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Lamprey Variable Lymphocyte Receptors Mediate Complement-Dependent Cytotoxicity

Fenfang Wu,*,† Liyong Chen,*,† Xin Liu,† Huaying Wang,† Peng Su,*,† Yinglun Han,*,† Bo Feng,*,† Xu Qiao,*,† Jing Zhao,*,† Ning Ma,*,† Huijie Liu,*,† Zhen Zheng,*,† and Qingwei Li*,†

An alternative adaptive-immune system is present in the most basal vertebrates—lampreys and hagfish—the only surviving jawless vertebrates. These eel-like fish use leucine-rich repeat–based receptors for Ag recognition instead of the Ig-based receptors used in jawed vertebrates. We report that in Japanese lamprey (Lampetra japonica), variable lymphocyte receptor (VLR)B interacts with C1q and C3 proteins to mediate complement-dependent cytotoxicity for bacteria and tumor cells. The immune-based lysis involves deposition of VLRB and C1q-like protein complex on the surface of target cells, activation of C3, and ultimate disruption of cell wall integrity. The demonstration of functional interaction between VLRB and complement components in lamprey provides evidence for the emergence of cooperative innate and adaptive-immune responses at a pivotal point in vertebrate evolution, before or in parallel with the evolution of Ig-based Abs and the classical complement-activation pathway. The Journal of Immunology, 2013, 190: 000–000.

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Materials and Methods

Cloning of VLRB, C3, and C1q-like cDNA and RT-PCR of C1q-like mRNA

The conserved C terminus of VLRB cDNA (European Molecular Biology Laboratory Release [Normal Divisions] ID: AB507270, nt 740–988) from lamprey leukocytes, the junctional region of α-chain and β-chain of C3 cDNA (GenBank accession number D10087; nt 1942–2857) from lamprey liver, and the C1q-like open reading frame without the signal peptides (GenBank accession number AB074155; nt 145–798) from lamprey intestine were separately cloned into pET-32a(+) vectors (Supplemental Figs. 1A, 2A, 3A). The tissue distribution of C1q-like mRNA in lampreys was determined by RT-PCR. Lamprey GAPDH was used as control. All primers are listed in Table I.

Preparation of lamprey antisera

Adult lampreys (200–220 g in weight, 49–52 cm in length) were divided into eight groups (10 fishes/group), and each group was immunized with a different Ag by four i.p. injections at 10-d intervals. The Ags used included 108 Escherichia coli, 107 Bacillus protein, 105 Staphylococcus aureus, 105 Mycobacterium smegmatis, 104 RBCs, 103 SRBCs, 105 NB4 cells, and 106 HeLa cells. RBC and tumor cells were injected as live cells, whereas all bacteria were inactivated by pasteurization (60°C for 30 min) before injections. The control animals were injected with 100 μl 0.9% NaCl. Three to four days after the fourth immunization, blood was col-

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lected from tail-severed lampreys. The sera and leukocytes were separated by centrifugation. The prepared serum was used as the antisera. For ease of referencing, the antisera obtained with the Ags E. coli, B. proteus, S. aureus, M. smegmatis, RRBC, SRBC, NB4 cells, and HeLa cells are hereafter referred to as t–anti–E. coli, t–anti–B. proteus, t–anti–S. aureus, t–anti–M. smegmatis, t–anti–RRBC, t–anti–SRBC, t–anti–NB4, and t–anti–HeLa, respectively.

Expression of recombinant proteins and production of Abs

Recombinant proteins with the 6-His tag were expressed in BL21(DE3) and affinity purified by Ni2+ Sepharose chromatography (GE Healthcare). To generate mouse anti-VLRB mAbs (M–anti–VLRB mAbs) and mouse anti–C1q-like mAbs, BALB/c mice were immunized with the recombinant VLRB (Universal Protein Knowledgebase ID: E0D2V5_LAMJA, Pro212 to Arg294) (Supplemental Fig. 1A) and C1q-like proteins (GenBank accession number BAD22833.1; Glu23 to Glu460) (Supplemental Fig. 2A). After four immunizations, spleen cells of immunized mice were fused with SP2/0 myeloma cells. Hypoxanthine, aminopterin, and thymidine culture medium was used for fused hybridoma cell screening. After the cell fusion 14 d later, the parent cells were diluted to 100 cells in 10 ml medium and cultured on 96-well cell culture microplate (0.1 ml per well). The Abs secreted by the different clones were then assayed for their ability to bind to the Ag using ELISA and immunodot blot. The most productive and GAPDH Abs secreted by the different clones were then assayed for their ability to bind to the Ag using ELISA and immunodot blot. The most productive and stable clone was selected for future use. To obtain a large number of Abs, hybridoma cells were injected into the peritoneal cavity of mice. Then, Ab-rich ascites fluid was collected, and the Abs were purified by protein G-Sepharose (GE Healthcare) chromatography.

