

ARTICLES

Dual nature of the adaptive immune system in lampreys

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Jawless vertebrates use variable lymphocyte receptors (VLR) comprised of leucine-rich-repeat (LRR) segments as counterparts of the immunoglobulin-based receptors that jawed vertebrates use for antigen recognition. Highly diverse VLR genes are somatically assembled by the insertion of variable LRR sequences into incomplete germline VLRA and VLRB genes. Here we show that in sea lampreys (*Petromyzon marinus*) VLRA and VLRB anticipatory receptors are expressed by separate lymphocyte populations by monoallelic VLRA or VLRB assembly, together with expression of cytosine deaminase 1 (CDA1) or 2 (CDA2), respectively. Distinctive gene expression profiles for VLRA⁺ and VLRB⁺ lymphocytes resemble those of mammalian T and B cells. Although both the VLRA and the VLRB cells proliferate in response to antigenic stimulation, only the VLRB lymphocytes bind native antigens and differentiate into VLR antibody-secreting cells. Conversely, VLRA lymphocytes respond preferentially to a classical T-cell mitogen and upregulate the expression of the pro-inflammatory cytokine genes interleukin-17 (IL-17) and macrophage migration inhibitory factor (MIF). The finding of T-like and B-like lymphocytes in lampreys offers new insight into the evolution of adaptive immunity.

The ability to mount specific immune responses to a virtually unlimited variety of antigens is apparently unique to vertebrates. The adaptive immune system in jawed vertebrates is characterized by antigen-specific cellular and humoral responses, which together provide durable protective immunity¹. This division of labour is conducted by developmentally separate, but functionally intertwined lineages of clonally diverse lymphocytes, named T and B cells because they are generated in the thymus or the avian bursa of Fabricius and mammalian bone marrow^{2,3}. For antigen recognition, both populations use the same type of immunoglobulin-based receptors. The T and B cell receptors (TCR and BCR, respectively) are assembled during lymphocyte differentiation by the recombination of different variable (V), diversity (D) and joining (J) immunoglobulin gene segments, imprecise V(D)J splicing, and insertion of non-templated nucleotides at the junctions⁴⁻⁷. The assembly process is usually completed only on one allele, so that each T and B cell expresses a different antigen recognition receptor as the basis for clonal diversity⁸. Although the TCR and BCR genes are remarkably similar, the antigen-binding repertoires of T and B cells are very different, primarily because immature T cells undergo clonal selection on the basis of how well they recognize major histocompatibility complex (MHC) class I or class II molecules and their self-peptide cargos⁹⁻¹². This intrathymic selection serves to eliminate T cells that can recognize and damage self tissues. The ensuing collaboration between T and B cells to achieve effective cellular and humoral immunity involves direct cell contact and indirect communication via cytokines and their receptors¹.

When and how this complex adaptive immune system arose are subjects of much conjecture. Even the most basal jawed vertebrates, the cartilaginous sharks, skates and rays, have a thymus in which T-cell progenitors assemble their TCR genes, and haematopoietic tissues in which B cell progenitors assemble their BCR genes^{13,14}. Although the extant jawless vertebrates lampreys and hagfish were noted previously to have lymphocyte-like cells and to produce antigen-specific agglutinins after immunization, neither a recognizable thymus, nor TCR, immunoglobulin or MHC genes have been found¹⁵⁻¹⁸. More recent studies indicate that these agnathans generate clonally diverse anticipatory receptors by

the assembly of genes for VLRs comprised of LRR subunits and an invariant membrane-proximal stalk region¹⁹. The two germline VLR genes, VLRA and VLRB, are incomplete in that they have coding sequences only for portions of the amino- and carboxy-terminal LRR subunits and for the stalk region¹⁹⁻²³. However, each germline VLR gene is flanked by hundreds of LRR-encoding sequences, and these are randomly used as templates to add the missing LRR segments needed for completion of a mature VLR gene²². A gene conversion mechanism has been postulated for VLR assembly^{21,23,24}, and candidate activation-induced deaminase (AID)/APOBEC participants have been identified in the lamprey²¹. VLRB assembly has been shown to be confined to one allele^{19,23}, ensuring that each VLRB lymphocyte expresses a receptor of unique sequence, but it is not yet known whether this is also true for VLRA.

In earlier studies, we found that the VLRB-bearing lymphocytes resemble B lymphocytes of jawed vertebrates. They can bind bacterial or erythrocyte antigens and respond by proliferation and differentiation into plasmacytes that secrete multimeric VLRB antibodies specific for protein or carbohydrate epitopes^{25,26}. In this study, we used VLRA-specific antibodies to identify a second population of lamprey lymphocytes and to determine their VLR gene assembly status, gene expression profile, tissue distribution, morphology and functional responses to antigens and mitogens in comparison with the VLRB lymphocytes. Our findings indicate that VLRA lymphocytes are surprisingly similar to the T cells in jawed vertebrates, and suggest functional interactions between the VLRA and VLRB lymphocyte populations.

Identification of VLRA⁺ and VLRB⁺ lymphocyte populations

The invariant stalk regions of the lamprey VLRA and VLRB proteins share less than 11% amino acid sequence identity, a difference that allowed the production of VLRB-specific monoclonal antibodies for use in characterizing the VLRB-bearing lymphocytes^{22,25}. To identify VLRA-bearing lymphocytes, we used a VLRA-immunoglobulin Fc fusion protein as an immunogen to produce two rabbit antisera and three mouse monoclonal antibodies. The VLRA specificity of the antibody preparations was established by comparative analysis with a

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VLRB monoclonal antibody in ELISA and immunoblot assays (Supplementary Fig. 1). Using these VLR-specific antibodies for immunofluorescence staining assays, we could identify discrete VLRA⁺ and VLRB⁺ lymphocyte populations in the principal lymphoid tissues of lamprey larvae: the blood, kidneys, typhlosole and gill region (Fig. 1a). VLRA-bearing lymphocytes were detected in the same relative frequencies by each of the VLRA-specific reagents (Supplementary Fig. 2). Notably, the VLRB⁺ lymphocytes significantly outnumbered VLRA⁺ lymphocytes in all tissues, except the gill region. The VLRB predominance was most marked in the blood and kidneys, in which the VLRB:VLRA ratio was approximately 8:1 (Fig. 1b); examination of adult blood samples indicated persistent VLRB predominance. These findings indicate that VLRA- and VLRB-bearing cells belong to separate lymphocyte populations that are dispersed throughout the body in a characteristic tissue distribution pattern.

