A major allogenic leukocyte antigen in the agnathan hagfish

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All vertebrates, from jawless fish to mammals, possess adaptive immune systems that can detect and inactivate non-self-antigens through a vast repertoire of antigen receptors. Unlike jawed vertebrates, the hagfish utilizes variable lymphocyte receptors (VLRs) that are unrelated to immunoglobulin molecules but are diversified by copy-choice gene conversion mechanism. Here, we report that hagfish VLRs react with allogenic leukocyte antigens but not with self-antigens. We found that a highly polymorphic membrane protein, NICIR3, is recognized by VLRs as an allogenic leukocyte antigen (ALA). In a serological cross-reactivity test, a close correlation was observed between the amino acid differences in the protein sequences and the VLR cross-reactivities. This leukocyte antigen was predominantly expressed in phagocytic leukocytes, where it was associated with phagocytosed protein antigens. These findings suggest that a polymorphic leukocyte antigen, NICIR3/ALA, plays a pivotal role in jawless vertebrate adaptive immunity.

Results

Serum VLRs recognize allogenic leukocytes. Three distinct types of VLRs are known in the sea lamprey; VLR-A, VLR-B and VLR-C. Each is expressed in three separate lymphoid cell subpopulations in a mutually exclusive and monoallelic manner. VLR-B molecules are secreted in blood and react to exogenous antigens, whereas VLR-A and VLR-C are expressed only in membrane-bound forms and VLR-Cs are not well-known. A functional VLR gene is generated by the assembly of multiple variable gene segments known as a copy-choice gene conversion mechanism. Thus, the evolutionary origin of adaptive immunity in jawless fish appears to be different from that of jawed vertebrates, in which the diversification process was acquired by an accidental insertion of transposons into the primordial Ig genes. Although jawless and jawed vertebrates possess distinct adaptive immune systems using different strategies for gene rearrangement, both have to eliminate self-reactive lymphocytes to establish immunological tolerance, as somatic diversification of antigen receptors occurs in a random fashion and in an antigen-independent manner producing potentially hazardous species. In this study, we investigate whether negative selection also takes place in the jawless fish VLRs. Furthermore, we studied a highly polymorphic leukocyte antigen, NICIR3/ALA, which may be involved in the recognition of phagocytosed protein antigens.

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Abbreviations: VLR, variable lymphocyte receptor; ELISA, enzyme-linked immunosorbent assay; PFA, paraformaldehyde; NICIR3, non-immune cell-specific receptor 3; SGP, small glycosylphosphatidylinositol; mAb, monoclonal antibody.

Figure 1 | Secreted VLR-Bs react with allogenic leukocytes but not with self-ones. (A) ELISA of hagfish serum with mouse anti-VLR-A (2A-1G) and anti-VLR-B (25D-9C) monoclonal antibodies (mAb). Error bars indicate the s.e.m. (n = 12 fish). These results show that hagfish VLR-Bs are secreted in blood. N.D. indicates less than 0.1. (B) Staining of VLR-B producing leukocytes. Buffy coat leukocytes from a hagfish were fixed on glass slides, masking VLR-Bs. Anti-VLR-B monoclonal antibodies (25D-9C) do not recognize VLR-B producing leukocytes only after antigen retrieval treatment (heating at 121°C for 20 min at 100 kPa in 0.01 M citric acid, pH 6). A scale bar is 100 μm. (C) Schematic diagram of the serological assay. Buffy coat leukocytes and serum containing VLR-Bs were separately collected from the hagfish Eptatretus burgeri. Buffy coat leukocytes from fish #X and fish #Y were fixed on glass slides and treated with complement-inactivated serum from fish #X. Binding of serum-derived VLR-Bs was detected with mouse monoclonal antibodies (25D-9C) against secreted VLR-Bs (bottom). (D) Leukocyte staining with VLR-Bs. Anti-VLR-B monoclonal antibodies (25D-9C) do not recognize VLR-B producing leukocytes after fixation (top panels). As a result, only secreted VLR-Bs from the serum is detected with this antibody. Secreted VLR-Bs react with buffy coat leukocytes from different individuals (bottom right, red), but not with those isolated from the same individual (bottom left), suggesting allogenic recognition of leukocyte antigens. A scale bar is 100 μm.

epitope for this antibody. Taking advantage of this observation, we examined the alloreactivity of VLR-Bs using serological assay described below. In the human, natural antibodies (IgM and IgGs) are known to react to allogenic, but not self antigens on the hematocytes, e.g., erythrocytes and leukocyte29,30. However, it has never been tested whether the agnathan VLRs recognize allogenic antigens.

