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Jawless vertebrates use variable lymphocyte receptors (VLRs) that are generated by RAG-independent combinatorial assembly of leucine-rich repeat cassettes for Ag recognition, instead of the Ig-based Ag receptors used by jawed vertebrates. The VLR genes encode for crescent-shaped proteins that use variable β-strands and a C-terminal loop to bind to Ags rather than the six CDR loops used by BCRs and TCRs. VLR mAbs have been isolated recently, which enabled the structure of VLR–Ag complexes to be defined. The jawless vertebrate adaptive immune system has many similarities to the Ig-based system of jawed vertebrates, including the compartmentalized development of B-like and T-like lymphocyte lineages that proliferate and differentiate into VLR-secreting plasmacytes and proinflammatory cytokine-producing cells in response to Ags. The definition of common features of the VLR-based and Ig-based systems offers fresh insight into the evolution of adaptive immunity. The Journal of Immunology, 2010, 185: 1367–1374.

Jawless vertebrates (agnathans) have been a focal point in the search for the evolutionary origins of adaptive immunity because of their unique position in chordate phylogeny (1). The first agnathans appeared in the Cambrian period >500 million years ago. However, lamprey and hagfish are the only groups of agnathans that have survived to the present. Prior to the agnathans, invertebrate and early chordate species relied on germline-encoded molecules for detection of infectious microorganisms and used phagocytes as the primary immune effector cell type (2). All of the surviving vertebrates (jawed and jawless) have evolved immune systems that utilize clonal and somatic selection mechanisms to generate highly diverse Ag receptors that are generated by somatic rearrangement of Ig V, D, and J gene segments during the development of clonally diverse lymphocytes.

In the 1960s and early 1970s, hints of an agnathan adaptive immune system were provided by experiments in which immunization of lampreys with particulate Ags, such as heat-killed Brucella abortus, bacteriophage f2, or mammalian erythrocytes, induced the production of specific Ab-like agglutinins (3–6). In addition to these humoral immune responses, delayed-type reactions to tuberculin and accelerated second set allograft rejection were suggestive of cell-mediated immunity (3). Despite the convincing evidence for Ag-specific agglutinins, biochemical evidence for Ig was not obtained, and the molecular basis for the lamprey humoral immune response remained elusive (5–8).

Sea lamprey development and anatomy

Sea lamprey (Petromyzon marinus) is the most studied jawless vertebrate species; larvae hatch from fertilized eggs in inland streams and burrow into the muddy streambed, where they filter feed on plankton. The wormlike larvae lack teeth and eyes and can grow up to 20 cm in length. After 3–14 y, the larvae metamorphose into parasitic adults that resemble eels and migrate to the sea. Adult sea lampreys develop their eyes and rows of teeth arranged in concentric circles, which they use to attach to fish and feed on their body fluids. Adult sea lampreys grow up to 100 cm in length and weigh ~1 kg. After 12–18 mo at sea, the lampreys return to freshwater streams to spawn and die shortly after mating. In the laboratory, lamprey larvae can be housed for >1 y in aerated, sand-lined aquariums maintained at 15–25°C and fed brewer’s yeast. Adult sea lamprey can also be maintained in aquaculture, but extended culture is more cumbersome because live fish are required for feeding.

Lampreys lack lymph nodes or a histologically recognizable thymus, but lymphoid cells are present in their blood and tissues including the typhlosole (an invagination of the dorsal epithelial wall of the intestine), kidneys, and gills (9, 10). The typhlosole is the earliest site of hematopoiesis in lamprey larvae and is sometimes referred to as the spleen (9). During metamorphosis, the typhlosole regresses, and hematopoiesis shifts to the dorsal fat body located above the spinal cord. The interstitial spaces of the anterior kidney are populated by lymphoid and myeloid cells, and the blood vessels of the gills contain many lymphocyte-like cells. In addition to nucleated erythrocytes and thrombocytes, the blood contains cells that are morphologically similar to the lymphocytes, plasma cells, monocytes, and granulocytes of jawed vertebrates (9–12).

