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Evolutionary implications of a third lymphocyte lineage in lampreys

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Abstract

Jawed vertebrates (gnathostomes) and jawless vertebrates (cyclostomes) have different adaptive immune systems^{1,2}. Gnathostomes use T- and B-cell antigen receptors belonging to the immunoglobulin superfamily^{3,4}. Cyclostomes, the lampreys and hagfish, instead use leucine-rich repeat proteins to construct variable lymphocyte receptors (VLRs), two types of which, VLRA and VLRB, are reciprocally expressed by lymphocytes resembling gnathostome T and B cells^{5–7}. Here we define another lineage of T-cell-like lymphocytes that express the recently identified VLRC receptors^{8,9}. Both VLRC⁺ and VLRA⁺ lymphocytes express orthologues of genes that gnathostome $\gamma\delta$ and $\alpha\beta$ T cells use for their differentiation, undergo VLRC and VLRA assembly and repertoire diversification in the ‘thymoid’ gill region, and express their VLRs solely as cell-surface proteins. Our findings suggest that the genetic programmes for two primordial T-cell lineages and a prototypic B-cell lineage were already present in the last common vertebrate ancestor approximately 500 million years ago. We propose that functional specialization of distinct T-cell-like lineages was an ancient feature of a primordial immune system.

The invariant stalk region of the sea lamprey VLRC shares approximately 20% sequence identity with the invariant VLRA and VLRB stalk regions, and distinguishing VLRC sequences are also present in the amino-terminal and carboxy-terminal leucine-rich repeat regions^{8,9}. Four mouse monoclonal antibodies specific for the VLRC protein were produced, all of which identified a third lymphocyte population in lampreys that did not express VLRA or VLRB (Fig. 1a and Supplementary Fig. 1). VLRC⁺ lymphocytes were more numerous than VLRA⁺ lymphocytes in the principal lymphoid tissues of lamprey larvae (blood, kidneys, typhlosole and gill region) and they constituted the majority of lymphocytes in typhlosole and gills, whereas VLRB⁺ lymphocytes predominated in blood and kidneys (Fig. 1a).

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Immunofluorescence analysis of tissue sections indicated a similar distribution pattern for VLRA⁺ and VLRC⁺ lymphocytes in the typhlosole (Fig. 1b), kidneys (Fig. 1c), gills (Fig. 1d) and hypopharyngeal fold (Fig. 1e). Both cell types were round or oval in shape within blood vessels and interstitial spaces of the kidneys and typhlosole, but were dendritic in shape in the gill and intestinal epithelium (see inset in Fig. 1d) where the VLRC⁺ cells were more numerous (VLRC/VLRA ratio of approximately 1.7/1) (Fig. 1b, f) and VLRB⁺ cells were infrequent. VLRC⁺ cells with inter-digitating morphology were the dominant lymphocyte type in the epidermal region of the skin (VLRC/VLRA ratio of 8/1) (Fig. 1f, g), a site in which VLRB⁺ cells were rarely observed. The predominance of VLRC⁺ cells in the epidermis is reminiscent of the dendritic epidermal T cells in mice, which express the same canonical $\gamma\delta$ T-cell receptor (TCR)¹⁰. When VLRC sequences for the VLRC⁺ cells in different tissues were compared, repetitive VLRC sequences were abundant in the skin, but rare in kidney and blood samples (Fig. 1h). We found several examples of identical or almost identical VLRC sequences in skin samples from different animals (Supplementary Fig. 2), suggesting that the VLRC repertoire in the skin is less diverse and more stereotypic than elsewhere. Importantly, restricted diversity was not observed for VLRA sequences isolated from the same skin samples (Fig. 1i).

Antigen-binding VLRB⁺ lymphocytes respond to immunization with proliferation and differentiation into plasma cells that secrete VLRB antibodies^{11,12}. VLRA⁺ lymphocytes also proliferate in response to immunization, but fail to bind unmodified immunogens, either before or after immunization, and do not differentiate into VLRA-secreting cells⁵. Proliferative responses to *Bacillus anthracis* exosporium were also observed for VLRC⁺ lymphocytes (Fig. 2a and Supplementary Fig. 3a). The VLRC⁺ lymphocytes also responded to the plant mitogen phytohaemagglutinin (PHA) with vigorous proliferation (Fig. 2b) and increased cell numbers (Fig. 2c and Supplementary Fig. 3b) and the activated VLRC⁺ cells resembled activated VLRA⁺ cells, in that they were large lymphoblasts with limited endoplasmic reticulum (Supplementary Fig. 3c). Nevertheless, plasma samples from naive or PHA-stimulated lampreys were devoid of VLRC protein (Fig. 2d) and VLRC transfectants did not secrete the VLRC protein (Supplementary Fig. 4).

