overlaps those recognized by camelid (80) and shark heavy chain–only (40) antibodies; a striking feature of this conserved interaction mode is that the loops of both the VLR (**Figure 1*c***) and the immunoglobulin antibodies project into the catalytic cleft of the enzyme.

Interestingly, a potential inhibitory receptor of VLRB antibodies was recently identified on lamprey myeloid cells (81). Although the structural determinants mediating this interaction remain to be defined, these results are reminiscent of functionally equivalent interactions between immunoglobulins and their Fc-based inhibitory receptors (82).

4.3. Structure of VLRAs

In contrast to VLRB antibodies, direct binding of antigens to VLRA-bearing cells could not be demonstrated (14), raising the possibility that VLRA-type receptors (and possibly VLRC-type receptors) are tuned to recognize processed antigens. On the other hand, it should be noted that high-throughput selection by an in vitro yeast surface display assay (25) allowed the identification of one VLRA molecule with nanomolar affinity to HEL (26). The HEL-contacting surface of this VLRA protein derives predominantly from the β strands furnished by the LRRV elements, whereas contacts between the LRRCT insert and the antigen are more prominent in an analogous VLRB-HEL complex (26). The binding mode of this particular VLRA is thus suggestive of direct recognition of unprocessed antigens [akin to the recognition mode of VLRB antibodies and γδ TCRs of jawed vertebrates (83)]. However, since this VLRA-antigen complex structure so far is the only one of its kind, it is unclear whether this recognition mode is the rule for VLRAs or merely an exception—a result of the in vitro selection process. This question has important implications for the interpretation of functional analogies of antigen recognition in jawless and jawed vertebrates.

4.4. Structure of VLRCs

VLRC molecules also form a solenoid structure encompassing a variable concave surface (32, 38), but several features distinguish them from VLRAs and VLRBs. For instance, the N-terminal region of lamprey VLRCs contains a loop insert of eight amino acids with limited sequence variability that connects two antiparallel β strands in LRRNT and projects toward the concave surface of the VLRC. This loop could potentially contact antigen or an antigen-presenting molecule and, despite high sequence similarity, the LRRNT loop inserts in the two known structures of

lamprey VLRCs adopt markedly different conformations, implying substantial structural flexibility (32, 38).

As discussed above, recognition of most protein and carbohydrate antigens by VLRBs depends on a distinctive insert in the LRRCT module that is highly variable in both sequence and length. In contrast, the average length of the LRRCT insert in VLRCs is usually much shorter (two residues) than that in VLRAs (ten residues) or VLRBs (eight residues); thus, it does not protrude from the LRRCT to form an antigen-binding loop with conformational flexibility. These structural characteristics of VLRCs were also observed in an earlier study of hagfish VLRs (27), although it should be noted that VLRs originally designated VLRA belong to the VLRC isotype (16). So far nothing is known about the nature of the antigen(s) recognized by VLRC receptors.

4.5. Isolation of Antigen-Specific VLRBs

Early studies indicated the possibility of cloning antigen-specific VLRBs from cDNA libraries of hyperimmunized lamprey larvae. This strategy led to the identification of antibodies directed against proteinaceous and carbohydrate antigens (19, 21, 22, 24, 25, 29, 31, 36, 37, 84). To circumvent the current inability to culture and/or immortalize individual lamprey lymphocytes, or the necessity to clone and test the specificities of monoclonal VLRs prevalent in immunized animals, several types of cell surface display systems were developed to obtain antigen-specific VLRs. The recently developed yeast surface display system is based on the expression of VLR sequences constructed from cDNA libraries of lamprey lymphocytes followed by affinity selection (25, 77); this system was successfully used to isolate VLRB antibodies directed against protein antigens and glycan epitopes. In a variation of this strategy, designer VLRs were constructed based on the consensus sequences of hundreds of known VLRB antibodies. Using this strategy, the resulting conserved scaffold was diversified only at those residues presumed to contribute most of the binding to antigens. The complex libraries of these artificially diversified VLRs, called repebodies, were subjected to affinity selection based on phage display. One implementation of the system resulted in the identification of repebodies directed against IL-6, with affinities in the range of ~50–120 nM (39); interestingly, this scaffold design afforded the rational design and successful construction of anti-MD2 and anti-HEL antibodies with micromolar affinities. When another designer VLR system was used (85), VLRB-like proteins recognizing HEL and S100A7 proteins were isolated with dissociation constants in the micromolar range.