Leukocytes of buffy coat fraction9,27,28 and serum samples were isolated from the peripheral blood of different hagfish. Leukocytes were then fixed on glass slides and treated with the complement-inactivated serum from self or non-self hagfish. We used anti-VLR-B monoclonal antibody mAb 25D-9C to detect the "serum-derived" VLR-Bs (Fig. 1A). It should be noted that mAb 25D-9C does not recognize VLR-Bs in fixed leukocytes (Fig. 1B). We found that VLR-Bs in the serum reacted to leukocytes from allogenic hagfish but not to autologous cells (Fig. 1C and D and supplementary Fig. 1E). VLR-Bs did not react to allogenic erythrocytes. These results indicate that VLR-Bs are negatively selected for self-leukocyte antigens in the hagfish, as has been known for immunoglobulins in jawed vertebrates22.

We then examined the types of molecules recognized by VLR-Bs as allogenic antigens. We first hypothesized that these molecules would be carbohydrates, as in the case of mammalian blood antigens29. However, VLR-B reactivity was not affected by glycosidase treatment. Because VLR-B reactivities varied among individuals, we assumed that this allogenic leukocyte antigen may be a polymorphic membrane protein.

A highly polymorphic leukocyte antigen. To identify such a polymorphic leukocyte antigen, we performed microarray analyses using mRNAs isolated from buffy coat leukocytes and erythrocytes, and searched for transmembrane proteins among the 10% most highly expressed proteins in leukocytes (but not in erythrocytes) as the initial criterion. A custom microarray was designed with approximately 24,000 hagfish leukocyte EST sequences31. After sequence analysis of 40 candidate genes, we found that a gene for immunoreceptor tyrosine-based activation motif-containing Ig superfamily receptor 3 (NICIR3)32 is the only polymorphic gene. NICIR3 was previously reported as a polymorphic transmembrane receptor with unknown functions by Haruta et al.32. Although the hagfish has threeNICIR family genes, our analysis of the NICIR sequences from 25 fish samples confirmed that only NICIR3 is highly polymorphic (Fig. 2A and supplementary Fig. 2A). The extracellular portion of NICIR3 consists of two Ig domains (V and C), and the variable residues are clustered in the extracellular region (Fig. 2B and C).

NICIR3 is a major allogenic leukocyte antigen for VLR-Bs. To examine the alloreactivities of NICIR3 proteins and serum samples, we prepared buffy coat leukocytes and sera (secreted VLR-Bs) from eight hagfish specimens and performed serological tests for all combinations of leukocytes and serum samples. Each serum sample demonstrated variable reactivities to leukocytes from different hagfish (Fig. 3A). To identify the NICIR3 haplotypes, the coding sequences of two alleles (paternal and maternal) were amplified by PCR and were sequenced for all eight specimens. The differences in the deduced amino acid sequences were analyzed for alloreactivity. For each serum sample (#X), the total residue differences with another individual (#Y) were counted for both the maternal and paternal alleles. For example, in the combination of serum from #X and buffy coat leukocyte from #Y, where #X has two allelic sequences, AAAAA and AABAB, and #Y has ABABA and BBBC, the difference (the expected antigenicity score) would be counted as 1 + 2 + 0 + 2 + 1 = 6. For the combination of serum #Y and buffy coat leukocyte #X, the difference would be 0 + 2 + 0 + 2 + 1 = 5. In this way, expected antigenicity scores were calculated for 64 different combinations of serum and buffy coat leukocyte samples isolated from eight different individuals (Fig. 3B). In Fig. 3C, the calculated amino acid differences among the different haplotypes are plotted against the observed alloreactivities (VLR-B binding). A positive correlation was
observed between the two (R = 0.69; Spearman’s correlation coefficient, p < 0.0001). These results indicate that the VLR-Bs of more distantly related haplotypes tend to possess higher reactivities to buffy coat leukocytes (supplementary Fig. 3). Therefore, we hereinafter refer to NICIR3 as the allogenic leukocyte antigen (ALA) in hagfish. To determine whether the allogenic NICIR3/ALA is directly recognized by VLR-Bs, an NICIR3/ALA was expressed in HEK293T cells and the interaction with allogenic VLR-Bs was examined using flow cytometry. Fig. 3D demonstrates the allogenic interaction between the NICIR3/ALA and the serum VLR-Bs.