Gene-expression profiles were compared for VLRA⁺, VLRB⁺, VLRC⁺ and triple-negative populations of cells with lymphocyte light-scattering characteristics by examining the expression levels of a selected panel of orthologous genes. Discriminating profiles that were observed for the different populations included genes for transcription factors, cytokines or chemokines and their receptors, integrins, Toll-like receptors (TLRs), and various signalling molecules (Fig. 3a and Supplementary Tables 1 and 2). This analysis confirmed the clear dichotomy of the VLRB⁺ B-like and the VLRA⁺ T-cell-like lymphocytes. Although the transcriptional profiles were more similar for VLRA⁺ and VLRC⁺ cells, they differed in certain aspects; genes were expressed preferentially by VLRA⁺ lymphocytes for the T-cell factor 1 (TCF1, also known as transcription factor 7), a key transcription factor for $\alpha\beta$ T-cell lineage determination, and CTLA4, an important regulatory co-receptor for mammalian T cells. Conversely, VLRC⁺ lymphocytes preferentially expressed the SRY-box containing gene 13 (SOX13) encoding a fate-determining transcription factor used for $\gamma\delta$ T-cell lineage commitment^{13,14}, an integrin α L (ITGAL) orthologue of one component of the heterodimeric lymphocyte function associated antigen 1 (LFA1), and integrins α 4 and β 1 (ITGA4 and ITGB1) orthologues of the two components of very late antigen 4 (VLA4), the expression of which was correlated with adherence of human $\gamma\delta$ T cells to epithelial cells¹⁵, TLR3 (ref. 16) and interleukin-16 (IL-16), a modulator of T-cell activation¹⁷.

The functional implication of the preferential TLR3 expression by lamprey VLRC⁺ lymphocytes was examined by comparing the VLRA⁺, VLRB⁺ and VLRC⁺ lymphocyte responses to poly(I:C), a structural mimic of double-stranded RNA. This analysis revealed a

preferential proliferative response by VLRC⁺ lymphocytes (Fig. 3b); VLRB⁺ lymphocytes also responded to a lesser extent. Poly(I:C) stimulation also enhanced the expression of *IL-16* transcripts by VLRC⁺ cells, while having only a slight effect on *IL-17* expression by the VLRA⁺ and VLRB⁺ cells and no effect on *IL-8* expression by VLRB⁺ cells (Fig. 3c). These results suggest that TLR3 expression by VLRC⁺ lymphocytes could potentially facilitate their participation in responding to infection with RNA viruses, akin to the modulation of $\gamma\delta$ T cell responses by TLRs in mammals¹⁸.

To determine whether VLRC assembly accompanies VLRA assembly in the thymus-equivalent region at the tips of the gill filaments¹⁹, we compared DNA samples obtained by laser capture micro-dissection of cells from the 'thymoid' region, which is characterized by ongoing CDA1 expression, and from blood where CDA1 expression is undetectable (Fig. 4a, b). Functional and non-functional VLRC assemblies were observed in both locations, although non-functional VLRC assemblies were present in higher frequency in the 'thymoid' region (14 non-functional out of 61 total distinct VLRC assemblies) than in blood (7 non-functional out of 100 total VLRC assemblies). Moreover, most of the non-functional VLRC sequences in cells within the 'thymoid' region consisted of incomplete intermediate-stage assemblies (9 out of 14), whereas incomplete VLRC assemblies were not found among the non-functional VLRC assemblies in blood (0 out of 7).

Transcription profiles for the VLR loci and their assembly status were examined in VLRA⁺, VLRB⁺, VLRC⁺ and triple-negative lymphocyte populations purified by fluorescence-activated cell sorting from pooled cell suspensions derived from the blood, gills, kidneys, typhlosole and skin of lamprey larvae. Each sample was divided into two aliquots, which were used for genomic DNA and RNA transcript analysis. The polymerase chain reaction (PCR) products for the assembled or mature VLRA and VLRC genes were larger than those for the germline genes (see Fig. 4c), thus allowing their discrimination, purification and sequence analysis. Both germline and assembled VLRB genes were transcribed in VLRB⁺ cells, whose VLRA and VLRC genes were transcriptionally silent (Fig. 4d). Conversely, VLRB transcription was not detected in VLRA⁺ or VLRC⁺ cells. However, both VLRA and VLRC loci were transcriptionally active in VLRA⁺ and VLRC⁺ lymphocytes. Hence, the expression of VLRA and VLRC genes was not lineage-specific, in contrast to the distinct cell surface phenotypes of VLRA⁺ and VLRC⁺ lymphocytes.