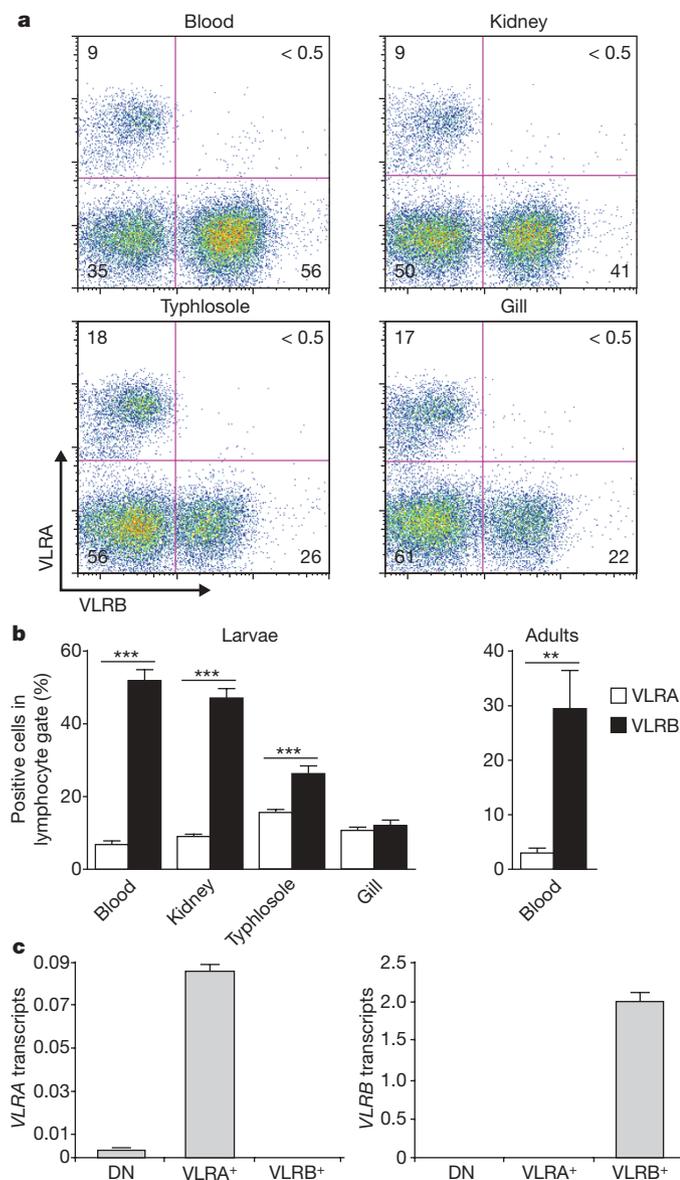


Figure 1 | VLRA and VLRB expression define distinct lymphocyte populations. **a**, FACS analysis of lymphocyte-gated cells stained with anti-VLRA (R110) and anti-VLRB (4C4) antibodies. **b**, Percentages of VLRA- and VLRB-expressing lymphocytes in lamprey tissues. Larvae (left) $n = 23$, except gill $n = 6$; adults (right) $n = 8$; $***P < 0.01$ and $***P < 0.001$. **c**, Lymphocyte-gated cells were stained as in **a** and separated on the basis of surface expression of VLRA and VLRB by FACS. Sorted cells were analysed for VLRA (left) and VLRB (right) transcripts by quantitative real-time PCR, $n = 3$. Error bars indicate s.e.m.

Genetic basis of VLRA versus VLRB expression

To explore the genetic basis for the differential expression of VLR types by the two lymphocyte populations, we used fluorescence-activated cell sorting (FACS) to purify VLRA⁺, VLRB⁺ and VLRA⁻ VLRB⁻ double-negative populations of cells having the light scatter characteristics of lymphocytes. The VLRA⁺ and VLRB⁺ lymphocytes could be separated cleanly (>95% purity), whereas the double-negative population included several cell types, most of which were nucleated thrombocytes. When VLR transcription was evaluated for each population by quantitative PCR (qPCR) analysis, the VLRA⁺ lymphocytes expressed VLRA transcripts exclusively, VLRB⁺ lymphocytes expressed VLRB transcripts exclusively, and the double-negative population expressed few or no VLR transcripts of either type. A representative experiment illustrating these results for adult blood cells is shown in Fig. 1c, and the same pattern of selective VLRA and VLRB expression was observed for the sorted populations of blood, kidney and typhlosole cells from larval stage lampreys 3–4 years of age (Supplementary Fig. 3).

When VLRA and VLRB gene configurations were examined for the VLRA⁺, VLRB⁺ and double-negative cells using paired 5' and 3' PCR primers (Fig. 2a, b), we observed mature VLRA assembly only in the VLRA⁺ lymphocyte population, and the same relationship held for VLRB assembly by VLRB⁺ lymphocytes. Mature and germline VLRA PCR products were observed in equal proportions (Supplementary Fig. 4), indicating that VLRA assembly occurs on one allele, a finding that parallels the monoallelic pattern of VLRB assembly demonstrated previously^{19,23}. Because the lamprey AID-like deaminases have been postulated to function in the gene conversion mechanism postulated for VLR gene assembly²¹, we examined the expression of CDA1 and CDA2 in the sorted cell populations. Notably, CDA1 expression was observed only in VLRA⁺ cells, whereas CDA2 expression was limited to the VLRB⁺ population (Fig. 2c). These findings suggest that the monoallelic VLRA versus VLRB gene assembly in the two lymphocyte populations is linked to expression of the genes for the CDA1 or CDA2 deaminases, respectively.