To produce polyclonal rabbit Abs against VLRB, C1q-like protein, and B. proteus, M. smegmatis, and tumor cells (NB4 and HeLa), Ag–Sepharose (GE Healthcare) chromatography. Recombinant proteins with the 6-His tag were expressed in BL21(DE3) and affinity purified by Ni2+ Sepharose chromatography (GE Healthcare).

for 30 min. After incubation, the cells were washed, resuspended in 0.9% NaCl, spread onto Lysogeny Broth agar plates, and incubated at 37˚C for 14 h. CFU were counted to determine the cell viability. The average number of colonies was estimated from three plates. The number of CFU at zero time (before incubation with antisera) was taken as 100%. NaCl solution (0.9%) was used as a blank control. Lamprey sera stimulated with 0.9% NaCl and naive serum were used as negative controls.

To determine the temperature dependence of the antisera, t–anti–RRBC was pretreated at various temperatures (4–65˚C) for 30 min and then incubated with RRBCs.

To determine the optimum concentration of t–anti–RRBC for inducing cytotoxicity, RRBCs (5 × 106 cells/ml) were mixed with t–anti–RRBC at concentrations ranging from 0.5 to 40% (v/v) at 4˚C for 20 min. Optimum time for the cytotoxicity to occur was investigated by incubating the antisera–cell mixtures at 4˚C for different periods of time (5–120 min).

Agglutination assay

The antisera were first heated at 56˚C for 30 min to inactivate the complement components and then subjected to 2-fold serial dilution in 0.9% NaCl. An aliquot (50 μl) of the diluted antisera from each dilution was added to 50 μl the corresponding Ag in a 96-well flat-bottom plate and incubated at 37˚C for 1 h, followed by incubation at 4˚C overnight before visual inspection of the Ag agglutination by light microscopy. The Ags included RRBCs, SRBCs, Gram-negative E. coli, B. proteus, Gram-positive S. aureus, M. smegmatis, and tumor cells (NB4 and HeLa). Ag agglutination was scored on an arbitrary scale from 0 to 4 (0, single 2–3 Ags; 2, clusters of 4–6 Ags; 3, clusters of 7–10 Ags; 4, large clusters >10 Ags) (4).

Electron microscopy

For transmission electron microscope (TEM) analysis, E. coli cells were treated with t–anti–E. coli at 4˚C for 30 min. Subsequently, cells were collected and fixed with 2.5% glutaraldehyde solution in 100 mM sodium phosphate buffer (pH 7.2) at room temperature overnight. After washing with 100 mM sodium phosphate, the cells were further fixed with 1% (w/v) osmium tetroxide in phosphate buffer at 4˚C for 2 h. The cells were then dehydrated successively in 70, 80, 90, and 100% ethanol; transferred into propylene oxide; and embedded in Epon812. Ultrathin sections were cut with a Leica EM UC6 ultramicrotome (Germany) and mounted on a formvar-coated brass grid. The sections were stained with 2% uranyl acetate (w/v) in 70% methanol (v/v) and 0.5% lead citrate. Observations and image recording of the cells were performed with a JEM-2000EX TEM.

E. coli treated with t–anti–E. coli were also observed by scanning electron microscope (SEM). E. coli were treated with t–anti–E. coli at 4˚C for 30 min. The cells were collected by centrifugation at 4000 × g for 10 min and then washed with 0.9% NaCl. Subsequently, the cells were fixed with 2.5% glutaraldehyde and successively dehydrated in 70, 80, 90, and 100% acetone. Thereafter, the cells were dried, mounted on aluminum stubs, and sputter coated with gold using an SBC-12 ion sputter coater for observation under a KYKY-100B SEM.