NICIR3/ALA is expressed in phagocytic cells. To study the localization of NICIR3/ALA, we generated anti-NICIR3/ALA polyclonal antibodies in mice (supplementary Fig. 2A–D). In jawless fish, blood cells can be sorted into at least three different subpopulations by flow cytometry: erythrocytes, lymphocytes, and myeloid cells (Fig. 4A). By staining the blood cells with anti-NICIR3/ALA antibodies, we found that leukocytes (i.e. lymphocytes and myeloid cells) expressed NICIR3/ALA, whereas erythrocytes did not (Fig. 4B) and that VLR-B positive lymphocytes expressed NICIR3/ALA more than VLR-A positive lymphocytes (Fig. 4C).

Figure 2 | Polymorphisms of the allogenic leukocyte antigen, ALA. (A) A phylogenetic tree of 50 different NICIR3/ALA alleles identified in 25 different individuals (#1–#25). The tree was constructed with the unrooted Neighbor-Joining method. Bootstrap support values over 50% are shown at branches. Amino acid sequences were compared using the Clustal W program. (B) The extracellular domains of NICIR3/ALA consist of two Ig domains, V (48–165 aa) and C (166–247 aa). The variability of amino acid (aa) residues (a–l) in 50 NICIR3/ALA sequences from 25 animals are shown. The ratio of atypical aa residues was calculated for each aa position as follows: the number of samples with atypical aa residues was divided by total number of samples. Vertical bars represent variable aa residues: red, > 0.25; pink, 0.04–0.25; and black, < 0.04. Variable aa residues (> 0.04) are indicated by alphabets, a–l. The schematic diagram of NICIR3/ALA is shown on the bottom. SP, signal peptide; TM, transmembrane domain; ITAM, immunoreceptor tyrosine-based activation motif. The V and C domains are in green and yellow, respectively. (C) Polymorphic aa residues in the NICIR3/ALA sequences. Variable aa residues (a–l) in Fig. 2B are shown on the bottom. Hydrophobic residues are shown in black color, hydrophilic residues in green, acidic residues in red and basic residues in blue. GenBank accessions of the NICIR3/ALA alleles are AB723629–AB723678.
leukocytes were stained for KLH, and immunoelectron microscopy analyses demonstrated that most of these KLH-positive phagocytic cells were monocytes and granule like cells (supplementary Fig. 4A). Immunocytochemistry with the anti-NICIR3/ALA antibodies then demonstrated that NICIR3/ALA colocalized with phagocytosed KLH proteins in the phagocytic cells (Fig. 4D and supplementary Fig. 5). The colocalization of NICIR3/ALA and phagocytosed proteins were observed within a lysosomal compartment by colloidal gold immunostaining (Fig. 4E and supplementary Fig. 4B).

### Discussion

In the present study, we found that hagfish VLR-Bs recognize an allogenic leukocyte antigen, ALA (previously reported as NICIR3). NICIR3/ALA is a transmembrane protein consisting of two domains, IgV and IgC, in the extracellular region and two immuno-receptor tyrosine based activation motifs (ITAM) in the intracellular region (supplementary Fig. 2). We assume that NICIR3/ALA transduces ligand signals to induce a set of genes for lymphocyte activation via ITAMs. From serological tests, NICIR3/ALA was