Distinctive gene expression profiles

Examination of the VLRA⁺, VLRB⁺ and double-negative populations for expression of a selected panel of genes indicated that they have different gene expression profiles, and the distinctions were particularly informative for the two lymphocyte populations. The discriminating genes expressed by VLRA and VLRB lymphocyte populations included genes for transcription factors, Notch1, cytokine/chemokines and their receptors, Toll-like receptors (TLRs), and various signalling molecules (Fig. 3, Supplementary Tables 1–3 and Supplementary Fig. 5). A surprising number of the genes that were selectively expressed by VLRA lymphocytes are orthologues of genes that are typically, although not exclusively, expressed by T lineage cells in jawed vertebrates. The genes expressed preferentially by VLRA lymphocytes included: several transcription factors that may be used for T-cell differentiation such as GATA2/3 (refs 27, 28), c-Rel²⁹, aryl hydrocarbon receptor (AHR)³⁰ and BCL11b³¹, the CCR9 chemokine receptor involved in lymphocyte progenitor homing to the thymus³², the T-cell fate-determining molecule Notch1 (ref. 33), the tyrosine phosphatase receptor protein CD45 that is essential for T-cell development³⁴, the IL-8 receptor CXCR2 (ref. 35), and two pro-inflammatory cytokines made by T cells—IL-17 (refs 36, 37) and MIP^{38,39}. The contrasting gene expression profile noted for the VLRB population included: the gene for the haematopoietic progenitor homing receptor CXCR4 (ref. 40), the herpesvirus entry mediator/tumour necrosis factor receptor superfamily, member 14 (TNFRSF14) that binds to LIGHT on T cells⁴¹, two components of the BCR-mediated signalling cascade, Syk⁴² and the B cell adaptor protein (BCAP)⁴³, the immunoglobulin superfamily member TCRL that has inhibitory potential⁴⁴, the chemotactic inflammatory cytokine IL-8 (ref. 45), the IL-17 receptor³⁶, and the Toll-like receptor orthologues TLR2abc, TLR7 and TLR10, the ligation of which is important for B cell activation^{46–48}. This analysis, albeit limited by the incomplete status of the lamprey

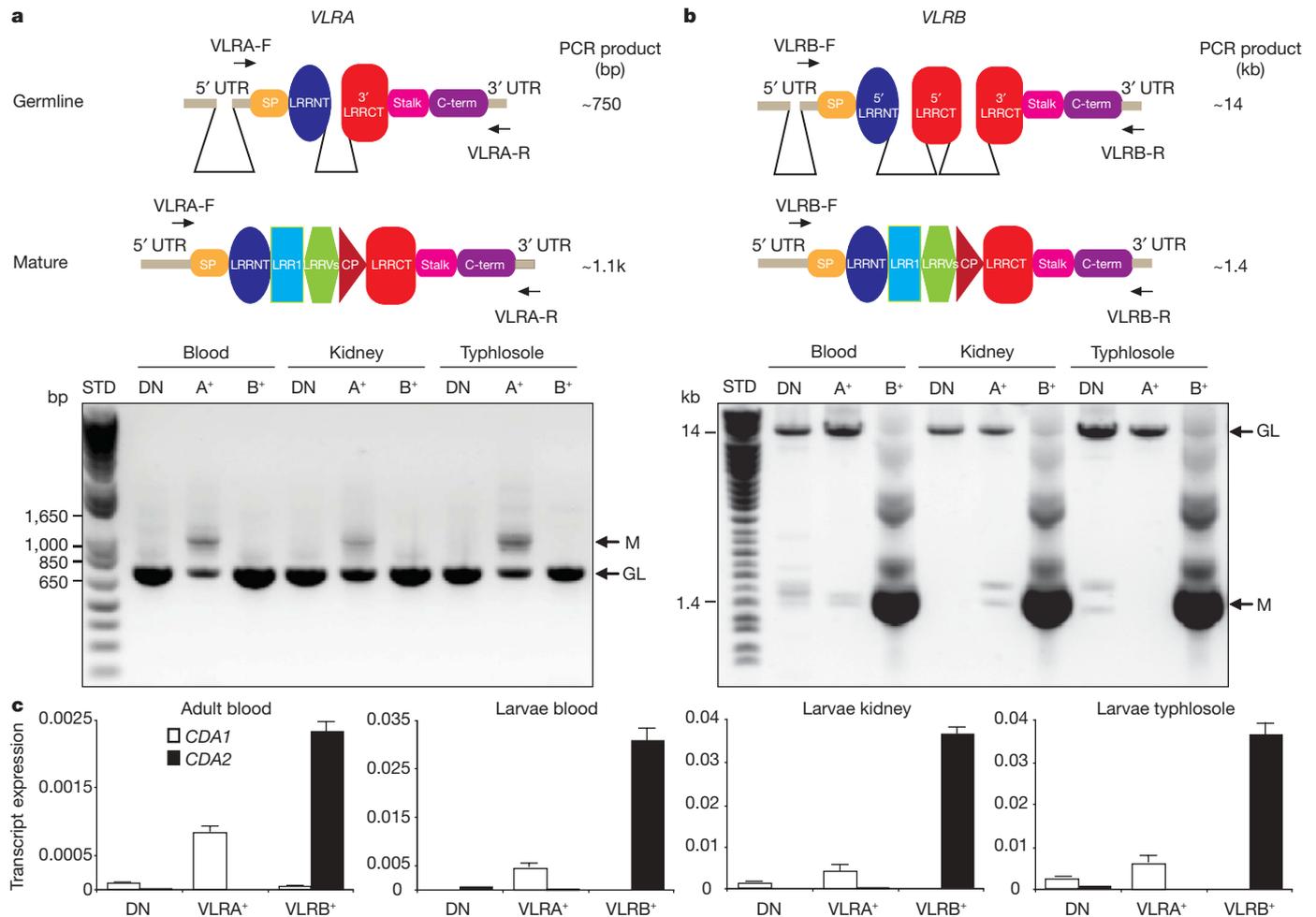


Figure 2 | Monoallelic assembly of *VLRA* and *VLRB* genes. **a, b**, Schematic of *VLRA* (**a**) and *VLRB* (**b**) genes before (top) and after (middle) gene assembly. Forward (F) and reverse (R) primer locations and the predicted sizes of PCR products are indicated. Lymphocytes from the indicated tissues were stained with anti-*VLRA* (R110) and anti-*VLRB* (4C4) antibodies, and lymphocyte-gated cells were FACS sorted into three populations:

VLRA⁻ *VLRB*⁻ (DN), *VLRA*⁺ (A⁺) and *VLRB*⁺ (B⁺). *VLRs* were amplified from genomic DNA of the sorted lymphocyte populations (bottom). Germline (GL) and mature (M) products were verified by sequence analysis of representative DNA clones. bp, base pairs; kb, kilobases. **c**, *CDA1* and *CDA2* expression in sorted lymphocytes was measured by qPCR. Error bars indicate s.e.m., n = 3.

genomic sequence analysis, reveals gene expression profiles for *VLRA* and *VLRB* lymphocytes that are surprisingly similar to those for T and B cells in jawed vertebrates.