4.6. Use of VLRBs as Analytical, Diagnostic, and Therapeutic Reagents

Lamprey antibodies offer several distinct advantages over the commonly used immunoglobulins as diagnostic reagents. First, the naturally selected VLRB-type antibodies are homo-oligomers with favorable biophysical characteristics, such as heat stability (24). Moreover, since VLRBs also bind antigens in monomeric structure, the in vitro selection of high-affinity variants is greatly simplified, akin to natural and synthetic single-chain immunoglobulins. Second, it appears that—in contrast to immunoglobulins—VLRBs are capable of distinguishing closely related carbohydrate moieties (77), offering detection of a previously largely inaccessible group of chemically distinct epitopes. Third, VLRB antibodies can also exhibit exquisite specificity for protein epitopes, such as BclA (24), a major coat protein of *B. anthracis* spores; and the signature VH CDR3 sequence of a B cell chronic lymphocytic leukemia (84).

The use of VLRB antibodies to purify (by immunoprecipitation) cell surface molecules was first demonstrated for human CD5 (86), in a study that also highlighted the avidity-enhancing nature of multimeric VLRBs. Monoclonal VLRB antibodies can also be generated against novel

antigenic epitopes, such as the multimeric enzymatically active form of the CD38 ectoenzyme on human plasmablasts and plasma cells (87). The rationale for this approach is to exploit the large evolutionary distance between lampreys and mammals, avoiding potential tolerance restrictions on the recognition of mammalian protein determinants by immunoglobulins resulting from coevolutionary constraints.

Chimeric antigen receptors have proven to be versatile and therapeutically useful modalities to direct the cytotoxic activity of T effector cells to cell surface antigens of choice (88). Whereas the classical chimeric antigen receptor (CAR) design encompasses antigen-specific ectodomains in the form of single-chain antibody heavy and light chain variable regions, the immunoglobulin-based ectodomain can be replaced by the ectodomain of monomeric VLRB chains. In a recently described implementation of this design, antigen-specific VLRB sequences were linked to a transmembrane region derived from CD28 and an intracellular CD3ζ signaling domain (89). If potential problems with antigenicity can be addressed, VLR-CARs could provide an alternative strategy to complement the classical CAR portfolio.

VLRB antibodies selected ex vivo for binding to an effector protein of a plant pathogen maintain their reactivity when expressed *in planta* (90), indicating the possibility to exploit antigen-specific VLRBs for plant protection or as flexible modules to bind other proteins or carbohydrates of interest in plants.

5. LAMPREY LYMPHOCYTE LINEAGES

5.1. Identification of Three VLR-Bearing Lymphocyte Lineages

The generation of monoclonal antibodies specific for the invariant stalk regions of the three known VLR isotypes provided a straightforward approach to identify and characterize lymphocyte lineages of lampreys. These studies revealed that the three VLRs are expressed in a mutually exclusive manner, thereby defining three major lymphocyte lineages (14, 30) (**Figure 3**). The VLR isotype–specific antibodies were used to examine the tissue distribution of the three principal lymphocyte lineages and to isolate them by flow cytometry for phenotypic and genotypic analyses. These studies revealed that VLRB$^+$ cells are the dominant type of lymphocytes in the blood and kidneys of sea lamprey larvae; VLRC$^+$ lymphocytes predominate in the typhlosole and gills and also outnumber VLRA$^+$ cells in the hematopoietic typhlosole and kidneys (30).