Discovery of variable lymphocyte receptors

The identification of a genetic basis for adaptive immunity in jawless vertebrates began with transcriptome analysis of\n
Abbreviations used in this paper: AID, activation-induced cytidine deaminase; CDA, cytosine deaminase; CP, connecting peptide; HEL, hen egg lysozyme; HP, hydrophobic peptide; LRR, leucine-rich repeat; LRR1, the first 24-residue leucine-rich repeat; LRRCT, C-terminal leucine-rich repeat; LRRNT, N-terminal leucine-rich repeat; LRRV, variable leucine-rich repeats; LRRVe, end leucine-rich repeat; SP, signal peptide; VLR, variable lymphocyte receptor.
lymphocyte-like cells isolated from the typhlosole of lamprey larvae (11, 13). The light-scattering characteristics of these lymphocyte-like cells were similar to mouse lymphocytes, which allowed their enrichment by flow cytometry-based cell sorting (11). The lamprey lymphocyte-like cells were morphologically similar to mammalian lymphocytes by light and electron microscopy. More than 8000 clones were sequenced from a cDNA library prepared from the sorted lymphocyte-like cells, and a basic local alignment search tool analysis of the resulting expressed sequence tag sequences was conducted to determine the identity of the orthologous genes (11, 13). Lamprey genes with homology to transcription factors (Spi B and Ikars) and signal transducing molecules (CD45 and B cell adaptor protein) that are important for lymphocyte development were present in the cDNA library. Nevertheless, the cardinal elements of adaptive immunity, namely Ig, TCR, RAG1 and 2, and MHC class I and II, were conspicuously absent.

In a revised strategy, lampreys were injected with an Ag and mitogen mixture that induced lymphoblastoid transformation of lymphocyte-like cells (12). The activated lymphoblastoid cells in blood samples were sorted by flow cytometry, and a cDNA library was prepared by subtracting lamprey myeloid cell and erythrocyte cDNA from lymphocyte-like cDNA. Of the >1500 subtracted cDNA clones that were sequenced, 239 uniquely diverse leucine-rich repeat (LRR)-containing transcripts were characterized that were exclusively expressed by lymphocytes, with highest expression being seen in the lymphoblasts. Due to the sequence diversity and lymphocyte-restricted expression, these receptors were named variable lymphocyte receptors (VLRs). Each VLR transcript encoded an invariant signal peptide (SP) followed by highly variable LRR modules: a 27–34 residue N-terminal LRR (LRRNT), the first 24-residue LRR (LRR1), up to eight 24-residue variable LRRs (LRRV), one 24-residue end LRRV (LRRVe), one 16-residue connecting peptide LRR (LRRCP), and a 48–63 residue C-terminal LRR (LRRCT) (Fig. 1A). Southern blotting of lamprey genomic DNA with probes specific for invariant 5′ and 3′ portions of the VLR sequences revealed that the highly variable VLRs were encoded by a single VLR gene (12).

The germline VLR gene was incomplete in that it consisted of sequences encoding for invariant N- and C-terminal portions of VLRs separated by noncoding intervening sequences. However, in lymphocyte genomic DNA, the noncoding intervening sequences were replaced by LRR-encoding sequences to form a functional, mature VLR gene. All of the data pointed to the astonishing conclusion that an alternative form of adaptive immunity comprised of LRR-based Ag receptors evolved before our Ig-based system.

Somatic generation of VLR diversity

After the discovery of a VLR gene in lamprey, two VLR genes (VLRa and VLRb) were identified in a subsequent analysis of hagfish (14). An additional VLR gene with homology to hagfish VLRa was later found in lamprey (15), and the original lamprey VLRgene was renamed VLRb because of its similarity to hagfish VLRb. The germline VLRa and VLRb genes are comprised of two exons, with the first exon encoding only a portion of the 5′ untranslated region (12, 14, 15). The second exon contains the rest of the 5′ untranslated region, a SP, a 5′ portion of the LRRNT, a 3′ portion of the LRRCT, and the stalk region. For hagfish VLRa and VLRb and for lamprey VLRA, the 5′ LRRNT sequence is separated from the 3′ LRRCT sequence by a short noncoding intervening sequence that lacks canonical splice donor and acceptor sites (Fig. 1B). The lamprey VLRb gene is more complex. The 5′ LRRNT coding sequence is separated from a segment encoding a 5′ portion of LRRCT by a 5.8-kbp intervening sequence, and a second 6.4-kbp intervening sequence separates the 5′ LRRCT sequence from the 3′ LRRCT and the stalk region (12). The germline configuration of the VLR genes in all cell types except lymphocytes lacks direct coding sequences for LRR1, LRRV, LRRVe, and LRRCP, as well as partial coding sequences for LRRNT and LRRCT (12, 14, 15). Remarkably, the incomplete germline VLR genes are flanked by an extensive array of partial LRR gene cassettes (12, 14–17). In lamprey, 393 VLRa-related LRR cassettes and 454 VLRb-related LRR cassettes have been characterized within a continuous locus of ~2 mega bp surrounding their respective VLR gene (15).