VLRA and *VLRB* lymphocyte responses to antigens

In response to immunization with *Bacillus anthracis* exosporium, antigen-binding *VLRB*⁺ members of the lymphocyte population undergo proliferation and differentiation into plasmacytes that secrete multivalent *VLRB* antibodies against BclA, a major protein in the outermost layer of *B. anthracis* spores²⁵. In the present experiments, we found that both the *VLRA* and *VLRB* lymphocyte populations responded to this antigen with comparable levels of proliferation (Fig. 4a). In contrast, *VLRA* lymphocytes that could bind anthrax spores were not demonstrable either before or after immunization (Fig. 4b); the same paradoxical results were obtained when *Escherichia coli* binding by *VLRA* and *VLRB* lymphocyte was examined before and after immunization (data not shown). Furthermore, the *VLRA* population failed to secrete *VLRA* proteins that could bind *B. anthracis*, *E. coli*, *Salmonella typhimurium* or *Streptococcus pneumoniae* after immunizations that led to the secretion of specific *VLRB* antibodies for each of these bacteria (Fig. 4c and Supplementary Fig. 6). Furthermore, *VLRA* proteins could not be detected in lamprey plasma by immunoblot analysis (Fig. 4d and Supplementary Fig. 7).

To determine whether an inherent difference in the two types of receptors could explain the failure to secrete *VLRA*, we transfected cells

of the 293T human embryonic kidney cell line with either *VLRA* or *VLRB* complementary DNAs to compare their patterns of intracellular synthesis and secretion. Although the *VLRB*-transfected cells secreted their *VLRB* protein products in the multimeric forms described previously²⁶, the *VLRA*-transfected cells expressed their *VLRA* protein products on the cell surface, but did not secrete them (Supplementary Fig. 8). These results indicate that *VLRA* lymphocytes respond to antigenic stimulation in a manner very reminiscent of the T-cell response, and confirm that *VLRB* lymphocytes respond to antigens much like B lymphocytes. In addition, the contrast between the abilities of *VLRA* and *VLRB* lymphocytes to bind native bacterial surface epitopes strongly suggests that they have different receptor repertoires.

Preferential PHA responsiveness of *VLRA* lymphocytes

Because of the resemblance between the *VLRA* lymphocytes and T lymphocytes in jawed vertebrates, we compared the responses of *VLRA* and *VLRB* lymphocytes to stimulation by the plant lectin phytohaemagglutinin (PHA), a classical T-cell mitogen. When larvae were injected with PHA, the *VLRA*⁺ lymphocytes responded vigorously by undergoing lymphoblastoid transformation and proliferation to become the predominant lymphocyte population (Fig. 5a, b). The activated *VLRA*⁺ cells were found by transmission electron microscopy to be large lymphoblasts with much less rough endoplasmic reticulum than the previously described *VLRB*-secreting plasmacytes (Fig. 5c)²⁵. Although *VLRB* lymphocytes also responded to PHA stimulation, their

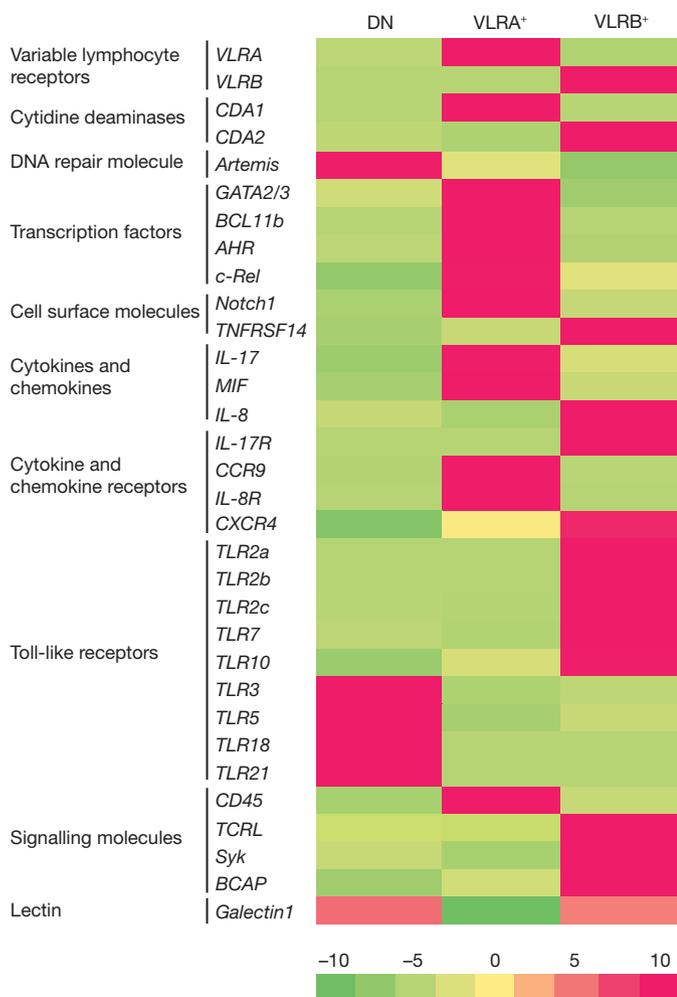


Figure 3 | Differential gene expression profiles of VLRA⁺ and VLRB⁺ lymphocytes. Lamprey lymphocytes were FACS sorted into three populations on the basis of VLRA and VLRB surface expression. Relative transcript levels of the indicated genes were measured by qPCR and compiled into a heat map as described in Methods.

response was much weaker in terms of cell size increases and the numbers of proliferating cells in this population. Nevertheless, PHA stimulation enhanced VLRB cell expression of *IL-8* transcripts, in contrast to its enhancement of VLRA cell expression of *IL-17* and *MIF* transcripts (Fig. 5d). These findings, coupled with the reciprocal expression of *IL-8R* by VLRA lymphocytes and *IL-17R* expression by VLRB lymphocytes (Fig. 3), are indicative of the potential for functional interactions between the two lymphocyte populations.