Both VLRA$^+$ and VLRC$^+$ T-like cells were found in tissue sections of the typhlosole, kidneys, gills, and hypopharyngeal fold; both cell types assume a round or oval shape within blood vessels and interstitial spaces of the kidneys and typhlosole but are dendritic shaped in the gills and intestinal epithelium (30). Interestingly, in the epidermal layer of the skin, the number of VLRC$^+$ cells is approximately ten times the number of VLRA$^+$ cells, whereas VLRB$^+$ cells are rarely found in the epidermis (30). This pattern of distribution, together with a restricted repertoire diversity of the epidermal VLRC$^+$ cells, is reminiscent of the dendritic epidermal T cell compartment in mice (91).

5.2. VLR$^-$ Lymphocytes in Lampreys

A substantial fraction of lymphocytes do not express any of the three VLR isotypes (30). These triple-negative (TN) lymphoid cells constitute ~25% of the lymphocyte fraction in the peripheral blood (30). Their abundance in blood suggests that many of them do not represent immature forms of the known lymphocyte lineages but rather belong to other lymphocyte-like lineages. Current analyses of transcriptomes of this cell population may allow the derivation of specific antibody

reagents for their isolation and further characterization. One distinct possibility (discussed below) is that the TN fraction of peripheral lymphocytes encompasses primordial versions of innate lymphocyte types.

5.3. Sites of Development of Lamprey Lymphocytes

Although lamprey larvae are not amenable to the sophisticated tissue ablation and transplantation procedures that played such an essential role in defining the functions of hematopoietic and lymphopoietic tissues in the immune system of jawed vertebrates, certain inferences can be made regarding the sites of lymphocyte development in lamprey larvae. Histological studies indicated that the typhlosole and the kidney are major sites of blood formation in lamprey larvae, whereas the supraneural body becomes the major hematopoietic site in metamorphosed animals (67). For more than a century, controversy surrounded the presence of a thymus equivalent in lamprey larvae (53). To resolve this issue, RNA in situ hybridization studies were conducted with probes derived from the genes encoding the presumptive antigen receptors of B- and T-like cells in conjunction with the lineage-specific *CDA* genes. These studies reaffirmed earlier conclusions of hematopoietic activity in the typhlosole and kidneys and also provided evidence for a thymus equivalent, termed the thymoid (53), which is located at the tip of the gill filaments; its distinctly metameric and inconspicuous appearance (53) contrasts with the more discrete structure of the thymus in jawed vertebrates (92, 93) and may explain why it was not identified earlier by classical histological means. Although we lack detailed information about the ontogeny of the three principal lymphocyte lineages, the preponderance of partial and nonfunctional *VLRA* and *VLRC* gene assemblies, in addition to the presence of large numbers of apoptotic cells, suggests that the thymoid is the site of development of the VLRA$^+$ and VLRC$^+$ lineages (30, 53). This conclusion is supported by the observation that the thymoid is the exclusive site of CDA1 expression, which is presumed to be involved in *VLRA/C* gene assembly; as in the thymus of jawed vertebrates, the lamprey thymoid is characterized by the presence of tight associations of lymphocytes and epithelial cells (53).

5.4. Functional Characteristics of Lymphocyte Lineages

The lack of transformed cell lines and protocols for long-term culture of primary cells is a major impediment to the analysis of lamprey lymphocyte lineages. Hence, characterization of the different lymphocyte lineages has relied heavily on determining expression levels of presumptive lamprey homologs of genes known to be important for lymphocyte lineage choice, development, and/or function in mammals (14, 30). However, the interpretation of results of this approach is often complicated by the difficulty of firmly establishing orthology for individual members of gene families. This is notoriously difficult for genes encoding highly related chemokines, cytokines and their respective receptors, cell surface molecules implicated in cell-cell communication (often members of the TNF superfamily) and their receptors, or pathogen-associated pattern recognition molecules (such as Toll-like receptors), but the problem also plagues the identification of lineage-specific transcription factors. Hence, the resolution of current ambiguities in gene designation awaits progress in the annotations of lamprey genomes and transcriptomes (94, 95).