The VLR gene assembly mechanism has been characterized by aligning genomic LRR cassettes to mature VLR sequences and by capturing VLR-assembly-intermediate genes that have LRR cassette inserts, but which have not completed the assembly process and still retain portions of the intervening sequence (15–18). These analyses have shown that the lamprey VLR genes are assembled in a stepwise, piecewise manner in which the intervening sequence is replaced by random and sequential copying of the flanking LRR cassettes into the incomplete VLR gene (Fig. 1C). The assembly process can be initiated at either the 5′ LRRNT or 3′ LRRCT ends and appears to be directed by short stretches of nucleotide homology (10–30 bp) between donor and acceptor sequences that guide the copying of flanking LRR segments into the germline gene (15–17). The VLR gene assembly mechanism has been described as gene conversion-like (15, 16, 19) because of the nonreciprocal insertion of LRR cassettes into the intervening sequence of the germline gene, or copy choice (17, 18) because short stretches of sequence homology are used to target donor LRR sequences, thereby resembling the mechanism used by yeast for mating-type switching.

VLR gene assembly occurs on only one allele at a time, a strategy that is reminiscent of the allelic exclusion of BCR and TCR gene assembly in jawed vertebrates (16, 17). VLR assembly is primarily monoallelic, but diallelic assembly has been detected in a small percentage of hagfish lymphocytes (7% VLRa, 4% VLRb) (18). In most cases, one of the diallelically assembled VLR genes contained base deletions or additions that resulted in frame-shift mutations, which rendered the gene nonfunctional. A computational assessment of mature VLR gene sequences and the LRR cassettes used to assemble them predicted a potential repertoire of >1014 distinct Ag receptors, which is comparable to the theoretical Ab repertoire diversity of mammalian adaptive immune systems (16).

In contrast to the Ig V, D, and J gene segments in jawed vertebrates, the LRR cassettes are not flanked by recombination signal sequences, and RAG1 and 2 have not been identified in lampreys or hagfish (11–13, 16). Avian and some mammalian species use a gene conversion mechanism catalyzed by activation-induced cytidine deaminase (AID) to generate Ab diversity (20–22). Two members of the AID-apolipoprotein B mRNA editing catalytic component family of DNA cytosine deaminases (CDA) have been identified in lamprey, and these may be involved in VLR assembly and diversification (15). CDA1 expression is...
detected in VLRA+ cells, whereas CDA2 expression is restricted to VLRB-expressing lymphocytes (23). CDA1 was shown to be highly mutagenic and increased the rate of intragenic mitotic recombination in yeast (15). These findings favor the conjecture that CDA1 may catalyze VLRA gene assembly by inducing DNA strand breaks that allow for homology-based pairing between donor LRR cassettes and the VLRA gene, whereas CDA2 may play a similar role in VLRB gene assembly.

VLRB mAbs

The tremendous sequence diversity generated by VLR assembly suggested they could be useful biomedical reagents if Ag-specific clones could be isolated. This achievement was hampered initially because of technical limitations, including the lack of a cell culture system or a means to immortalize primary lamprey lymphocytes. However, VLRs are readily amenable to rDNA techniques, because they are composed of a single polypeptide, and two methods have been developed to produce VLR mAbs.