Discussion

This unforeseen genotypic and functional division of lymphocyte differentiation in lampreys offers an intriguing new piece to the puzzle of how the adaptive immune system may have evolved. Our initial definition of the VLRA and VLRB lymphocyte populations also offers interesting clues about key aspects of their differentiation and function, which are at present unresolved.

A question that is immediately raised by our findings concerns the anatomical location(s) in which VLRA lymphocytes begin their differentiation. Indeed, one of the reasons why the remarkable resemblance of VLRA lymphocytes to T lymphocytes was so surprising is the thus far fruitless quest to identify a thymus equivalent in lampreys^{16,17}. However, the relatively high concentration of VLRA lymphocytes in the gill region could favour the branchial region as a potential site for their generation. Furthermore, our gene profile analysis indicates that VLRA lymphocytes express an array of transcription factors,

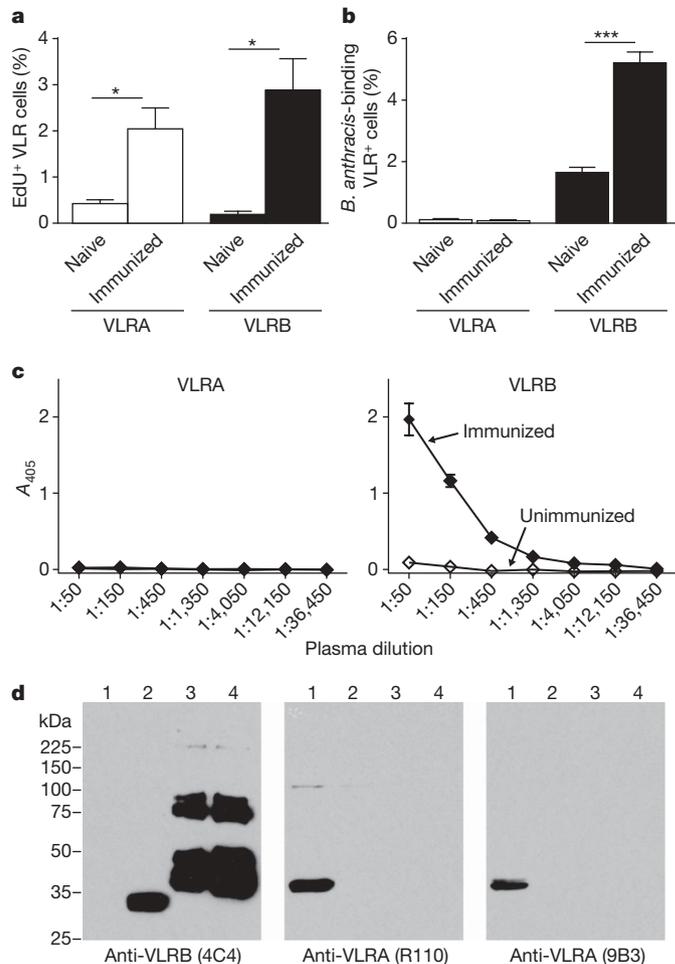


Figure 4 | Antigen-activated VLRA⁺ lymphocytes do not secrete their receptors. **a**, Immunization with *B. anthracis* exosporium induces proliferation of VLRA⁺ and VLRB⁺ lymphocytes; $n = 5$; $*P < 0.05$. EDCU, 5-ethynyl-2'-deoxyuridine. **b**, VLRA⁺ lymphocytes do not bind to *B. anthracis* spores before immunization or 14 days after booster immunization; $n = 5$; $***P < 0.001$. **c**, **d**, VLRB, but not VLRA, is secreted. **c**, *Bacillus-anthracis*-specific VLRA and VLRB reactivity was evaluated by ELISA with anti-VLRA (9A6) and anti-VLRB (4C4) monoclonal antibodies. **d**, Immunoblot of VLRA (lane 1) and VLRB (lane 2) HEK-293T cell transfectant lysates, naive lamprey plasma (lane 3), and immunized plasma (lane 4) under reducing conditions. 9B3 denotes anti-VLRA monoclonal antibody; R110 denotes anti-VLRA rabbit antiserum. Error bars indicate s.e.m.

chemokine receptors and Notch1 that lymphocyte progenitors use for homing to the thymus and T lineage commitment in jawed vertebrates. Analysis of the expression of the corresponding chemokines and Notch1 ligands in the pharyngeal pouch-derived epithelial cells and the neighbouring VLRA lymphocytes could therefore facilitate the identification of a thymus equivalent in lampreys.

Solving the issue of where the VLRA and VLRB genes are assembled will also require better understanding of the molecular mechanisms involved in this process. The donor and recipient LRR sequences used in VLR assembly lack recombination signal sequences that characterize the immunoglobulin variable, diversity and joining gene segments, and the RAG1 and RAG2 recombination activation genes used for TCR and BCR gene assembly have not been found in lampreys¹⁸. The features of VLR gene assembly known at present instead suggest that a gene conversion mechanism is used to copy the donor LRR sequences^{21,23,24}. In accord with this hypothesis, two AID/APOBEC family orthologues, CDA1 and CDA2, have been identified in the lamprey. CDA1 has been shown to have an AID-like DNA deaminase mutator function in bacteria and yeast lacking uracil DNA glycosylase activity²¹. Although the functional competency of CDA2 has not yet been elaborated, it shares the DNA deaminase catalytic motif with