Nonetheless, even if orthology cannot be established with certainty in many cases, expression patterns of operationally defined genes can be used to clarify shared and distinct expression profiles among the presently identifiable lymphocyte lineages (14, 30). Using a selected panel of presumptive orthologous genes, discriminating expression profiles were observed for VLRA$^+$, VLRC$^+$, VLRB$^+$, and TN populations, with respect to genes encoding transcription factors, cytokines, or chemokines and their receptors; integrins; Toll-like receptors; and various signaling molecules

(14, 30). For instance, the two T-like lineages share the expression of homologs of *GATA2/3* and *BCL11B* genes, whereas the VLRB$^+$ cells exhibit high levels of homologs of *PAX5* and *SYK*. Interestingly, high transcription levels for a *Sox13* homolog, a transcription factor that is specifically expressed in γδ T cells of jawed vertebrates, distinguish VLRC$^+$ cells from their VLRA$^+$ cousins.

Collectively, the transcriptome analyses not only confirm a clear relationship of VLRA$^+$ and VLRC$^+$ lymphocytes but also reaffirm the dichotomy between these two T-like lineages and the distinct VLRB$^+$ B-like lineage.

5.5. Functional Networks in Lamprey Immunity

When lamprey larvae are immunized, a characteristic lymphocyte response is observed that features enhanced cell proliferation and morphological transformation of small lymphocytes into large blast-like cells (13). Early studies of the lamprey immune response to human erythrocytes indicated that lampreys (5) produce specific agglutinins for the human H antigen with an apparent molecular weight of 300 kDa (24, 36). Other investigators (3) reported that the erythrocyte-specific agglutinins are heat stable and suggested that, together with heterologous complement, these agglutinins can induce lysis of the erythrocytes. This early work was reaffirmed by recent studies focusing on the complement components in lampreys. The C3 component evolved well before the emergence of vertebrates and hence is also present in lampreys (96). Lamprey C1q homologs have also been identified (97) and shown to interact with VLRB antibodies to promote lysis of target erythrocytes and tumor cells (98). These findings suggest that a variant of the classical complement pathway in lampreys can be activated by VLRB antibodies and their cognate antigens.

The transcriptomes of purified lymphocyte populations in conjunction with studies on the presence of homologs of immune effector cells have begun to shed light on the intercellular communication systems of lampreys (14, 30). Notably, several chemokines and their putative receptors have been identified (99, 100); moreover, members of several cytokine and cytokine receptor families were discovered, although their functional characterization is just beginning. For instance, lampreys possess five distinct IL-17 genes and six IL-17 receptors; analyses of their expression patterns suggest a potential role for IL-17 in coordinating interactions between T-like cells and other cells of the adaptive and innate immune systems (101).

6. HAGFISH IMMUNE SYSTEM

Compelling evidence for similar developmental programs for the lamprey and hagfish immune systems has come from the identification of homologs of the three VLR isotypes (16–18, 26, 27, 32, 38). Given that the last common ancestor of lampreys and hagfishes lived ~480 million years ago (1), it is not surprising that there are qualitative and quantitative differences between their immune systems. For instance, hagfishes have relatively fewer VLRB$^+$ cells and less robust antibody responses than lampreys (7, 35).

Many features of hagfish *VLR* gene structures and assembly and LRR module sharing between VLRA and VLRC receptors mirror those of their lamprey orthologs (16–18, 26, 27, 32, 38). The hagfish genome has not been sequenced yet, so it is not known whether *VLRA* and *VLRC* genes are arranged in the same interspersed manner as their lamprey orthologs. *VLR* gene assembly in hagfishes appears to follow the rules established for lampreys. For instance, most *VLRB* and *VLRC* assemblies in individual lymphocytes are monoallelic, and when biallelic assemblies occur, one is invariably defective (23). Hagfish *VLRB* and *VLRC* assemblies also never occur in the same cell

(23). Hagfish *CDA* orthologs exist but have not yet been well defined (M. Hirano, unpublished work).