In the first method, VLRB cDNA libraries were prepared from the lymphocytes of lampreys immunized biweekly for 6 wk with Bacillus anthracis exosporium or human O type erythrocytes (24). Individual clones from the VLRB cDNA library were transiently transfected into HEK-293T cells, which secreted the VLRB proteins as multivalent Abs into the tissue culture supernatant. The VLRB-containing supernatants were then screened for Ag-binding by ELISA, immunofluorescence staining, or agglutination assays. VLRB mAbs were isolated against BclA, the major spore coat protein of B. anthracis (25), and the H-trisaccharide of human erythrocytes. Fourteen VLRB clones specific for BclA were isolated out of the 212 tested clones (6.6%) (24). Although all of the VLRB clones were unique, they shared notable sequence similarity, even in hypervariable positions. All but one of the clones contained the same number of LRR modules, whereas non-binding VLRB clones from the same library varied greatly in the number of LRR modules. VLRB mAbs were highly specific as evidenced by their ability to discriminate B. anthracis from Bacillus cereus T spores that differ in BclA sequence at 14 of 134 amino acids, only 9 of which are solvent exposed. VLRB mAbs isolated using this method were of low affinity (micromolar $K_D$) but displayed remarkable avidity to Ags with repetitive epitopes due to their multivalency. For example, an anti-BclA VLRB mAb (VLR4) agglutinated B. anthracis spores at a 1000-fold higher dilution than a corresponding high-affinity mouse IgG mAb for the same Ag (24).

The VLRB mAbs performed as well as Ig-based Abs did in a variety of Ab-based assays including ELISA, immunofluorescence staining, and Western blotting. rVLRB mAbs purified by Ag affinity chromatography required extreme conditions for elution (pH 3.11), yet the Abs retained the ability to bind to Ag despite the harsh elution conditions (24). rVLRB Abs are also remarkably stable at a variety of temperatures. They have been stored at 4°C (either purified or in crude tissue culture supernatant) for >2 y, room temperature for >1 mo, and 56°C for 36 h without loss of Ag-binding ability. Incubation

FIGURE 1. VLR gene assembly. A, Mature VLR genes consist of coding sequences for a SP, an LRRNT, an LRR1, up to eight LRRV cassettes, an LRRVe, a CP LRR, an invariant stalk region, and a C-terminal HP, which is preceded by a GPI cleavage site (arrow). B, Two germline VLR genes, VLRA and VLRB, are present in hagfish and lamprey. The germline VLR genes are incomplete and consist of invariant regions encoding for portions of the LRRNT and LRRCT separated by noncoding intervening sequences. C, The germline VLR genes are flanked by hundreds of LRR cassettes. During lymphocyte development, the noncoding intervening sequence is replaced by random and sequential copying of sequences from the flanking LRR cassettes into the germline VLR gene. VLR gene assembly is initiated at either the LRRNT or LRRCT end and proceeds in a stepwise manner that is guided by short stretches of sequence homology between the donor and acceptor LRR sequences until a mature VLR gene is generated. Illustrations not drawn to scale. CP, connecting peptide; HP, hydrophobic peptide; SP, signal peptide.
at >70°C for 1 h was required to denature the VLRB Abs and prevent Ag binding (B.R. Herrin, unpublished observations). Molecular weight analysis of monomeric and multimeric VLRB Abs indicated that the multimers are composed of 8–10 identical subunits, and the dimeric forms observed under partial reducing conditions suggested that the multimers are composed of four to five dimeric subunits (10, 24). Visualization of an rVLRB Ab by electron microscopy revealed that the pentameric and tetrameric pairs of the VLRB solenoids connected at a central point by the thin and highly flexible stalk region (24).

A high-throughput yeast display library screening method has recently been developed to isolate VLRB mAbs (19). VLRB proteins were expressed on the surface of yeast by fusion to the yeast flocculation protein Flo1p, which contains a stalklike structure and a C-terminal GPI linkage (26, 27). Ag-specific clones were isolated by sorting the VLRB-expressing yeast that bound to fluorescently labeled soluble Ags, including hen egg lysozyme (HEL), R-PE, β-galactosidase, cholera toxin subunit B, and blood group A and B trisaccharides (19). The yeast display method allowed for greater breadth of screening, which enabled VLRB Ag-specific clones to be isolated from both immunized and nonimmunized libraries and for the selection of higher affinity clones by in vitro mutagenesis. Three HEL-specific VLRB clones with high nanomolar affinities (KD = 455–117 nM) were isolated from a VLRB cDNA library constructed from the lymphocytes of HEL-immunized lamprey larvae. Although high-affinity VLRB clones could not be isolated from the HEL-immunized cDNA library, higher affinity mutants (KD = 55–4.3 nM) of a low-affinity anti-HEL clone, VLRB.2D (KD = 455 nM), were selected from a mutant library generated by error-prone PCR (19). Notably, all of the high-affinity mutants contained substitutions in the hypervariable LRRCT loop region. This finding suggested a strategy of selecting for high-affinity mutants by swapping the LRRCT region of a low-affinity anti-HEL clone with LRRCT regions PCR amplified from a large pool of VLRB cDNAs. Remarkably, a mutant clone was isolated using this approach with 1300-fold higher affinity (KD = 119 pM from 155 nM) than the parent clone (19). These data demonstrate that there are no inherent limitations that prevent VLRs from reaching affinities comparable to mammalian IgG Abs and that a limited number of mutations are required to obtain high affinity for both types of Ab.