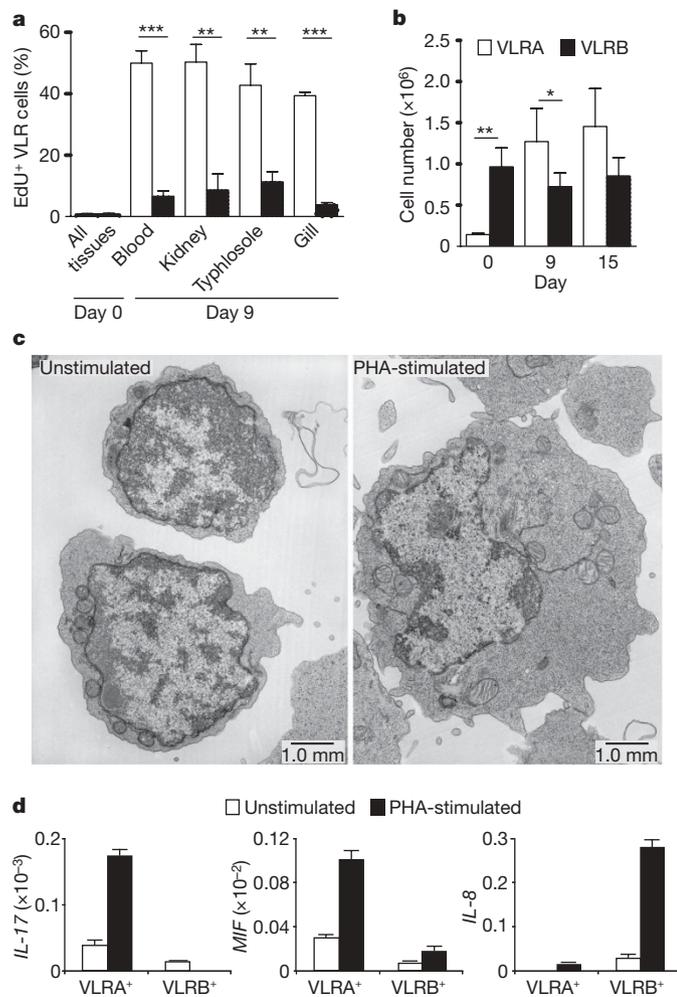


Figure 5 | PHA preferentially stimulates VLRA⁺ lymphocytes. **a, b**, *In vivo* PHA stimulation. Lampreys were given 25 μg of PHA by intracoelomic injection on day 0. Proliferation (**a**) and absolute numbers (**b**) of VLRA⁺ and VLRB⁺ cells were determined on days 9 and 15 after injection; $n = 4$ (**a**) and 7 (**b**); * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. **c**, Transmission electron microscopy imaging of unstimulated (left panel) and PHA-stimulated (day 9) (right panel) VLRA⁺ cells. PHA-stimulated cells have a larger diameter (mean \pm s.e.m., naive = 3.8 ± 0.1 μm ($n = 7$) versus PHA-stimulated = 7.4 ± 0.3 μm ($n = 7$, $P < 0.01$)) and increased cytoplasm. **d**, Cytokine expression by VLRA⁺ and VLRB⁺ lymphocytes. FACS-sorted blood lymphocytes were isolated from PHA-stimulated (day 7, $n = 3$) or unstimulated lampreys ($n = 3$), and analysed for expression of *IL-17* (left panel), *MIF* (middle panel), and *IL-8* (right panel) by qPCR. Error bars indicate s.e.m.

CDA1 and also has an extended C terminus containing a small AT-hook peptide motif that may confer DNA binding specificity. These distinctive features, and the restriction of CDA1 and CDA2 expression to VLRA and VLRB lymphocytes, respectively, thus imply the potential for selective involvement of these enzymes in VLRA and VLRB assembly during lymphocyte development.

One of the most provocative issues raised by our findings concerns the mode of antigen recognition by VLRA-bearing lymphocytes. We expected to find comparable antigen binding and response patterns for the two lymphocyte populations, because the VLRA and VLRB proteins are very similar and have comparable diversity. The proliferative responses of VLRA and VLRB lymphocytes to immunization indeed were found to be equivalent. Surprisingly however, antigen-binding VLRA lymphocytes could not be found before or after immunization, whereas specific antigen-binding VLRB lymphocytes were easily detected in naive animals, and immunization increased their frequency. A possible explanation for the inability of VLRA lymphocytes to bind native epitopes on immunogens that induce

their proliferation is that VLRA lymphocytes recognize processed antigens and undergo selection in a manner analogous to the T-lymphocyte repertoire selection in jawed vertebrates. Lampreys lack the MHC genes for MHC class I and class II molecules that accessory cells use to present peptide fragments to T cells in jawed vertebrates¹⁸. However, a precedent for an alternative mechanism for self recognition is provided by the recent identification of a highly polymorphic fusion/histocompatibility gene locus in *Botryllus schlosseri*⁴⁹, a colonial tunicate member of a vertebrate sister group in the new chordate phylogeny⁵⁰. The nature of the antigens recognized by VLRA anticipatory receptors remains an unresolved enigma at present.

The failure of VLRA cells to secrete their receptors as soluble proteins was the most notable functional difference observed between the VLRA and VLRB lymphocytes. This feature could reflect an inability of VLRA cells to develop the necessary secretory machinery. However, when VLRA proteins were produced by a human cell line that can secrete VLRB proteins, the transfected cells produced cell surface VLRA proteins, but did not secrete them. The VLRA proteins, like the TCR proteins, thus seem to be expressed exclusively as transmembrane molecules. The preferential expression of *TLR2*, *TLR7* and *TLR10* orthologues by the VLRB lymphocytes suggests that TLR ligands may facilitate activation of this population of lymphocytes in a manner similar to their roles in activating B lymphocytes. The development of appropriate conditions for culturing lamprey lymphocytes and cell lines will facilitate the future analysis of the signalling mechanisms used by VLRA and VLRB lymphocytes.

Many of the elaborate cytokine networks that T and B cells use to coordinate effective inflammatory responses in jawed vertebrates are not evident in the more basal jawless vertebrates. Agnathan orthologues have not been identified for the interferons and interferon receptors *IL-2/IL-2R*, *IL-4/IL4R*, *IL-7/IL-7R* and other cytokine/cytokine receptor pairs. Nevertheless, the VLRA lymphocytes preferentially express *IL-17* and *MIF* transcripts, and their enhanced expression after activation suggests that VLRA lymphocytes may use these pro-inflammatory cytokines to engage the *IL-17R*-bearing VLRB lymphocytes for defence purposes, in a manner similar to their T cell counterparts. Conversely, activated VLRB lymphocytes upregulate their expression of *IL-8* transcripts, suggesting that they may use this cytokine to attract and engage *IL-8R*-bearing VLRA lymphocytes and other cell types to amplify the response to potential pathogens.