With respect to the predicted protein structures of hagfish VLRs, one interesting distinguishing feature has been observed for VLRCs. As discussed above, all known lamprey VLRC sequences exhibit an eight-residue insert loop of low sequence diversity linking strands β1 and β2 in LRRNT, whereas in hagfish VLRCs this loop consists of only four residues, just as in both lamprey and hagfish VLRA sequences (16–18, 26, 27, 32, 38). Importantly, a loop size of four residues is insufficient to form a protrusion in LRRNT that could possibly contact antigen.

The hematopoietic and lymphoid tissues are not well defined for hagfishes. For instance, the location and histomorphological structure of a thymoid have not been elucidated. Nonetheless, mixed leukocyte reactivity has been described in a hagfish, *Eptatretus stoutii*, wherein a small leukocyte population (presumably encompassing lymphocytes) responded to allogeneic stimulation and adherent cells functioned as stimulator cells (102), reminiscent of the situation in jawed vertebrates. Note, however, that uncertainty remains about the effector cell type during skin rejection in lampreys, where myeloid cells are suggested to be the main driver of the allogeneic response (8). Hagfishes respond to immunization with an increase in VLRB levels (35); the identification of a C1q molecule (103) and the presence of the C3 component (96) suggest that hagfishes, like lampreys, might also be able to exploit a variant of the classical pathway for immune defense.

The possibility of allogenic responses in hagfishes was explored in studies using a monoclonal antibody against a hagfish VLRB peptide epitope which is destroyed by paraformaldehyde fixation. Naturally occurring serum VLRB antibodies in individual hagfishes reportedly recognized allogeneic but not autologous determinants (104) of a previously identified polymorphic membrane protein (NICIR3) (105). This finding was interpreted to indicate that self-reactive VLRB specificities are purged, analogous to the situation for immunoglobulins in jawed vertebrates.

With respect to the molecular underpinnings of the hagfish immune system, similarities to that of lampreys are beginning to emerge. A *GATA3*-like transcription factor gene and a *BTK*-like gene have been identified in a cDNA sequence analysis for leukocytes of inshore hagfish *E. burgeri* (106).

7. EVOLUTIONARY TRAJECTORY OF ADAPTIVE IMMUNE SYSTEMS

7.1. Evolutionary Origins of VLRs

An analysis of amphioxus genes encoding VLR-like proteins (107) led to the identification of nine hypothetical ancestral VLR candidates. The crystal structure of one of these representatives, containing one LRRV element and one LRRVe element, was noted to be VLRC like, a conclusion based mainly on the presence of a characteristic insert in the LRRNT region and the absence of an LRRCT insert. Interestingly, the reactivity of the amphioxus VLR-like protein with gram-positive bacteria was mediated by two negatively charged amino acid residues in the LRRVe element of the protein; expression of a closely related molecule in the gill region of a related amphioxus species was taken to support the notion that this molecule could have an immunity-related function (107). Of note, the amphioxus VLR-like genes are complete genes and thus unlikely to undergo somatic modification; they are therefore reminiscent of structurally invariant predecessors of vertebrate antigen receptors. In another analysis of the evolutionary origins of cyclostome VLRs (15), a platelet glycoprotein receptor complex gene was proposed as a possible VLR ancestor based on its structural similarity, particularly with the LRRCT insert region of VLR proteins, which at some point became interrupted by intervening sequences and dependent on gene conversion for functional assembly.