Table I. Sequence of primers used for PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Cloning conserved C terminus of VLRB gene</td>
<td>5′–CGGATCCACAACACCCTGCTCACTGCTACGACG–3′</td>
</tr>
<tr>
<td>Cloning full-length C1q-like gene</td>
<td>5′–GAGAGGCAGCCTCCCTCAGGCACGACG–3′</td>
</tr>
<tr>
<td>Cloning C3 gene fragment</td>
<td>5′–GAGAGGCAGCCTCCCTCAGGCACGACG–3′</td>
</tr>
<tr>
<td>RT-PCR of C1q-like mRNA</td>
<td>5′–GAGAGGCCAGCCTCCCTCAGGCACGACG–3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′–GAGAGGCCAGCCTCCCTCAGGCACGACG–3′</td>
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RRBCs treated with l-anti-RRBC were observed by SEM. The cells were treated with l-anti-RRBC at 4°C for 12 min and then collected by centrifugation at 1000 x g/4°C for 5 min. The cells were washed with 0.9% NaCl, fixed with 2.5% glutaraldehyde, and successively dehydrated in 70, 80, 90, and 100% acetone. Subsequently, the cells were dried, mounted on aluminum stubs, and sputter coated with gold using an SBC-12 ion sputter coater. Observation and photography were performed with a KYKY-1000B SEM.

Coimmunoprecipitation, Western blot assays, and depletion of VLRB, C1q-like protein, and C3

To perform coimmunoprecipitation and Western blot assays, the antiserum was incubated with 1 µg M-anti-VLRB mAbs, R-anti-C1q-like pAbs, or 0.01 M PBS and control Abs at 4˚C for 2 h. After incubation, protein G agarose was added to each sample. The mixture was incubated at 4˚C for 4 h, followed by centrifugation to collect the precipitated proteins. The precipitated proteins were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). After incubating with M-anti-VLRB mAbs or R-anti-C1q-like pAbs, the membrane was probed with HRP-labeled goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) or goat anti-rabbit IgG (Jackson ImmunoResearch) and detected by ECL (Pierce).

For depletion of VLRB, C1q-like protein, and C3, 4 µl antiserum was incubated with 10 µg M-anti-VLRB mAbs, 8 µg R-anti-C1q-like pAbs, or 20 µg R-anti-C3 pAbs at 4˚C overnight. Protein G agarose was then added to the sample, and it was incubated at 4˚C for 4 h. Subsequently, the sample was centrifuged, and the supernatant was collected and assayed for its cytolytic effect. The irrelevant IgG was used as control.

Affinity purification of VLRB from lamprey sera

M-anti-VLRB mAbs (10 mg) were immobilized on CNBr-activated Sepharose matrix (2 ml) according to the instructions provided by the manufacturer (Amersham Biosciences). After immobilization, the Ab matrix was packed in a chromatography column. Then, 100 ml lamprey antiserum was applied to M-anti-VLRB mAb–immobilized CNBr matrix pre-equilibrated with equilibration buffer (75 mM Tris-HCl [pH 8]). The adsorbed VLRB was eluted with elution buffer (100 mM glycine-HCl containing 0.5 M NaCl [pH 2.7]) and immediately neutralized with 1 M Tris-HCl (pH 9). The purified VLRB was then dialyzed with 0.01 M PBS, and the protein concentration was measured. The Ab preparation was stored at 4˚C. The purity of VLRB was checked by SDS-PAGE and Western blot (Fig. 3B).

Determination of cytolytic effects of lamprey sera after addition of affinity-purified VLRB

Different amounts (0.1, 0.5, 1, 1.5, or 2 µg) of the affinity-purified VLRB were added to 20 µl VLRB-depleted antiserum or naive serum. The mixture was incubated with 10⁷ RRBCs, 10⁸ E. coli, or 10⁶ HeLa cells.
at 4°C for 30 min, and the cell-killing activity (killing rate) for each sample was determined as described above.

Cleavage and deposition of C3

L-anti-RRBC and serum from naive lamprey sera were incubated with RRBCs for 30 min at 4°C. The RRBCs were collected and lysed with SDS-PAGE sample loading buffer. The deposition and cleavage of C3 into C3b on the surface of RRBCs were detected by Western blot using R-anti-C3 pAbs.