Consistent with previous studies indicating that mono-allelic assembly is the rule for VLRA⁺ lymphocytes⁵, we observed an approximately 50/50 ratio of assembled versus non-assembled VLRA genes in VLRA⁺ cells (Fig. 4e) and sequence analysis indicated that 94% (99 out of 105) of the VLRA assemblies in VLRA⁺ cells were functional. VLRC assemblies were also detected in the VLRA⁺ cells (Fig. 4e), but 79% (46 out of 58) of these were non-productive assemblies. The VLRA and VLRC assembly status was more complex for the VLRC⁺ cells, as indicated by the 65/35 ratio of assembled versus non-assembled VLRC genes (Fig. 4d, e). The predominance of assembled gene products in VLRC⁺ cells suggested the presence of bi-allelic VLRC assemblies in a fraction of these cells, and approximately 15% (20 out of 132) of the VLRC assemblies in VLRC⁺ cells were non-productive. Conversely, VLRA assemblies were rarely detected in VLRC⁺ cells, and almost all of these were non-productive (54 out of 55). Most non-functional VLRA and VLRC sequences contained frame-shift mutations (Supplementary Fig. 5), and stop-codon mutations were less common. These composite results suggest a differentiation model in which bi-potential precursor cells begin to undergo VLRC or VLRA assembly in the 'thymoid', and the assembly process may proceed to involve both alleles and even both loci to achieve a productive VLRC or VLRA assembly in order to survive as receptor positive cells (Fig. 4f).

The present studies demonstrate that lampreys possess two T-cell-like lineages that exhibit mutually exclusive expression of their VLRA and VLRC surface proteins and have overlapping but distinct gene expression profiles. Their currently defined characteristics are reminiscent of the two principal T-cell lineages in jawed vertebrates. In several aspects, the gene-expression profiles of VLRA⁺ and VLRC⁺ cells resemble those of mammalian TCRαβ⁺ and TCRγδ⁺ T cells, although these lymphocyte lineages clearly cannot be considered to be precise equivalents. In addition to the convergent evolution of entirely different types of recognition receptors, other divergent modifications have almost certainly occurred during the approximately 500 million years of evolution since jawless and jawed vertebrates last shared a common ancestor. It will thus be particularly important to establish the degree of functional similarity between these T-cell lineages in future studies.

Our present results firmly establish the ‘thymoid’ region of the gill tips as the site of VLRA and VLRC assembly, and indicate that the epithelial microenvironment of this primary lymphoid organ provides the necessary signals to support the development of the two T-cell-like lineages of lymphocytes identified here. The thymoid thus parallels the jawed vertebrate thymus in its support of both TCRαβ⁺ and TCRγδ⁺ T-cell lineage differentiation. Incomplete intermediate VLRC assemblies were found only in ‘thymoid’ cells, as shown previously for VLRA assemblies¹⁹. These findings also indicate that VLRA and VLRC gene assemblies are both associated with *CDA1* expression in the ‘thymoid’ region of the gills, whereas *CDA2* expression and *VLRB* assembly occur in the haematopoietic typhosole and kidney tissues in lamprey larvae¹⁹. Our analysis of VLRA and VLRC expression and assembly in ‘thymoid’ cells and in VLRA⁺ and VLRC⁺ lymphocytes throughout the body suggests that bi-potent lymphoid progenitors undergo a complex pattern of VLRC versus VLRA assembly in which VLRC assembly precedes VLRA assembly in the ‘thymoid’.

The findings here reveal functional similarities and differences between the three lymphocyte lineages of lamprey larvae. Unlike VLRB⁺ cells, the VLRC⁺ and VLRA⁺ lymphocytes preferentially populate the pharyngeal and intestinal epithelium. Both thymoid-derived lymphocyte types can be activated by antigen stimulation or by the polyclonal mitogen PHA to undergo lymphoblastoid transformation and cell division, but do not differentiate into VLR-secreting cells, a chief difference to VLRB⁺ cells. Notably, the VLRC⁺ cells constitute the predominant lymphocyte population in the lamprey epidermis, and the repertoire of VLRC⁺ cells derived from skin samples is restricted relative to that for VLRC⁺ cells in other tissue sites. These interesting findings, which bring to mind the canonical γδ TCR expressed by dendritic epithelial T cells in mice¹⁰ and the antigen-specific αβ T cells that patrol the skin of mice after herpes virus infection²⁰, emphasize the need to elucidate the mode of antigen recognition by the VLRC- and VLRA-bearing cells.