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**Figure 3 | NICIR3/ALA haplotypes and allogenic reactivities.** (A) Quantitative analysis of binding between VLR-B samples and buffy coat leukocytes. The buffy coat leukocytes and sera were isolated from eight different individuals (#1–#8). Serological tests shown in Fig. 1D were performed for all 8 \times 8 combinations. The resulting signal intensities are shown as a heat map. (B) Haplotypes and expected antigenicities of NICIR3/ALA. Expected antigenicity scores were calculated for each pair of serum and buffy coat leukocyte for both alleles. A correlation between the alloreactivities (serum) and the amino acid residue differences (leukocyte) is shown as a heat map for all 8 \times 8 combinations. The calculation of the number of residue differences is schematically shown on the right, where arrows indicate a given subtype of VLR-B reacting with a non-self-NICIR3/ALA. Antigenicity scores were calculated for both allele 1 and 2 by counting how many allogenic residues are contained in NICIR3/ALA without considering positions and species of aa residues. We also assumed that two alleles of NICIR3/ALA equally and independently contribute to the antigenicity. (C) Correlation between the antigenicity scores of NICIR3/ALA and VLR-B binding for allogreactivities. The allogenic antigenicity of NICIR3/ALA and binding score of VLR-B are shown in closed circles (○). Self-self reactivities are shown in open circles (○). A positive correlation \( R = 0.69; \) Spearman’s correlation coefficient) was observed between the expected antigenicity scores for NICIR3/ALA and VLR-B binding scores. (D) Alloreactivities of VLR-Bs to hemagglutinin (HA)-tagged NICIR3/ALA molecules expressed in HEK293T cells (left). Three different haplotypes from three different animals, #1, #2 and #3, were analyzed. Samples treated with the serum from animal #1 were applied (right), and the binding of VLR-Bs was measured by flow cytometry. The mock-transfected HEK293T sample (mock-293T) was used as a negative control. The #1 serum reacted with the #2 allele-1 and #3 allele-1 (allogenic) but not with the #1 allele-1 NICIR3/ALA (self). Allele numbers, #1–#8, are the same in Fig. 2.
shown to be a major allogenic antigen for the hagfish VLR-Bs (Fig. 3 and supplementary Fig. 3). Because the VLR-B genes are randomly assembled in an antigen-independent manner\textsuperscript{16}, self-reactive VLR-Bs must be generated in the hagfish. However, no VLR-Bs were found to react to autologous antigens. Thus, it is conceivable that the VLR-B repertoire is negatively selected for self antigens including NICIR3/ALA. Flow cytometric analysis showed that NICIR3/ALA is expressed in myeloid cells and lymphocytes (Fig. 4B). We also found that NICIR3/ALA colocalized with that of phagocytosed proteins within leukocytes (Fig. 4D and E and supplementary Fig. 4B). Furthermore, using the proximity ligation assay, we demonstrated close association of NICIR3/ALA and phagocytosed protein antigens both in the cytoplasm and on the cell surface (supplementary Fig. 7 and 8). Because NICIR3/ALA-protein antigen association requires both the polymorphic V and C domains, these domains may together constitute a ligand-binding site for various protein antigens (supplementary Fig. 8).

It was reported that VLR-A cells might be selected in the gill, a thymus like tissue\textsuperscript{36}. A search for the thymus in hagfish was unsuccessful, most likely because the thymus like tissue is lost in the adult. We also analyzed the hematopoietic nest\textsuperscript{37} a candidate lymphoid organ, located around intestine where expression of VLR-A and VLR-B was prominent (supplementary Fig. 10A–C). We examined whether NICIR3/ALA is expressed in the hematopoietic nest, and found that NICIR3/ALA positive cells are located in close proximity to VLR-positive cells (supplementary Fig. 10D and E). It is tempting...
to assume that NICIR3/ALA is involved in the elimination of self-reactive VLR cell. In the hagfish, it has been assumed that VLR-A function like mammalian TCRs. It is interesting to study whether NICIR3/ALA is also involved in the VLR-A selection.

The evolutionary origin of adaptive immunity represents one of the greatest biological enigmas. An obvious advantage of adaptive immunity is the capacity to cope with novel pathogens using a wide variety of antigen receptors and it is widely held that this emerged in vertebrates during the Cambrian explosion approximately 500 million years ago. For diversification of antigen receptor genes, jawless vertebrates employ a copy-choice mechanism, whereas jawed vertebrates utilize V(D) recombination, a reverse reaction involving the accidental insertion of transposons that occurred during their evolution. Although both systems diversify antigen receptor genes by entirely different mechanisms, the random generation of a large variety of antigen receptors potentially produces hazardous self-reactive repertoire in both systems. Through this study, we demonstrated that VLR-B molecules in jawless fish undergo negative selection to control the self-reactive repertoire in both systems. By comparing the results of unmanipulated hagfish with those of the NICIR3/ALA transgenic hagfish, we found that hagfish express NICIR3/ALA endogenously to control self-reactivity.

**Methods**

**Hagfish samples.** Adult hagfish (Eptatretus burgeri) were captured in the Pacific Ocean off the coast of Mutsu, Aomori Prefecture, Japan, and were cultured at 15°C in artificial sea water (Marine Art HG, Kobayashi). The hagfish were anesthetized in 500 mg/L of MS-222 (ethyl maminobenzoate methanesulfonate; Nacalai Tesque). Peripheral blood samples were collected from the caudal subcutaneous sinus and placed in hagfish phosphate-buffered saline (hagfish PBS) containing 30 mM EDTA. Leukocytes were prepared from a hagfish blood cell fraction as previously described. The serum was prepared from peripheral blood clotted at 4°C overnight. To label the phagocytic cells, 100 μg of keyhole limpet hemocyanin (KLH) (Sigma) and 100 μl of Freund’s complete adjuvant (Thermo) were intraperitoneally injected into the hagfish, and buffy coat leukocytes were collected one day after injection.