Flow cytometry analysis

HeLa cells were treated with 2% L-anti-HeLa for 30 min at 4°C and then blocked with 3% BSA in 0.01 M PBS at room temperature for 30 min. Thereafter, cells were incubated with 1 μg M-anti-VLRB mAbs and R-anti-C1q-like pAbs and stained with FITC-conjugated goat anti-mouse IgG (1.5 mg/ml, 1:100 dilution; Jackson ImmunoResearch) or R-Phycocerythrin-labeled goat anti-rabbit IgG (1:100 dilution; HangZhou HuaAn Biotechnology, Hangzhou, China). Cell nuclei were stained with DAPI (Sigma). Fluorescent images were taken using a Nikon confocal laser microscope.

Laser scanning confocal microscopy

HeLa cells were treated with 2% L-anti-HeLa for 30 min at 4°C and then blocked with 3% BSA in 0.01 M PBS at room temperature for 30 min. Thereafter, cells were incubated with 1 μg M-anti-VLRB mAbs and R-anti-C1q-like pAbs and stained with FITC-conjugated goat anti-mouse IgG (1.5 mg/ml, 1:100 dilution; Jackson ImmunoResearch) or R-Phycocerythrin-labeled goat anti-rabbit IgG (1:100 dilution; HangZhou HuaAn Biotechnology, Hangzhou, China). Cell nuclei were stained with DAPI (Sigma). Fluorescent images were taken using a Nikon confocal laser microscope.

Flow cytometry analysis

HeLa cells were treated with 2% L-anti-HeLa for 30 min at 4°C. After incubation with M-anti-VLRB mAbs (1:100 dilution) and R-anti–C1q-like pAbs (1:100 dilution), the cells were stained with FITC-conjugated goat anti-mouse IgG (1.5 mg/ml, 1:100 dilution; Jackson ImmunoResearch) and PE-conjugated goat anti-rabbit IgG (1:100 dilution; Hanzhou HuaAn Biotechnology) and analyzed by flow cytometry (BD Biosciences). Data analysis was performed using FlowJo software (TreeStar). HeLa cells treated with naive serum were incubated with primary Abs and secondary Abs as negative control. For blank control, HeLa cells were incubated with secondary Ab only.

Statistical methods

SPSS software and the Student two-sample t test were used for statistical analysis.

Results

Cytolytic and agglutination effects of lamprey antisera

We generated lamprey antisera by immunizing animals with RRBCs, SRBCs, Gram-negative E. coli, B. proteus, Gram-positive S. aureus, M. smegmatis, or tumor cells (NB4 and HeLa) (Fig. 1A). Sera from naive lampreys or those injected with 0.9% NaCl also showed some degree of cytolytic effect on these cells, possibly because of complement activation via lectin and the alternative pathways. The specific cytolytic effects of the individual Ag-specific generated antiserum were statistically significant (p < 0.01).

However, the antiserum prepared against the Gram-positive bacteria S. aureus or M. smegmatis did not exert any detectable cytotoxic effect (Fig. 1A), despite the fact that VLRB agglutination responses against these bacteria were of the same magnitude as those observed against Gram-negative bacteria (Fig. 1B).

SEM analysis revealed that the antiserum-treated RRBCs and E. coli were swollen and deformed, which was indicative of cytolyis (Fig. 1C). Further TEM examination of the cytolyis process of E. coli showed that, after incubation with antiserum, the cells underwent a rapid necrosis process, exhibiting swelling and loss of membrane integrity. Some prominences and channels were formed at the surface of the membrane, and the cell content was released to the external environment through the channels. With time, the cytoplasmic leakage and vacuole formation resulted in complete cell lysis (Fig. 1D).

Characteristics of bacteriolytic and hemolytic activity of lamprey antisera

The cytotoxic effect of L-anti-RRBC against RRBCs was dose dependent, with 80% of the cells showing lysis when the concentration of L-anti-RRBCs reached 4%, whereas further increases in L-anti-RRBC concentrations up to 40% had no additional effect (Fig. 2A). In addition, the cytotoxic effect caused by L-anti-RRBCs was temperature sensitive, with maximum lysis occurring between 4 and 20°C. The cytotoxic effect gradually diminished with increasing temperatures; it underwent a rapid decline at temperatures >50°C and was largely lost at 56°C (Fig. 2B). Furthermore, the cytotoxic effect of L-anti-RRBC was time dependent, reaching a maximum within 20 min, while showing no further increase in intensity with prolonged incubation for up to 120 min (Fig. 2C).