Structural basis for VLR Ag recognition

Crystal structures have been reported for hagfish VLRA and VLRB without Ag and for lamprey VLRB in complex with Ags (28–30). VLRA and VLRB encode for crescent-shaped, solenoidal proteins that resemble a cupped hand with parallel β-strands lining the palm. VLRB contains a thumblike C-terminal variable loop that projects back toward the palm of the hand. The overall VLR structure is typical of LRR receptor family members in that the “LxxLxLx” motifs in LRR1, LRRV, LRRVe, and LRRCP form parallel β-strands on the concave surface (Fig. 2A). The structure is stabilized by conserved leucines and phenylalanines that form the hydrophobic core of the solenoid. The hydrophobic core is capped at the N terminus and C terminus by LRRNT and LRRCT modules. The LRRNT module contains a β-hairpin structure in both VLRA and VLRB. The LRRCT is comprised of an α-helix at the base of the solenoid and a loop positioned on the concave surface just below the LRRCP β-strand. The LRRCT loop in VLRB varies greatly in length and sequence composition and often forms an extended loop that projects back toward the concave surface (16, 29, 30). Both LRRNT and LRRCT contain four conserved cysteines that form two disulfide bridges that further stabilize the structure (28–30).

The VLRA and VLRB sequence diversity is primarily concentrated in the β-strands and the LRRCT loop on the concave surface that combine to form a continuous hypervariable surface (12, 16, 28–30).

A crystal structure has been reported for a VLRB mAb (RBC36) that specifically binds to the H-trisaccharide (29). The H-trisaccharide contacted amino acid residues on the
β-strands of LRR1, LRRV1, LRRV2, LRRV3, LRRVe, LRR-CP, and LRRCT but not the LRRNT of RBC36 (Fig. 2B). The specificity of RBC36 for the H-trisaccharide was primarily mediated by hydrogen bonds contributed by Asp103 of LRRV2 and Asp152 and Gln153 of LRRVe. The H-trisaccharide interaction was also stabilized by van der Waals contacts contributed by eight other residues located on the β-strands. In addition to the β-strands, a tryptophan residue located at the tip of the LRRCT loop contributed a key interaction by stacking its indole group parallel to the plane of the galactose ring of the H-trisaccharide (29). The concave surface area of RBC36 was estimated to be ~1720 Å², which is much larger than the ~700–1000 Å² Ag binding site of a typical mammalian Ab. However, the relatively small H-trisaccharide buried only ~300 Å² of the VLRB surface.

A crystal structure has been solved for another VLRB mAb (VLRB.2D) in complex with a protein Ag, HEL (30). The interaction is mediated by 19 VLRB.2D residues forming contacts with 20 residues on the surface of HEL. Similar to the RBC36-H-trisaccharide complex, HEL makes contacts with amino acids in LRR1, LRRV, LRRVe, LRRCP, and LRRCT of VLRB.2D, but not the LRRNT (29, 30). The most notable feature of the structure is the insertion of the LRRCT loop into the catalytic cleft of HEL, where it forms a hydrogen bond with the catalytic residue (Fig. 2C) (30). Single-chain Abs, cameld VHH (31) and shark IgNAR (32), recognize almost the same epitope as VLRB.2D by inserting one of their Ig loops into the catalytic cleft of HEL (Fig. 2D). In contrast, Vγ1Vλ mouse Abs that have been crystallized with HEL invariably bind to relatively flat surfaces away from the catalytic cleft (Fig. 2E) (33). An in vitro affinity-matured mutant (VLRB.2DMut13) with ~10-fold higher affinity for HEL was also crystallized (30). Superposition of the high-affinity mutant with wild-type VLRB.2B revealed that the mutations did not cause substantial structural changes. Surprisingly, the two residues mutated in the LRRCT of VLRB.2DMut13 did not make contact with HEL, and the total surface area buried by the VLRB.2DMut13-HEL complex was ~100 Å² less than the wild-type complex. The greater affinity of VLRB.2DMut13 was the result of improved shape and electrostatic complementarity to HEL rather than formation of additional hydrogen bonds or van der Waals contacts (30). To examine the role of conformational changes in Ag binding by VLRB, the structures of bound and unbound VLRB.2D were compared by superposition. The concave surface of VLRB.2D maintained almost the same conformation after HEL binding; however, a hinge movement of ~1 Å occurred in the LRRCT loop in response to the HEL interaction (30). The structural rigidity of the LRR β-strands may make VLRB less prone to induced-fit conformational changes that can lead to nonspecific Ag binding by the CDR loops of Ig-based Abs.