**Generation of antibodies against VLRs and NICIR3/ALA.** Two monoclonal antibodies were produced in mice for VLR-A and VLR-B using synthetic peptides conjugated with bovine serum albumin (BSA). Peptides used are listed in Supplementary Table 1. Lymph node cells were cultured and fused with Ig-negative myeloma cells. Hybridoma clones producing antibodies against VLR-A (2A-IG; IgG2b, κ) and VLR-B (2SD-9C; IgG1, κ) were identified by ELISA, immunoblotting and immunocytochemistry. Anti-VLR-A and anti-VLR-B polyclonal antisera were produced by immunizing rabbits with BSA-conjugated synthetic peptides listed in Supplementary Table 1. Anti-NICIR3/ALA polyclonal antisera were produced in mice. Antibody reactivities were examined for 16 different haplotype NICIR3/ALAs expressed in HEK293T. All antibodies were affinity-purified with peptide antigens.

**Enzyme-linked immunosorbent assay (ELISA).** Diluted hagfish sera and VLR-A or VLR-B peptides (50 μg/well) in 20 mM phosphate saline were loaded on 96-well plates (Nunc) pretreated with 1% (w/v) BSA in PBS for 1 hr. Diluted samples were incubated for 2 hrs at 37°C. VLRs were detected with anti-VLR-A (2A-IG) or anti-VLR-B (2SD-9C) monoclonal antibodies and then with HRP-conjugated goat anti-mouse IgG (1:4000, Molecular Probes) at 37°C for 1 min, with a 15 s wash after each step. Absorbance at 405 nm was quantified with BertholdTech TriStar LB941 multimode plate reader.

**Serological tests for buffy coat leukocytes and serum.** Buffy coat leukocytes were placed on glass slides (2 × 105 cells per slide), treated with 4% (v/v) paraformaldehyde in hagfish PBS for 5 min, and subsequently fixed with 2% (v/v) glutaraldehyde in hagfish PBS for 20 min. The slides were dehydrated in TBS (50 mM Tris and 150 mM NaCl, pH 7.6) three times. Samples were blocked with 5% (w/v) skim milk in TBS and incubated with inactivated hagfish serum (20%) in TBS overnight at room temperature. The slides were washed three times and treated with primary antibodies (anti-VLR-B; 2SD-9C) for 1 hr at room temperature. Signals were visualized with secondary antibodies conjugated with Alexa Fluor 555 (Molecular Probes). Nuclear counterstaining was performed with DAPI (Roche). The stained cells were analyzed with an Olympus Model IX70 fluorescence microscope using a cooled CCD camera (model ORCA-R2, Hamamatsu Photonics). Region of interest (ROI) was selected by DAPI. Fluorescent intensity (VLR-B binding) per leukocyte was measured in ROI using ImageJ software (version 1.46, http://rsweb.nih.gov/ij/) (n > randomly chosen 300 cells/sample). Mean of fluorescence intensities scores are shown in boxes (Fig. 3A).

**Interactions of serum VLR-Bs to NICIR3/ALA expressed in HEK293T cells.** The NICIR3/ALA coding sequence was amplified by PCR and fused to the Igx signal sequence and hemagglutinin (HA) tag. HEK293T cells expressing NICIR3/ALA pEX expression vector was transfected into HEK-293T cells (Invitrogen). The cells were cultured in DMEM supplemented with 10% (v/v) FBS. To avoid nonspecific binding, the serum was pre-incubated overnight with HEK293T cells transfected with a mock vector (pDisplay). HEK293T cells expressing NICIR3/ALA were treated with 10% (v/v) pre-absorbed serum for 1 hr, anti-VLR-B monoclonal antibody (2SD-9C) for 1 hr, and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:400, Molecular Probes) at 37°C. HA-tagged proteins were stained with Alexa Fluor 488-conjugated anti-HA monoclonal antibody (1:200, Molecular Probes) for flow cytometric analysis (BD FACS Calibur).

**Microarray analysis.** A custom microarray (4 × 44 K probes, Agilent) was made, using approximately 24,000 EST sequences from hagfish leukocytes. Total RNA was purified from leukocytes or erythrocytes of the hagfish buffy coat (n = 4). Cy3- and Cy5-labeled cRNA probes were prepared with Agilent Low-RNA Input Linear Amplification Kit (Agilent) and hybridized to the custom microarray according to the manufacturer’s instruction (Agilent). The hybridized arrays were scanned and quantified by Agilent DNA microarray scanner and Feature Extraction (Agilent). Data analysis was performed with Microsoft Excel.