Identification of Abs

All of the primers for cloning of VLRB, C3, and C1q-like cDNA and RT-PCR of C1q-like mRNA are listed in Table I. The Ab specificity of M-anti-VLRB mAbs (5G12 and 4G10) and mouse anti–C1q-like mAbs (3G4) was assayed by ELISA (Supplemental Figs. 1B, 2B). 5G12 or 3G4 reacted with specific generated antiserum were statistically significant (p < 0.01).
5G12 was shown to recognize VLRB on the surface of lamprey lymphocytes (Supplemental Fig. 1F).

The titer of R–anti-VLRB pAbs, R–anti–C1q-like pAbs, and R–anti-C3 pAbs was assayed by ELISA (Supplemental Figs. 1C, 2C, 3B). The specificity of these Abs was confirmed by Western blot (Supplemental Figs. 1E, 2E, 3C).

VLRB plays a key role in the cytotoxic effects of lamprey antisera

To investigate whether the Ag-specific cytolytic effects of the different antisera were mediated by VLRB, VLRB in the antisera was depleted with M–anti–VLRB mAbs (Fig. 3A). The depletion of VLRB resulted in a reduction of the cytolytic activity in the

FIGURE 3. Function of VLRB to the cytotoxic effects of lamprey antisera. (A) VLRB in the lamprey sera was depleted by treatment with anti-VLRB mAbs and protein G agarose beads. Non-VLRB Abs were used as control. (B) Affinity purification of VLRB from lamprey antisera by anti-VLRB Ab–coupled CNBr-activated Sepharose. C1q-like protein partially complexed with VLRB was coprecipitated with M–anti-VLRB. (C) Requirement of VLRB for the cytotoxicity of the antisera. The RRBC–, E. coli–, or HeLa cell–specific antiserum was subjected to no treatment, depletion of VLRB, depletion of VLRB followed by readdition of purified VLRB, or depletion of non-VLRB, and the cytotoxic activity toward the corresponding cell was determined. Data are the mean percentage ± SE from three independent experiments. (D) Effect of VLRB on the cytotoxic effect of naive serum. VLRB was added to the naive serum, and the cytotoxic effect of the serum toward the corresponding cell was determined. Data are the mean percentage ± SE from three independent experiments. **p < 0.01.

FIGURE 4. C1q-like protein is required for the cytotoxic effect of antiserum and shares a similar tissue distribution with VLRB+ lymphocytes. (A) Western blots showing C1q-like protein depletion by anti–C1q-like Abs. (B) C1q-like protein is required for the cytotoxic effect of the Ag-specific antiserum. Antiserum directed against RRBCs, E. coli, or HeLa cells was subjected to no treatment or treatment as indicated, followed by evaluation of its cytotoxic effect against the corresponding Ag. Naive serum was used as control. Data are mean percentage ± SE from three independent experiments. (C) Tissue-specific expression of C1q-like mRNA. Total RNA from various lamprey tissues was extracted and used as template for RT-PCR using C1q-like protein specific primers. GAPDH served as control. **p < 0.01.
case of RRBCs, E. coli, and HeLa cells (Fig. 3C). The extent of cell killing was also reduced to the level observed from naive serum (Fig. 3D). Adding back the affinity-purified VLRB (Fig. 3B) to the VLRB-depleted antisera restored their cytolytic activity (Fig. 3C). Also, the addition of affinity-purified VLRB to naive serum resulted in intensified cytolytic activity (Fig. 3D). These results indicated that VLRB is essential for the cytotoxicity of the Ag-specific lamprey antiserum.

**C1q-like protein is required for the cellular toxic effects, and C1q-like mRNA shared similar tissue distribution as VLRB* lymphocytes**

In lampreys, C1q-like protein can act as a GlcNAc-specific lectin and associates with a serine protease of the MASP/C1r/C1s family to activate C3 in the lectin pathway (5, 10). To investigate whether C1q-like protein is required for the cytotoxic effect in various lamprey antisera, C1q-like protein in the antisera was depleted with R–anti–C1q-like polyclonal Abs (Fig. 4A). After depletion of C1q-like protein, the cytolytic effect of the antisera was significantly reduced (Fig. 4B), suggesting that C1q-like protein was also critical for the cytotoxic effect of the antisera. Analysis of the tissue distribution of C1q-like mRNA in lampreys showed the intestine had the highest level of C1q-like mRNA, followed by the heart, gills, and kidney, whereas no C1q-like mRNA was detected in the leukocytes and liver cells (Fig. 4C).