The VLR stalk region

An invariant threonine/proline-rich stalk region that is germline encoded connects the C terminus of the LRR solenoid to the cell membrane (12). Crystal structure data are not available for the stalk region, but computer modeling predicts that it forms an extended unstructured region with proline residues functioning to prevent secondary structure formation. The stalk regions of VLRA and VLRB end with a hydrophobic tail that fits consensus sequences of GPI-linked receptors (12, 14, 15). However, only the GPI-linkage of lamprey VLRB has been verified experimentally (12). Interestingly, VLRA and VLRB do not encode for cytoplasmic amino acids, a finding that implies VLR signal transduction is mediated by transmembrane receptors that function analogously to Igα/Igβ and CD3/ζ-chain in jawed vertebrates. In lampreys, VLRB is secreted into the blood as a disulfide-linked, multivalent Ab, whereas VLRA is expressed only as a cell-surface receptor (10, 16, 23). The hydrophobic tail downstream of the lamprey VLRB GPI-cleavage site is unusual in that it is rich in cysteine residues that are not present in VLRA or hagfish VLRB (12, 14, 15, 24). VLRB cDNAs expressed in mammalian cell lines, such as HEK-293T cells, are secreted as disulfide-linked multimers similar to VLRB Abs in lamprey plasma (24). Deletion of the cysteine-rich hydrophobic tail of the stalk region results in secretion of monomeric VLRB by transfected HEK-293T cells. This suggests a mechanism for switching to VLRB secretion by modulating GI addition.

Tissue distribution of lamprey lymphocytes

The development of Abs specific for invariant portions of the VLRA and VLRB receptors allowed the cellular basis of the lamprey adaptive immune response to be characterized. Surface expression of VLRA and VLRB was detected on distinct lymphocyte populations by flow cytometry (23). Analysis of the VLRA and VLRB genomic loci in cells sorted for VLRA and VLRB surface expression revealed that VLRA gene assembly only occurs in the VLRA+ cells, and VLRB gene assembly only occurs in VLRB+ cells. Furthermore, CDA1 expression was restricted to VLRA+ cells, whereas CDA2 expression was detected only in VLRB+ cells (23). The restricted expression of CDA1 and CDA2 may account for the mutually exclusive assembly of VLRA and VLRB genes. The two lineages of lamprey lymphocytes have distinct tissue distribution patterns, with VLRB cells being the dominant population in the blood and kidneys, where they outnumber VLRA cells by an 8:1 ratio. The VLRB to VLRA cell ratio is 2:1 in the typhlosole, and VLRB and VLRA cells are present in equivalent numbers in the gill region (23). VLRA+ cells are found by immunohistochemical analysis to be located within the blood vessels of the gills, in the interstitial spaces between renal tubules of the anterior kidney, and interspersed among hematopoietic cells in the typhlosole (10).

Characterization of B-like and T-like lymphocyte populations

Lamprey VLRB lymphocytes have many morphological and functional similarities to the B cells of jawed vertebrates in keeping with their function as the humoral arm of the lamprey adaptive immune system. Immunization of lampreys with bacteria, viruses, or mammalian cells induces VLRB lymphocytes to undergo lymphoblastoid transformation, proliferation, and differentiation into plasmacytes that secrete their Ag receptors as multivalent VLRB Abs (Fig. 3A) (10). In naive lamprey larvae, <1% of the VLRB cells in blood are dividing versus 3–5% of the circulating VLRB cells 14 d post-immunization with B. anthracis exosporium (23). After booster immunization with B. anthracis exosporium, Ag-specific VLRB-secreting cells can be detected in the blood, kidneys, and, to a lesser extent, the typhlosole (10). Electron microscopy analysis indicates that the VLRB Ab-secreting cells have...
extensive cytoplasm and rough endoplasmic reticulum, much like plasma cells in jawed vertebrate animals.