In conjunction with the previously documented dichotomy of Band T-cell-like lymphocytes in lamprey, these results offer fresh insight into the evolution of the alternative adaptive immune systems of jawless and jawed vertebrates. Representative species of the two vertebrate lineages use different sets of genes and different combinatorial mechanisms to construct diverse antigen recognition receptors, but they show the same basic principle of lymphocyte differentiation along two distinct T-cell-like lineages and one B-cell-like lineage. These composite findings suggest that the basic genetic program regulating the developmental pathways of the three lymphocyte lineages must have already existed in a common ancestor for all vertebrates (Supplementary Fig. 6) and this constitutes a fundamental organizing principle for lymphocyte-based adaptive immune systems.

METHODS

Animals, antigens and mitogens

Two outbred species of lamprey larvae (*Petromyzon marinus* and *Lampetra planeri*) were captured in the wild and housed in sand-lined aquaria. *Bacillus anthracis* exosporium was provided by C. L. Turnbough Jr. Lampreys were immunized with 10 µg of *B. anthracis* exosporium. Animals were injected with 25 µg poly(I:C) (Sigma) or 25 µg phytohaemagglutinin (PHA-L, Sigma).

Production of anti-VLRC monoclonal antibodies

VLRC complementary DNA constructs⁹ were cloned into a fusion vector to produce chimaeric proteins of the VLRC antigen-binding domain and stalk region fused to the constant region of human immunoglobulin-G1 (IgG1). Lymphocytes from mice immunized with the chimaeric proteins were used for hybridoma production, and supernatants were screened for antibody reactivity against recombinant VLRC. Monoclonal antibodies specific for VLRC were used together with anti-VLRA and -VLRB antibodies for characterizing and purifying VLR-expressing cells.

ELISA

ELISA (enzyme-linked immunosorbent assay) plates were coated with VLRA-IgG1-Fc⁵ or VLRC-IgG1-Fc fusion proteins for 12 h at 4 °C and blocked with 1% BSA in PBS for 3 h at 37 °C. Hybridoma culture supernatants were added for 2 h at 37 °C. VLR reactivity of antibodies was detected using alkaline phosphatase-conjugated goat anti-mouse immunoglobulin antibodies (Southern Biotech), developed with phosphatase substrate (Sigma) and read at 405 nm (Versamax microplate reader, Molecular Devices).

Flow cytometric analysis and sorting of VLRA, VLRB and VLRC lymphocytes

Leukocyte isolation from blood and tissues and staining for flow cytometry proceeded as described⁵. In brief, leukocytes from blood and tissues were stained with anti-VLRA rabbit polyclonal serum (R110), anti-VLRB mouse monoclonal antibody (4C4) and biotinylated anti-VLRC mouse monoclonal antibodies (1B4, 3A5, 10A5, 11B5) and matched secondary reagents. Flow cytometric analysis was performed on a CyAn ADP (Dako) or Accuri C6 (BD Biosciences) flow cytometers. VLRA⁺, VLRB⁺, VLRC⁺ and VLR triple-negative cells in the lymphocyte gate were sorted on BD FACS Aria II (BD Bioscience) for genomic and quantitative RT-PCR analysis. The purity of the sorted cells was 96.5 ± 1.5% (VLRA⁺), 96.1 ± 1.4% (VLRB⁺), 98.7 ± 1.3% (VLRC⁺) and 97.9 ± 1.4% (triple-negative).

Immunofluorescence microscopy

Tissue samples were prepared and stained as described previously¹⁹ using anti-VLRA rabbit serum (R110) and anti-VLRC monoclonal antibodies (3A5). Fluorescence microscopy was performed with a Zeiss Axiovert 200M and images were processed with Adobe Photoshop (Adobe Systems). For cell-number counts from intestinal epithelium and epidermis, the total numbers of positive cells from single 7-µm sections were counted for both tissues in a total of five larvae.