**C3 is activated and cleaved into C3b and fixed to the surface of target cells in the cytolytic process**

C3 plays a central role in the activation of complement system in that its activation is required for all three complement-activation pathways (11). To investigate whether C3 is involved in the cytotoxic process in lamprey antiserum, C3 was depleted in the antiserum with the R–anti-C3 pAbs (Fig. 5A). After depletion of C3, the cytolytic effects were reduced significantly (Fig. 5B). Both the α-chain and β-chain of C3 were present in the antiserum, indicating that C3 was not activated. However, after incubating with RRBCs, only the β-chain was detected, indicating that C3 was activated and deposited on the surface of target cells (Fig. 5C). In contrast, a much smaller amount of β-chain was detected on the surface of RRBCs treated with naive serum, indicating that only

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**FIGURE 5.** Activation of C3 protein and its cleavage into C3b. (A) C3 protein in lamprey antiserum was depleted by anti-C3 Abs and protein G agarose beads. (B) Antiserum directed against RRBCs, E. coli, or HeLa cells was subjected to no treatment or treatment as indicated, followed by evaluation of its cytotoxic effect against the corresponding Ag. Naive serum was used as control. Data are the mean percentage ± SE from three independent experiments. (C) Deposition and cleavage of C3 into C3b on the surface of RRBCs treated by l–anti-RRBC sera. **p < 0.01.
a low level of C3 was activated and deposited on the surface of RRBCs. These results indicated that activated C3 might participate in the cytotoxic effect exerted by Ag-specific antisera.

The interaction of VLRB and C1q-like protein

To investigate the interaction between VLRB and C1q-like protein, naive serum and t-anti-HeLa treated and not treated with HeLa cells were each subjected to immunoprecipitation with M-anti-VLRB mAbs or R-anti-C1q-like pAbs, followed by Western blot using R-anti-C1q-like pAbs or M-anti-VLRB mAbs, respectively. A 26-kDa band was recognized by R-anti-C1q-like pAbs when immunoprecipitation was carried out with anti-VLRB mAbs, whereas a 35-kDa band was recognized by M-anti-VLRB mAbs when immunoprecipitation was carried out with R-anti-C1q-like pAbs (Fig. 6A). This suggested that VLRB and C1q-like protein could associate with each other in a complex, both in naive sera and antisera. However, a large number of VLRB and C1q-like proteins remained free in the sera (naive or Ag specific) (Supplemental Fig. 4). Interestingly, the precipitated complex of C1q-like protein and VLRB by anti-VLRB mAb or by anti-C1q pAb was less in the t-anti-HeLa incubated with HeLa cells than in naive serum and antisera without incubating with HeLa cells (Fig. 6A), which implied that most of the VLRB and C1q-like protein was deposited on the surface of HeLa cells. In fact, fluorescent confocal and flow cytometry analysis further confirmed the interaction of VLRB and C1q and their deposition on the surface of target cells (Fig. 6B, 6C).

Discussion

Lectin and alternative complement-activation pathways, including the key complement components MBL, MASP, Bf, C3, and C1q-like proteins, have been described in lampreys (4–7). However, these animals appear to lack Ig-based Abs and many of the components of an Ig-mediated classical complement-activation pathway. Our present study demonstrates that antisera obtained from lampreys immunized with Gram-negative bacteria, RBCs, or NB4 or HeLa cells displayed clear cytolytic effects on the corresponding bacteria or cells, resulting in rapid cell lysis and ultimate disruption of cell wall integrity; however, antisera cannot effectively kill Gram-positive bacteria. This discrepancy may due to the fact that the cell wall of Gram-positive bacteria is made up of a dense layer typically composed of numerous rows of peptidoglycan and molecules of lipoteichoic acid, wall teichoic acid, and surface proteins, which can protect them against complement attack (12, 13). Importantly, the cytolytic effect requires both VLRB and C1q-like proteins. Depletion of either protein resulted in a significant reduction in the cytotoxicity.
In jawed vertebrates, C1q can activate the classical complement-activation pathway by binding to the Fc portion of the Ag-bound IgG or IgM. The classical pathway in jawed vertebrates is triggered by activation of the C1 complex, which is composed of one molecule of C1q, two molecules of C1r, and two molecules of C1s. This activation occurs when C1q binds to the C region 3 (CH3) of the H chain in the Fc region of IgM or to the C region 2 (CH2) of the H chain in the Fc region of IgG complexed with Ags. This pathway can be triggered by a single IgM or multiple IgGs. Moreover, Glu318-Lys320-Lys322 has been identified as a key binding motif for C1q (14, 15). In contrast, a number of non-Ig components binding to C1q can also lead to activation of the classical complement pathway; these molecules include bacterium (16, 17), protozoa (18), tobacco glycoprotein (19), virus-infected cells (20), apoptotic cells (21), lipoteichoic acid (22), and pentraxin 3 (23). In addition, SIGN-R1 lectin can capture microbial polysaccharides in the spleen and bind C1q to initiate assembly of C3 convertase, without requiring either Ab or factor B (24, 25). The lamprey C1q-like protein may also have dual functions. Acting as a GlcNAc-specific lectin, the C1q-like protein associates with a serine protease of the MASP/C1r/C1s family to activate C3 in the lectin pathway (10). In addition, our study showed that C1q-like protein associated with VLRR in the antiserum, forming complexes on the surface of target cells that led to complement activation and subsequent cells lysis. However, the molecular basis of the interaction between VLRR and C1q-like protein is still unknown.