The evolution around 500 million years ago of two very different anticipatory receptors of comparable diversity in jawless and jawed vertebrates, while conserving similar compartmentalization of lymphocyte differentiation, strongly attests the survival value of adaptive immunity. The evolution of an LRR-based adaptive immune system in lampreys and hagfish, apparently before the second round of whole genomic replication, and an immunoglobulin-based adaptive immune system in jawed vertebrates, after a second round of genomic duplication⁵⁰, suggests that these very different types of anticipatory receptors were convergent evolutionary acquisitions¹⁹. The failure so far to find relics of the VLR genes in jawed vertebrates is also consistent with an independent evolution of clonally diverse LRR-based receptors versus immunoglobulin-based receptors for otherwise similar adaptive immune systems. An obvious advantage of adaptive immunity over the elaborate invertebrate strategies for innate immunity is the capacity for memory of previous encounters with infectious agents. On the other hand, the development of a randomly generated anticipatory repertoire of great diversity inevitably creates the hazard of self-reactivity and attendant autoimmunity. The orchestration of cellular and humoral immune responses by T cells, whose TCR repertoire is shaped during development to discriminate between self and non-self antigens, provides an important safeguard against autoimmunity in jawed vertebrates. The potential for autoimmunity theoretically would necessitate a similar solution for the adaptive immune system in jawless vertebrates. Our findings thus suggest that dual recognition and response arms with intertwined function were fundamental to the evolution of adaptive immunity in vertebrates.

METHODS SUMMARY

Sea lamprey larvae (8–15 cm, 2–4 years of age) were collected from tributaries to Lake Michigan (Lamprey services) and housed in sand-lined aquaria at 20 °C. Larvae anaesthetized with 0.1 g l⁻¹ MS222 (Sigma) were given intracoelomic injections of antigens or mitogens (25 µg phytohaemagglutinin (PHA)-L, Sigma) prepared in 60 µl of sterile 0.67× PBS. Injections were administered on days 0 and 14, and tissues were collected from lampreys euthanized with 1 g l⁻¹ MS222 on day 28 (unless otherwise indicated). Blood was collected in 30 mM EDTA, 0.67× PBS. Buffy coat leukocytes were separated from red blood cells by centrifugation at 50g. Leukocytes were isolated from kidney, typhlosole and gills by disrupting tissues between frosted glass slides.

For VLRA antibody production, four VLRA cDNAs isolated from lamprey lymphocytes were cloned in-frame with the constant region of human IgG1 (IgG1-Fc). The VLRA-IgG1-Fc fusion proteins were expressed in HEK-293T cells and purified from tissue culture supernatants by protein A (GE Healthcare) chromatography. Monoclonal anti-VLRA antibodies were produced by immunization of BALB/c mice with VLRA-IgG1-Fc proteins emulsified in TiterMax Gold adjuvant. Lymphocytes from the draining lymph nodes of immunized mice were fused with the Ag8.653 myeloma cell line using PEG-1500 (Roche). Three VLRA-specific hybridoma clones (9A6 (IgG1), 9B3 (IgG1) and 2A5 (IgG1)) were identified by ELISA with VLRA recombinant protein and immunoblotting of VLRA transfectants. Anti-VLRA polyclonal antisera were produced by immunization of rabbits with the VLRA-IgG1-Fc proteins (PickCell Laboratories BV).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 19 February; accepted 14 April 2009.

Published online 27 May 2009.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank M. N. Alder and G. R. A. Ehrhardt for suggestions and discussion; C. L. Turnbough, Jr for providing *B. anthracis* spores and exosporium; D. E. Briles and W. H. Benjamin, Jr for *E. coli*, *S. pneumoniae* and *S. typhimurium*; H. Yi for help with electron microscopy; S. A. Durham and R. E. Karaffa, II for help with cell sorting; M. Flurry for help with preparation of figures. This work is supported by the National Institutes of Health and the Georgia Research Alliance.

Author Contributions P.G., M.H., B.R.H., J.L., C.Y., A.S. and M.D.C. designed the research, analysed data and wrote the paper; P.G., M.H., B.R.H., J.L., C.Y. and A.S. performed the research.

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METHODS

Antigens. *Escherichia coli* (ATCC 25922 strain), *Salmonella enterica* Typhimurium (WB99-SR11 strain) and *Streptococcus pneumoniae* (R36A strain) were provided by D. E. Briles and W. H. Benjamin, Jr (University of Alabama at Birmingham). Bacteria were heat-killed by incubation at 60 °C for 1 h. Five-hundred-million heat-killed *E. coli* and *S. typhimurium*, and 1×10^8 *S. pneumoniae* were used for immunization. *Bacillus anthracis* (spores and exosporium) and *Bacillus cereus* spores were provided by C. L. Turnbough, Jr (University of Alabama at Birmingham). Lampreys were immunized with 10 µg of *B. anthracis* exosporium.

Flow cytometric analysis of VLRA and VLRB lymphocytes. Leukocytes from blood and tissues were stained with anti-VLRA rabbit polyclonal serum (R110 or R111) or mouse monoclonal antibodies (9A6, 9B3, 2A5, IgG1) and anti-VLRB mouse monoclonal antibody (4C4, IgG2b) for 10 min on ice, washed twice and stained with fluorescent-conjugated secondary antibodies. For anti-VLRA (IgG1) and anti-VLRB (4C4, IgG2b) antibody staining combinations, R-phycoerythrin (PE)-conjugated goat anti-mouse IgG1 antibodies (Southern Biotech) and FITC-conjugated goat anti-mouse IgG2b antibodies (Southern Biotech) were used. When staining with anti-VLRA rabbit serum and anti-VLRB monoclonal antibody (4C4), RPE-conjugated goat anti-rabbit Ig antibodies (Southern Biotech) and allophycocyanin (APC)-conjugated goat anti-mouse Ig antibodies (Southern Biotech) were used. Cells were analysed on a CyAn ADP high-performance flow cytometer (Dako). Staining and washes were in 0.67× PBS with 1% BSA.