Proliferation assay

Lampreys stimulated with antigen, mitogen or poly(I:C) were injected with 5 µg of 5-ethynyl-2'-deoxyuridine (Invitrogen) in 40 µl 0.67 × PBS and returned to aquaculture for 24 h before collecting leukocytes for flow cytometry analysis as described⁵.

Lymphocyte counts

Lymphocytes were prepared from lamprey blood and kidneys by passage through 70 μ m cell strainers (BD Biosciences), producing single-cell suspensions. Cells were washed and resuspended in 1 ml 0.67 $\times$ PBS. Total cells were counted in 25 μ l of each sample on an Accuri C6 flow cytometer. The total number of cells in the 'lymphocyte gate' was calculated by the formula: total lymphocyte number = number of cells in the lymphocyte gate $\times$ 40 (dilution factor). Lymphocytes were then stained with anti-VLRA, -VLRB and -VLRC antibodies to determine the percentages of VLRA⁺, VLRB⁺ and VLRC⁺ cells, which were used to calculate the numbers of lymphocytes of each type.

VLR expression in transfected HEK-293T cells

VLRB- and VLRC-pIRESpuo2 plasmids were transfected into HEK-293T cells cultured in DMEM containing 5% FBS using linear polyethylenimine (PEI), MW 25,000 (Polysciences) 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.

Immunoblot analysis

Samples were separated on 11% non-reducing SDS-PAGE gels before transfer to polyvinylidene fluoride membranes (Millipore). Membranes were blocked overnight with 5% skimmed milk (US Biological) and incubated with anti-VLRB (4C4) or anti-VLRC (3A5) antibodies for 1 h. After five washes with 0.5% Tween-20 in 1 $\times$ PBS, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin polyclonal antibodies (Dako) and washed. Blots were developed using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific).

Genomic PCR

Genomic DNA was extracted from VLRA⁺, VLRB⁺, VLRC⁺ and VLR triple-negative-sorted lymphocytes using DNeasy kit (QIAGEN). Genomic PCR was carried out using primers VLRA-F and VLRA-R, VLRB-F and VLRB-R or VLRC-F and VLRC-R (Expand Long Template, Roche). Primers are listed in Supplementary Table 1.

RT-PCR

Total RNA was extracted from sorted cells of lamprey blood and tissues (kidneys, typhlosole, gill and skin) and reverse-transcribed using random hexamers (Invitrogen). KOD Hot Start DNA Polymerase (TOYOBO) and Ex Taq were used to amplify the VLR genes. When necessary, the PCR products were cloned into pBluescript (Stratagene) or pCR4-TOPO (Invitrogen) vector and sequenced. Primers used for PCR are listed in Supplementary Table 1.

VLRC assemblies from *Lampetra planeri*

Genomic DNA was procured from the thymoid region of gill filaments of *Lampetra planeri* larvae by laser-capture micro-dissection, essentially as described¹⁹. VLRC genes were amplified using high-fidelity Phusion Taq polymerase (Invitrogen) and primers VLRC5.1 and VLRC3 (Supplementary Table 1), cloned into the pGEM-T vector (Promega) and sequenced; in some cases, sequences were cloned after a second amplification step using primers VLRC5.2 and VLRC3. Non-functional sequences exhibited either internal stop codons, frame-shift mutations, or consisted of partial assemblies.

RNA *in situ* hybridization analyses

RNA *in situ* hybridization analyses were performed on paraffin-embedded tissue sections of *L. planeri* larvae, essentially as described¹⁹. The *CDA1* probe has been described previously¹⁹.

Quantitative real-time PCR

Target gene sequences were obtained from the lamprey genome database²¹ and subsequent *in silico* analysis (including phylogenetic analysis) was performed as described previously⁹. RNA was extracted from each population using RNeasy kits 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 carried out with SYBR Green on a 7900HT ABI Prism (Applied Biosystems). Quantitative real-time PCR reactions were performed to evaluate the expression of each gene orthologue. Five separate determinations were carried out for heat-map analysis. The value of the target gene expression was normalized to β -actin. The normalized value for each gene was compiled into a heat map (z -scores) with three-colour scale. Red, $z = 1.5$; blue, $z = -1.5$; white, $z = 0$ ($z = (\text{each value} - \text{average})/\text{standard deviation}$). Primers and the values of the target gene are described in Supplementary Tables 1 and 2, respectively.

Electron microscopy

Tissue lymphocytes from naive or PHA-stimulated lamprey larvae were sorted on a BD FACS Aria II (BD Biosciences). The VLRC-positive cells were prepared for transmission electron microscopic analysis as described previously⁵.