Lampreys lack lymphatic organs, spleen, and thymus. C1q-like mRNA is distributed in the intestine, gill, heart, and kidney. It differs from mammals, wherein C1 levels are highest in peritoneal monocyte-derived macrophages (26–28), with lower levels present in spleen, thymus, heart, and brain; only trace amounts of C1q mRNA were observed in liver, kidney, lung, and small intestine (29). Interestingly, Cooper and colleagues (30) reported that the Ag-binding VLRR cells are distributed in the taphosole, gill region, and kidneys in larval lampreys. The similar distribution of C1q-like protein and VLRR suggested that these tissues may be the primary sites where defensive action against foreign invading organisms takes place, as well as the sites where the lectin pathway mediated by C1q–MASP-A may play a crucial role in innate immunity, through activation of C3 (11). Therefore, the interaction between VLRR and C1q-like protein would initiate the complement-activation cascade, producing an enhanced adaptive-immune response.

C3 plays a central role in the activation of complement system. Its activation is required for all three complement-activation pathways (15). In lampreys, C3 is the essential mediator for opsonization in phagocytosis (31). It can be cleaved and activated by the serine protease MASP-A during the activation of lamprey innate-immune response (11). Our data show that depletion of C3 in lamprey antisera directed against RRBCs, E. coli, or HeLa cells resulted in a significant reduction in its cytolytic effect toward the corresponding cells. Furthermore, the activation of C3 resulted in its cleavage into C3b and subsequent deposition on the surface of target cells. Taken together, the results suggested that the C1q-like protein participates in both innate and adaptive-immune defense mechanisms in lampreys. The lectin pathway mediated by C1q-MASP-A could serve a crucial role in the innate immunity by acting as a first line of defense through activating C3 to counter the invasive pathogens. The animals would later mount an Ag response and produce Ag-specific VLRR Abs. Upon repeated encounter with the same invading pathogens, the interaction between VLRR and C1q-like molecule could activate C3, thereby activating the complement cascade, and produce complement-dependent cytotoxicity, which would ultimately cause the effective destruction of the invaders. Such an immune mechanism has proven effective, as reflected by the long history (500 million years) of lampreys. Thus, the coordinate function of VLRR and components of the complement system provide evidence for the merging of the innate and adaptive-immune responses as an additional safeguard against invasive pathogens. This probably occurred at a pivotal point in the evolution of vertebrates, before the emergence of the Ig and classical complement pathway. The combination of such powerful defense mechanisms might play an important role in ensuring the survival of lampreys by providing them with an effective immune defense.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

SI Figure Legends

Fig. 1. Production and specificity of mouse anti-VLRB mAb and PAbs.
(A) Schematic representation of the cloning of VLRB c terminus, expression of TRX-VLRB recombinant proteins and antibodies production. (B) ELISA assay of M-anti-VLRB mAb. (C) ELISA assay of M-anti-VLRB PAbs. (D) Western blot analysis of VLRB in lamprey sera. Lamprey sera was treated with increasing concentrations of 2-mercaptoethanol (2-ME) and then subjected to western blot analysis using M-anti-VLRB mAb 5G12. (E) Detection of VLRB in lamprey sera by western blot using R-anti-VLRB PAbs. (F) Detection of VLRB on the surface of lymphocyte by flow cytometry. 5G12 was used as primary antibody (1:100 dilutions).