Immunoblotting. Samples were separated on 10% SDS-PAGE gels with or without 5% (v/v) 2-mercaptoethanol before transfer onto nitrocellulose membranes. Membranes were blocked overnight with 5% milk and incubated with anti-VLRA or anti-VLRB antibodies for 1 h. After five washes with PBS-0.5% Tween-20, membranes were incubated with HRP-conjugated goat anti-rabbit or mouse Ig polyclonal antibodies (Southern Biotech) and washed. Blots were developed using SuperSignal West Pico chemiluminescent substrate (Pierce).

Genomic DNA and PCR. Genomic DNA was extracted from VLRA⁺, VLRB⁺ and VLR double-negative-sorted cells in blood and tissue lymphocytes using DNeasy kit (QIAGEN). Genomic PCR was carried out using primers VLRA-F and VLRA-R (Expand High Fidelity, Roche, and Ex Taq, Takara) or VLRB-F and VLRB-R (Expand Long Template, Roche). Primers are listed in Supplementary Table 1.

Quantitative PCR. Target gene sequences were obtained from the National Center for Biotechnology Information database or the lamprey genome database of the Genome Sequencing Center at Washington University. Cells in the lymphocyte gate were sorted into VLRA⁺, VLRB⁺ and VLR double-negative populations on a BD FACS Aria II. RNA was extracted from each population using RNeasy kit with on-column DNA digestion by DNase I (QIAGEN). First-strand cDNA was synthesized with random hexamer primers and Superscript III (Invitrogen). Quantitative real-time PCR was done with SYBR Green on a 7900HT ABI Prism (Applied Biosystems). Three independent quantitative real-time PCR reactions were performed at different times for each sample. The value of the target gene was normalized to β-actin. The normalized value of each gene was compiled into a heat map (z -score $\times 10$). Magenta: $z > 0$, green:

$z < 0$ and yellow: $z = 0$ [$z = (\text{each value} - \text{average})/\text{s.d.}$]. Primers are described in Supplementary Table 1.

Proliferation assay. Lampreys stimulated with antigen or mitogen were injected with 5 µg of 5-ethynyl-2'-deoxyuridine (Invitrogen) in 60 µl 0.67× PBS and returned to their tanks for 24 h before collection of leukocytes for staining with anti-VLRA or anti-VLRB antibodies. Stained cells were fixed in 4% paraformaldehyde for 15 min at room temperature, then resuspended in 100 µl of the 1× saponin-based permeabilization and wash buffer for 10 min on ice. EdU⁺ cell detection was performed according to the manufacturer's protocol (Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit, Invitrogen). In brief, cells were incubated with the EdU reaction cocktail, which contains Alexa Fluor 488-azide, for 30 min at room temperature, washed twice in 1× saponin-based permeabilization and wash buffer, and analysed by flow cytometry.

ELISA. For animals immunized with *B. anthracis* exosporium, 10^6 *B. anthracis* spores or *B. cereus* strain T spores per well were applied on poly-L-lysine-coated plates as described previously²². For animals immunized with bacteria, 5×10^6 heat-killed bacteria per well were applied on poly-L-lysine-coated plates. Plates were blocked with 1% BSA in PBS for 1 h. Serially diluted plasma was applied for 2 h at 37 °C. VLRA were detected with anti-VLRB (4C4) or anti-VLRA (9A6) monoclonal antibodies and alkaline phosphatase-conjugated goat anti-mouse Ig antibodies (Southern Biotech). ELISAs were developed with phosphatase substrate (Sigma) and read at 405 nm (Versamax microplate reader, Molecular Devices).

Lymphocyte antigen-binding assay. One million lamprey leukocytes were incubated with 10^6 Alexa-Fluor-488-labelled spores or 10^7 Alexa-Fluor-488-labelled bacteria for 20 min on ice. Spores and bacteria were labelled using the Alexa Fluor 488 Protein Labelling Kit (Invitrogen). Cells were stained with anti-VLRA (R110) and anti-VLRB (4C4) antibodies followed by APC-conjugated goat anti-mouse Ig antibodies (Southern Biotech) and RPE-conjugated goat anti-rabbit Ig antibodies (Southern Biotech), then analysed by flow cytometry.

VLRA expression in HEK-293T cells. VLRA and VLRB plasmids were transfected into HEK-293T cells cultured in DMEM containing 5% FBS using linear polyethylenimine (PEI), MW 25,000 (Polysciences, Inc.) at a 3:1 PEI:DNA ratio. Cells were separated from supernatants 48 h after transfection by centrifugation at 300g and lysed in 1% NP-40 lysis buffer.

Lymphocyte counts. Lymphocytes were extracted from lamprey kidney and typhlosol by passage through a 70-µm cell strainer (BD Bioscience) to obtain a single cell suspension. Cells were washed and resuspended in 1 ml 0.67× PBS. Total cells were counted in 25 µl of each sample on an Accuri C6 flow cytometer (Accuri Cytometers Inc.). The total number of cells in the 'lymphocyte gate' was calculated by the formula: total lymphocyte number = number of cells in the lymphocytes gate $\times 40$ (dilution factor: 1,000 µl/25 µl). Lymphocytes were then stained with anti-VLRA and -VLRB antibodies to determine the percentage of VLRA⁺ and VLRB⁺ cells in the samples. These percentages were used to calculate the total number of lymphocytes of each type.

Electron microscopy. Blood lymphocytes from naive or PHA-stimulated lamprey were sorted on BD FACS Aria II (BD Bioscience). The VLRA-positive cells were prepared for transmission electron microscopic analysis as described²².

Statistical analysis. A two-sample Student's *t*-test was used for statistical analysis.

CORRIGENDUM

doi:10.1038/nature08354

Dual nature of the adaptive immune system in lampreys

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Nature 459, 796–801 (2009)

In this Letter, the scale bars in Fig. 5c are incorrectly listed as 1 mm, the correct size of the scale bars is 1 μm .