**RT-PCR and sequence analysis.** NICIR3/ALA genes were amplified by PCR from cDNA prepared from buffy coat leukocytes under the following condition: 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final hold at 72°C for 5 min. NICIR3/ALA primers used in this study are listed in Table S2. Cloning and sequence analysis of the genes were performed as described previously. Ten clones were sequenced for each allele. Genbank accession numbers are AB723629–AB723678.

**Phylogenetic tree of NICIR3/ALA-related proteins.** Amino acid sequences were compared using the Clustal X program. A phylogenetic tree was made using the Neighbor Joining (NJ) method with bootstrap distances were calculated with the JTT matrix-based method. Both NJ analysis and bootstrap analysis were carried out with MEGA 5.05.

**Sorting of hagfish blood cells.** Hagfish blood cells were sorted into three discrete subpopulations (lymphocytes, myeloid cells and erythrocytes) based on the forward vs. 90° light-scattering profiles from flow cytometry. Cells were collected from each subpopulation using FACS Aria (BD).

**Flow cytometric analysis.** Buffy coat leukocytes (and erythrocytes, representing 10% of the cells) from peripheral blood samples were fixed with hagfish PBS containing 10% formaldehyde for 5 min. Samples were washed twice and stained with mouse anti-NICIR3/ALA polyclonal antibodies for 1 hr on ice. The samples were then washed twice and stained with fluorescein-conjugated secondary antibodies, Alexa Fluor 488-conjugated goat anti-mouse IgG (1:400, Molecular Probes). Data were obtained using FACS Aria (BD) and analyzed with FlowJo software (Treestar). For staining and washing, PBS with 0.5% BSA and 2 mM EDTA (pH 7.4) was used.

**Unimmunized mouse serum was used as a negative control for primary antibodies.**

**Confocal imaging of phagocytosed proteins.** To label the phagocytic cells, 100 μg of proteins (KLH or OVA) and 100 μl of Freund’s complete adjuvant (Thermo) were intraperitoneally injected into the hagfish. Buffy coat leukocytes were collected one day after injection. The cells were fixed with 4% (v/v) PFA for 1 hr and collected. 100 μl of Freund’s complete adjuvant (Thermo) was added to the cells with 4% PFA for 1 hr and embedded in LR white resin after dehydration with ethanol. Samples were sliced in 60–70 nm sections, incubated with rabbit anti-KLH antibodies (Rockland) for 2 hrs at room temperature, and then with goat anti-rabbit-IgG labeled with 6 nm colloidal gold (1:30, Jackson Immuno Research Laboratories, Inc.) for 1 hr. After washing, the samples were incubated with 0.1% Triton-X100 PBS, confocal images were obtained with an upright confocal microscope, Olympus Model FV500.

**Immunoelectron microscopy of phagocytosed proteins.** To label the phagocytic cells, 100 μg of KLH proteins and 100 μl of Freund’s complete adjuvant (Thermo) were intraperitoneally injected into the hagfish. Buffy coat leukocytes were collected one day after injection. The cells were fixed with 4% (v/v) paraformaldehyde in hagfish PBS for 5 min, and subsequently fixed with 2% (v/v) glutaraldehyde in hagfish PBS for 20 min. The cells were dehydrated in TBS (50 mM Tris and 150 mM NaCl, pH 7.6) three times. Samples were blocked with 5% (w/v) skim milk in TBS and incubated with inactivated hagfish serum (20%) in TBS overnight at room temperature. The slides were washed three times and treated with primary antibodies (anti-VLR-B; 2SD-9C) for 1 hr at room temperature. Signals were visualized with secondary antibodies conjugated with Alexa Fluor 555 (Molecular Probes). Nuclear counterstaining was performed with DAPI (Roche). The stained cells were analyzed with an Olympus Model IX70 fluorescence microscope using a cooled CCD camera (model ORCA-R2, Hamamatsu Photonics). Region of interest (ROI) was selected by DAPI. Fluorescent intensity (VLR-B binding) per leukocyte was measured in ROI using ImageJ software (version 1.46, http://rsweb.nih.gov/ij/) (n > randomly chosen 300 cells/sample). Mean of fluorescence intensities scores are shown in boxes (Fig. 3A).