Fig. 2. Production and specificities of M-anti-C1q-like mAb and R-anti-C1q-like PAbs.
(A) Schematic representation of the cloning C1q-like, expression of TRX-C1q-like recombinant proteins and production of antibodies. (B) ELISA assay of M-anti-C1q-like mAb. (C) ELISA assay of R-anti-C1q-like PAbs. (D) Analysis of C1q-like in the three randomly selected lampreys naïve sera by western blot using M-anti-C1q-like mAb, 3G4. (E) C1q-like in the three randomly selected lampreys naïve sera was analyzed by western blot using R-anti-C1q-like PAbs.

Fig. 3. Production and specificity of R-anti-C3 PAbs.
(A) Schematic representation of the cloning of the junctional zone of α chain and β chain of C3, expression of TRX-C3 recombinant proteins and production of anti-TRX antibodies. (B) ELISA assay of R-anti-C3 PAbs. (C) Detection of C3 in three randomly selected lampreys naïve sera by western blot using R-anti-C3 PAbs.

Fig. 4. Immunoprecipitation of VLRB and C1q-like protein from naïve sera and antigen-specific antisera.
Lamprey Naïve serum, L-anti-Hela serum and L-anti-Hela pre-incubated with Hela cells were each incubated with 1 µg M-anti-VLRB mAb or R-anti-C1q-like pAbs at 4°C for two hours. Protein G agarose was then added. The mixture was incubated at
4°C for four hours, followed by centrifugation. The supernatant and pellet were both analyzed by SDS-PAGE using 10% gel, and the protein bands were then transferred to PVDF membrane (Millipore). The membrane was incubated with R-anti-C1q-like pAb or M-anti-VLRB mAb, followed by probing with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., USA) or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., USA), and then detected by enhanced chemiluminescence (ECL) (Pierce). Three types of sera are used in the assay. IP, immunoprecipitate. IB, immuno-dot blot.
Supplemental Figure 1

A. Diagram showing the process of recombinant and expressed TRX-VLRB fragment fusion proteins. The pET32a (+) vector is used for immunization. Rabbit polyclonal antibody and Mouse monoclonal antibody are used for further analysis.

B. Graph showing the OD values for Anti-VLRB monoclonal antibody with dilutions of 5G12, 4G10, and Control.

C. Graph showing the OD values for Anti-VLRB polyclonal antibody with dilutions of Anti-VLRB antibody and Control.

D. Western blots showing the protein bands at 118kDa, 90kDa, 50kDa, and 34kDa with 2-mercaptoethanol concentrations of 0.25%, 0.5%, and 5%.

E. Western blots showing Lamprey sera with dilutions of 0.5μl, 1μl, 2μl, and 4μl.

F. Flow cytometry histograms showing lymphocyte counts for Control and Anti-VLRB groups with M1 markers indicating 36%.
A. pET32a(+) vector

Signal peptide

C1q-like

Recombined and expressed

Thioredoxin (TRX)

TRX-C1q fusion proteins

His-tag

Immunized

Rabbit polyclonal antibody & Mouse monoclonal antibody

B. Anti-C1q monoclonal antibody

OD

3G4
Control

C. Anti-C1q polyclonal antibody

OD

Anti-C1q antibody
Control

D. Sera1 Sera2 Sera3

26kDa

Anti-C1q monoclonal antibody

E. Sera1 Sera2 Sera3

26kDa

Anti-C1q polyclonal antibody
Supplemental Figure 3

A

pET32a(+) vector

Thioredoxin (TRX)

Recombined and expressed

TRX–C3 fragment fusion proteins

Immunized

His-tag

Rabbit polyclonal antibody

B

OD

Anti-C3 antibody

Control

1.2000
1.4000
1.6000
1.8000
2.0000
2.2000
2.4000
2.6000
2.8000
3.0000

1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
1:128000

C

Sera 1  Sera2  Sera3

118kDa

90kDa

50kDa

C3 α chain

C3 β chain
Supplemental Figure 4

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IP: Anti-VLR
IB: Anti-C1q-like
IP: Anti-C1q-like
IB: Anti-VLR

S: Supernatant
P: Precipitation