The VLRB Ab response evolves more slowly than the Ab responses of mammals and is more similar to the IgM responses of cartilaginous and bony fishes (10, 34). The peak Ab levels observed in the lamprey VLRB response are also lower than in the mammalian Ab response. The highest Ag-specific plasma VLRB titers obtained after repeated immunization is ~1:10,000 in lamprey larvae, whereas mammalian IgG titers may exceed 1:100,000. Lampreys immunized with *B. anthracis* exosporium produced VLRB Abs that reacted with the BclA spore coat protein, which is also the dominant Ag recognized by Abs in mice immunized with *B. anthracis* exosporium (10, 25). VLRB Abs specific for BclA were detectable by day 7 postimmunization, and their levels peaked by day 26, although appreciable anti-BclA titers persisted beyond day 50 (10). A second immunization with *B. anthracis* exosporium induced several-fold higher titers of VLRB Abs for BclA relative to lampreys given a single immunization. Similar kinetics were observed for the VLRB Ab response in lampreys immunized with human O-type erythrocytes. VLRB agglutination titers peaked at day 19 after a single immunization, and a second immunization resulted in a 20-fold enhancement in agglutination titer (10).

VLRA cells also undergo lymphoblastoid transformation and proliferation postimmunization with viruses, bacteria, and mammalian cells, but they do not produce soluble VLRA proteins either pre- or postimmunization with bacteria or stimulation with the classical T cell mitogen, PHA (Fig. 3) (23). Moreover, the VLRA molecules are not secreted by HEK-293T cells transfected with VLRA cDNAs, whereas VLRB Abs are secreted by transfected heterologous cells (23, 24). PHA also does not induce VLRA secretion, but it preferentially stimulates vigorous proliferation of VLRB cells, leading them to become the dominant lymphocyte population by 9 d postinjection into lamprey larvae (23). The activated VLRB lymphoblasts are more than twice the size of naive VLRA cells and have expanded cytoplasm, mitochondria, and endoplasmic reticulum. Importantly, the activated VLRA cells express higher transcript levels for proinflammatory cytokines, macrophage migration inhibitory factor, and IL-17 (23). These findings suggest that Ag-activated VLRA cells differentiate into cytokine-secreting effector cells that may direct the immune response.

It is not yet clear whether VLRA cells primarily recognize native or processed Ags. Yeast display experiments have identified one example of VLRA clones that can bind to HEL with high affinity ($K_D = 26–0.27$ nM) (19). Thirteen unique HEL-specific VLRA clones were isolated from a single animal that differed from each other at only 15 out of 244 aa positions. The high degree of sequence similarity between the clones raises the intriguing possibility that the clones were derived from a single lymphocyte precursor that diversified its receptor by somatic mutation, perhaps catalyzed by the AID-like enzyme CDA1 (15, 19). In contrast, VLRA cells that bind bacteria could not be demonstrated pre- or postimmunization, whereas the numbers of bacteria-binding VLRB cells increased postimmunization (10, 23). If the VLRB cells recognize native Ags, like $\gamma\delta$ T cells, they may function to coordinate and regulate the immune response via cytokines and costimulatory molecules. If VLRA cells instead detect processed Ags displayed on the surface of infected cells, like $\alpha\beta$ T cells, they may function to monitor intracellular compartments that are inaccessible to VLRB Abs. MHC receptors have not been found in EST sequences (11–13) or in draft sequences of the lamprey genome (B.R. Herrin, unpublished observations). Therefore, if VLRA cells detect processed Ags, the peptides are likely presented by a non-MHC receptor(s) that arose by convergent evolution. Genes with homology to a TAP-like peptide transporter and the LMP7/X proteasome subunit have been characterized, which could suggest the possibility that lamprey cells have the capacity to generate peptides and transport them for loading onto a putative receptor for presentation (35, 36).