7.2. Shared and Distinguishing Features of Vertebrate Adaptive Immune Systems

Although different in molecular detail, the design principles of adaptive immune systems of jawless and jawed vertebrates appear to be surprisingly similar (reviewed in 92, 108–110). The principal dichotomy between T- and B-like cells therefore must have been established in a common vertebrate ancestor before the divergence of the two extant branches of vertebrates, whereas the implementation of somatic diversification of antigen receptor genes may have occurred independently in the two lineages. It seems unlikely that a common ancestor of vertebrates possessed both LRR- and immunoglobulin-based antigen receptor systems, given the complexity of mechanisms that would have been required to contain potential self-reactivity of lymphocytes simultaneously expressing receptors of the two different emerging repertoires. Nonetheless, it is conceivable that the facility for genome modifications via the activities of cytidine deaminases is an ancient feature of metazoans; AID-like cytidine deaminases are known to have diverse roles in the adaptive immune system of jawed vertebrates, from generation of primary receptor repertoires to secondary modifications of antigen receptor specificities via somatic hypermutation and functional variation via class-switch recombination of immunoglobulins (92, 108–110). By contrast, the origin of the principal components of V(D)J recombination, the *RAG* genes, is less clear. Recently, it has been shown that primordial forms of the *RAG1/2* genes exist in the genome of the basal chordate amphioxus; however, no such genes are found in the genomes of tunicates or jawless vertebrates (111, 112). While it is possible the *Rag* genes were lost from these genomes, the current evidence is also compatible with the idea of repeated introduction of *RAG*-like sequences via horizontal gene transfer.

Although the chemical nature of antigen receptors is completely different for jawless and jawed vertebrates, certain features of the loci encoding these receptors are surprisingly similar. For instance, the genomic structure of the *VLRA/C* locus in lampreys (33) (**Figure 2*c***) is reminiscent of the interspersed nature of the *TCRA/D* locus in jawed vertebrates that also allows sharing of variable gene segments during the recombinatorial assembly of TCR genes (46). Notably, VLRAs and VLRCs are expressed by two different T-like lineages of lampreys (**Figure 3**), much like the TCRA and TCRD components of the heterodimeric TCR$\alpha\beta$ and TCR$\gamma\delta$ receptors. It is possible, therefore, that this evolutionarily conserved interspersion of genetic elements is indicative of an as yet unappreciated functional interdependence of the two principal T cell lineages in both jawless and jawed vertebrates.

Many similarities are also evident between jawless and jawed vertebrates with respect to the location of hematopoietic and lymphopoietic tissues. For instance, in all vertebrates, dedicated thymopoietic microenvironments in the pharyngeal region support T cell development; another shared characteristic is the apparent designation of general hematopoietic tissues (albeit occurring in many different anatomical sites) for B cell development (93).

8. OUTLOOK: CELLULAR COMPONENTS OF THE INNATE SYSTEM

Jawless vertebrates have several principal nonlymphoid cell types, such as granulocytes, macrophages, and monocytes, that closely resemble, both in morphology and apparent function, their counterparts in jawed vertebrates (113, 114).

It is also likely that a degree of similarity extends to the diverse components of the lymphoid innate immune system, which have risen to prominence in recent years (115). As noted above, it is likely that several different cell types are currently lumped together in the VLRA$^-$VLRB$^-$VLRC$^-$ (TN) population of lymphocyte-like cells found in all hematopoietic tissues of lamprey larvae.

For example, high levels of homologs of *PLZF/ZBTB16* and *ID2* genes were found in the TN population of cells (116; M. Hirano, J. Li, & M.D. Cooper, unpublished work), thus suggesting the presence of innate lymphocytes. Moreover, the identification of cells with large granular lymphocyte morphology suggests the presence also of natural killer (NK)-like cells in lampreys, a notion that is supported by the gene expression profile marked by high levels of homologs of *GZMA/K* and *RAB27A* by the NK-like cells (116; M. Hirano, M. Li, & M.D. Cooper, unpublished work), the latter encoding an essential component of the machinery transporting cytotoxic granules to the immunological synapse (117). If confirmed by further analyses, these results point to a surprisingly complex set of adaptive and innate lymphocytes in lampreys.