Statistical analysis

Statistical significance was determined by a two-sample Student's *t*-test and Fisher's exact test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Tissue distribution of VLRA⁺, VLRB⁺ and VLRC⁺ lymphocytes

a, Flow cytometric analysis of lymphocyte-gated cells stained with monoclonal antibodies specific for VLRA (R110), VLRB (4C4) and VLRC (3A5) (left). Lymphocyte population in lamprey larvae (right); $n = 11$.

b–g, Immunofluorescence staining of VLRC⁺ (green) and VLRA⁺ (red) lymphocytes in larval tissue sections, and DAPI counterstaining of nuclei (blue); scale bars, 50 μ m (**b–e**, **g**). Shown are typhlosome surrounded by intestinal epithelium (intraepithelial lymphocytes) (**b**, arrows), kidneys (**c**), gill filaments (**d**; inset is a magnification of the area indicated by the dashed box), hypopharyngeal fold (**e**), skin (**g**). Lymphocyte distribution in the intestinal epithelium and skin epidermis is shown in **f**; $n = 5$ larvae. *** $P < 0.0001$; error bars, s.e.m.

h, Frequency of replicate VLRC sequences in indicated tissues from two larvae; replicate VLRC sequences were not shared by different tissues. **i**, Frequency of VLRA replicates. The numbers of clonal replicates are colour-coded: red, 22; blue, 7; green, 5; pale orange, 4; yellow, 3; orange, 2.

Figure 2. Antigen and mitogen responses

a, Lymphocyte proliferation in typhlosole before ($n = 6$) and 28 days after ($n = 3$) *B. anthracis* exosporium immunization measured by EdU (5-ethynyl-2'-deoxyuridine) incorporation. **b**, Lymphocyte proliferation 9 days after PHA stimulation ($n = 7$). **c**, Lymphocyte numbers in blood after PHA stimulation ($n = 7$). **d**, Western blot (WB) analysis of plasma before (day 0 (D0)) and 9 days after (D9) PHA stimulation. kDa, kilodaltons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; error bars, s.e.m.

Figure 3. Gene-expression profiles of VLRA⁺, VLRB⁺ and VLRC⁺ lymphocytes and their poly(I:C) responses

a, Relative transcript levels of the indicated genes were measured by quantitative PCR for purified VLRA⁺, VLRB⁺, VLRC⁺ and triple negative (TN) lymphocyte populations and compiled into a heat map ($n = 5$). **b**, Proliferative responses to *in vivo* poly(I:C) stimulation, measured by EdU incorporation ($n = 3$). **c–e**, Cytokine expression of purified blood lymphocytes before and 9 days after poly(I:C) treatment of lamprey larvae measured by quantitative PCR ($n = 3$): **c**, IL-16, **d**, IL-17 and **e**, IL-8. * $P < 0.05$, ** $P < 0.01$; error bars, s.e.m.

Figure 4. Analysis of *VLRC*, *VLRA* and *VLRB* transcription and assembly

a, Thymoid procurement site before (top) and after (middle) laser-capture micro-dissection; *CDA1*-expressing cells (blue) were detected by RNA *in situ* hybridization in an adjacent section (bottom). **b**, Proportion of non-productive sequences among assembled *VLRC* genes (top) and partially assembled genes among non-productive *VLRC* sequences (bottom). **c**, Schematic of *VLR* genes before (top) and after (bottom) assembly. Forward (F) and reverse (R) primer locations and predicted PCR product sizes are indicated. The F2 and R2 primer pair (grey) was used to amplify *VLRB* transcripts. **d**, Total RNA extracted from purified lymphocyte populations was amplified by RT-PCR (PCR with reverse transcription). bp, base pairs; GL, germline transcripts; M, transcripts of assembled *VLR* genes. **e**, Assembly of *VLRB*, *VLRA* and *VLRC* genes identified by PCR of genomic DNA. **f**, Schematic model of *VLRA* and *VLRC* assembly during the development of bi-potent precursor cells in the 'thymoid'. Orange bars indicate *VLRC* assembly, green bars indicate *VLRA* assembly (solid coloured bars, productive assembly; bars with a cross, non-productive assembly). Green and orange chains represent leucine-rich repeat modules of *VLRA* and *VLRC*, respectively. Red text represents genes that are assembled. Grey text represents genes that are not assembled. Dagger symbols represent cell death.