### Differential gene expression by VLRA and VLRB lymphocytes

VLRA and VLRB lymphocytes have been shown to have different gene expression profiles by quantitative analysis of the transcripts for a selected panel of genes (23). VLRA lymphocytes express a number of genes that are associated with T cell development in jawed vertebrates. These include several transcription factors (GATA2/3, c-Rel, aryl hydrocarbon receptor, and BCL11b), the T cell fate-determining receptor Notch1, the receptor protein tyrosine phosphatase CD45 that is essential for TCR signaling, and the chemokine receptor CCR9 that promotes homing of T cell progenitors to the thymus, although a thymus equivalent has not yet been identified in lamprey (Fig. 3C). The VLRA cells also express the

![Figure 3](http://www.jimmunol.org/DownloadedFrom/1372_BRIEF_REVIEWS_ALTERNATIVE_ADAPTIVE_IMMUNITY)

**FIGURE 3.** B-like and T-like lymphocytes in lamprey. **A** and **B**, Particulate Ags stimulate lymphoblastoid transformation and proliferation of VLRB$^+$ and VLRA$^+$ cells. VLRB$^+$ cells differentiate into VLRB Ab-secreting cells that resemble plasma cells in jawed vertebrates (Δ). In contrast, activated VLRA$^+$ cells differentiate into proinflammatory cytokine-producing effector cells that do not secrete their Ag receptors. **C**, VLRA and VLRB cells express many genes that are required for B cell and T cell development in jawed vertebrates. The reciprocal expression of cytokines and cytokine receptors by VLRB$^+$ and VLRA$^+$ cells suggests the possibility of functional interactions between the two lymphocyte lineages.
proinflammatory cytokines, macrophage migration inhibitory factor, and IL-17, as well as the IL-8R (CXCR2). In contrast, the VLRL lymphocytes express many genes that are required for B cell activation and differentiation in jawed vertebrates, including the protein tyrosine kinase Syk and the B cell adaptor protein that transduce signals initiated by BCR ligation, the TNFR superfamily member 14/Herpesvirus entry mediator that binds to LIGHT or B and T lymphocyte attenuator on the surface of T cells, and the chemokine receptor CXCR4 (23).

Like mammalian B cells, the VLRL lymphocytes also preferentially express several TLRs (TLR2, 7, 10) that may synergize with Ag receptor signals to promote activation. One of the most notable findings for the VLRL cells is their expression of the chemotactic and inflammatory cytokine IL-8 and the receptor for IL-17, which complements the expression of IL-8R and IL-17 by VLRA cells to suggest the possibility of cooperative functional interactions between the two lymphocyte lineages.

Conclusions

There is much left to be learned about the adaptive immune system in agnaths before we can understand the survival advantage that favored its acquisition, presumably to augment innate mechanisms of protective immunity against pathogens. Although the Ig-based and VLR-based adaptive immune systems in jawed and jawless vertebrates use different genes and assembly mechanisms, both systems generate diverse repertoires of anticipatory receptors capable of recognizing almost any Ag through the combinatorial assembly of large arrays of partial gene segments. The development of clonally diverse lymphocytes allows for Ag-specific responses and memory, which are lacking in innate immunity. However, these advantages must offset the inherent danger associated with the stochastic assembly of anticipatory receptor genes by effecter lymphocytes, namely the potential to generate receptors with specificity for self Ags. Although we know that the Ig-based system has evolved multiple mechanisms to ensure tolerance to self, the tolerance phenomenon has not yet been explored in the VLR-based system. It will be fascinating to learn how this fundamental immunological dilemma is resolved in jawless vertebrates.

Hybridoma technology enabled the discovery of the diverse mammalian B cell repertoire to be harnessed to produce mAbs with high affinity and specificity for widespread use as experimental, diagnostic, and therapeutic reagents. rDNA techniques and Ab display technologies have facilitated the production of VLRL mAbs and revealed several advantages, including broad pH and temperature stability, lack of tolerance to mammalian Ags, and modular, single peptide composition that make them an attractive alternative to mammalian V H V L mAbs. Although artificial alternatives to Ig Abs have been constructed using protein A domains, ankyrin repeats, fibronectin domains, and lipocalin scaffolds among others (57), VLRS are the only naturally occurring Ig alternative for which the Ag recognition proficiency has been honed by hundreds of millions of years of natural selection. Only 6 y after their discovery, VLRs already have demonstrable biotechnology potential, and the quest to exploit these ancient Abs has just begun.

Disclosures

The authors have no financial conflicts of interest.

References