A potential strategy for studying cellular heterogeneity of lamprey lymphocyte populations lies in the derivation of antibody reagents specifically recognizing cell surface molecules to mark innate cell types for purification and subsequent transcriptome analysis. Besides examination of differentially expressed genes in RNA populations obtained from TN cells, a potentially promising means to disentangle the cellular complexity of this population is the use of single-cell RNAseq, perhaps coupled to other types of omics approaches (118). However, it should be recognized that the deficiencies of genomics resources and the lack of validated gene models directly affect the use of single-cell techniques to define cell lineages and/or determine their developmental trajectories.

9. CONCLUSIONS

Although evidence for the adaptive immunological capacity of jawless fishes was obtained several decades ago, its molecular underpinnings are only recently beginning to be revealed. We now know that a somatically generated family of diverse *VLR* genes encode the antigen-specific agglutinins and are clonally expressed by B-like lymphocytes. Since the description of the initial antibody-like VLR isotype now referred to as VLRB, two more VLRs have been discovered: VLRA and VLRC are expressed in a mutually exclusive manner by two distinct T-like lymphocyte lineages that bear similarities to the canonical $\alpha\beta$ and $\gamma\delta$ TCR–expressing cells in jawed vertebrates. Indeed, discriminating expression profiles confirm a clear relationship of VLRA$^+$ and VLRC$^+$ lymphocytes and reaffirm the dichotomy between these two T-like lineages and the VLRB$^+$ B-like lineage.

The VLRs are membrane-bound proteins of conserved general structure, but only the VLRBs can be secreted. Crystallographic studies of VLRB antibodies in complex with their cognate proteinaceous and carbohydrate antigens reveal the importance of residues located on the concave surface of the VLRB solenoid and the flexible loop insert in the C-terminal LRR. *VLR* genes are incomplete in their germline configuration and thus incapable of encoding any functional proteins. Functional *VLR* gene assemblies are generated by serial stepwise replacement of the intervening sequences in the germline *VLR* genes by employing flanking variable *LRR* (*LRRV*) sequences as templates. This assembly process appears to be mediated by different types of cytidine deaminases, CDA1 for the T-like lineage and CDA2 for B-like cells, in the lineage-specific deployment of this ancient facility for genome modifications.

Neither *VLRA* nor *VLRC* assemblies are observed in lymphocytes expressing VLRBs, and vice versa, indicating a lineage-specific execution of the assembly process that has hallmarks of allelic exclusion first described for immunoglobulin genes in jawed vertebrates.

With respect to the number of LRRVs found in mature VLRs, they are typically fewer in *VLRB* assemblies than in *VLRA* and *VLRC* assemblies, both of which have a distinct preference for four LRRVs, very likely as the result of postassembly selection.

VLRB$^+$ cells develop in the typhlosole and kidneys, the major sites of blood formation in lamprey larvae, and are the dominant lymphocyte type in blood and kidneys of sea lamprey larvae. By contrast, lamprey T-like cells begin their development in the thymoid, which is located at

the tip of the gill filaments. In larval tissues, VLRC$^+$ lymphocytes predominate over the VLRA$^+$ cells in the typhlosole, gill region, mucosal epithelium, and epidermis, wherein they constitute the major lymphocyte type. This distribution pattern, together with an apparently restricted receptor diversity of *VLRC* assemblies, is reminiscent of the $\gamma\delta$ TCR population in mammals.

In summary, research in the last decade has demonstrated that, despite differences at the molecular level, the overall design principles of adaptive immune systems of jawless and jawed vertebrates are surprisingly similar. The dichotomy between T- and B-like cells is clearly an ancestral vertebrate feature, whereas the implementation of somatic diversification processes of the structurally distinct antigen receptor genes of jawless and jawed vertebrates evolved independently in the two lineages.

DISCLOSURE STATEMENT

M.D.C. is a cofounder and shareholder of NOVAB, which produces lamprey antibodies for biomedical purposes. The other authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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