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The role of serpin protein on the natural immune defense against pathogen infection in *Lampetra japonica*

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ABSTRACT

Serine protease inhibitors (serpins) are a large protein family that is involved in various physiological processes and is known to regulate innate immunity pathways.

However, research for the functional study of serpins in lamprey is limited. In the present study, a serpin gene was cloned and characterized from *Lampetra japonica* at molecular, protein and cellular levels, named L-serpin which belongs to family F serine protease inhibitors (serpin family). The L-serpin includes a serpin domain in the N-terminus. The mRNA transcript of L-serpin was extensively expressed in kidney, supraneural body, intestine, liver, heart, gill and the highest expression in leukocytes. The mRNA expression level of L-serpin increased significantly after *Vibrio anguillarum, Staphylocccus aureus* and Poly I:C stimulation and dramatically peak at 8 h. It is demonstrated that the L-serpin protected cells from lethal Gram-negative endotoxemia through associating with inhibition of lipopolysaccharide (LPS)-triggered cell death and inflammatory factors expression. Surface plasmon resonance (SPR) and the microbe binding assay were used to determine that L-serpin interacts directly with LPS (KD = 6.14×10^{-7} M). Furthermore, we confirmed L-serpin is a major inhibitor of complement activation by inactivating lamprey-C1q protein (KD = 2.06×10^{-6} M). Taken together, these findings suggest that L-serpin is a endogenous anti-inflammatory factor to defend against Gram-negative bacterial challenge and involved in lamprey innate immunity.

1. Introduction

Leukocytes and complement serine proteases play an important role as effectors of the immune system by killing invading pathogens or by destroying infected, abnormal or foreign cells. Members of the serine protease inhibitors (serpins) superfamily regulate such proteases to prevent both tissue damage and the premature death of immune cells [1]. The serpin genes superfamily has been divided into 16 clades based on phylogenetic and similarity analyses of sequences, and the A-I clades were preferentially observed in vertebrates [2,3]. And based on the variance in gene structure, the vertebrate serpins have also been classified into six groups (V1–V6) [4–6]. Up to now, more than 3000 serpin genes have been identified from various species, including animals, plants, prokaryotes, and virus [7]. The average number of amino acids in serpin proteins is 350–400 amino acids, with molecular weight being approximately 40–50 kDa [8]. All serpins are known to share similar tertiary structures with three β-sheets (β-sheet A, β-sheet B and β-sheet C), 8–9 α-helices, and an exposed reactive center loop (RCL). The RCL contains a scissile bond between two residues which serves as a bait for proteolytic attack, named P1 and P1' [9]. Once the target protease recognizes and cleaves RCL, the serpin undergoes a typical serpin stressed-to-relaxed (S-to-R) transition with the insertion of the cleaved RCL into the central β-sheet as a middle strand, leading to translocation and inactivation of the covalently linked protease [10,11]. Serpin proteins involve in multiple basic biology processes, including complement activation, blood coagulation, cell migration, apoptosis, inflammation, tumor suppression and immunology by inhibiting the activity of serine proteases or cysteine proteases [12].

Serpins are an important component of the vertebrate immune response. For example, it has been reported that serpinA1 (α1-antitrypsin) is a potent inhibitor of neutrophil elastase which induces lung tissue damage, liver disease and chronic inflammation [13–15]. Recent

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Table 1
Primers Used in this Study.

Species	Primer name	Nucleotide sequence (5′-3′)	Purpose
Lethentron japonicum	L-serpin-1F	TCAGGTGGAGACCAGAGCGAC	Gene cloning
	L-serpin-1R	ATCATCCCGTCTGACCCCAAT	
	L-serpin-2F	CGCGGATCCATGGCTCGCCTCTCCCCTCTCTT	Protein expression
	L-serpin-2R	CCCAAGCTTCTAATGATGATGCTTCTTTCCC	
	L-serpin-3F	AGCGAAACCGCAGAACTTGAT	Q-PCR
	L-serpin-3R	AAGAAAATGGCGCTCACGAT	
	L-IL-8-F	CACAGAGCTCCAACTGCAAG	Q-PCR
	L-IL-8-R	AATGTGGCTGATCACCTTCC	
	L-IL-17-F	CTGGTTCTGAGACGCACGAA	Q-PCR
	L-IL-17-R	TGTCTCTAGGTCCGGCTTGTAAA	
	L-HMGB1-F	CCCGTCGGCTTTCTTCATC	Q-PCR
	L-HMGB1-R	TTCCACATCTCACCCAGTTTCTT	
	L-LECT-2-F	GTGGCTTGCGATGGGAAGAACG	Q-PCR
	L-LECT-2-R	TGGTTAGAGGGTCCCACTGAG	
	L-GAPDH-F	AACCAACTGCCTGGCTCCT	Q-PCR
	L-GAPDH-R	GTCTTCTGCGTTGCCGTGT	
Homo sapiens	TNF-α-F	TGTAGCCCATGTTGTAGCAAACC	Q-PCR
	TNF-α-R	GAGGACCTGGGAGTAGATGAGGTA	
	IL-1β-F	CTGAGCACCTTCTTTCCCTTCA	Q-PCR
	IL-1β-R	TGGACCAGACATCACCAAGCT	
	IL-6-F	TGGCTGAAAAAGATGGATGCT	Q-PCR
	IL-6-R	TCTGCACAGCTCTGGCTTGT	
	GAPDH-F	AGGTGAAGGTCGGAGTCAACGGA	Q-PCR
	GAPDH-R	TCAAAGGTGGAGGAGTGGGTGTC	

evidence suggests that serpinA1 may act as a signaling molecule by modulating inflammation independently of its protease inhibitory function. SerpinA1 reduces levels of lipopolysaccharide (LPS)-mediated proinflammatory mediators and increases anti-inflammatory cytokines [16,17]. SerpinA3 (α1-antichymotrypsin) prevents degradation of extracellular matrix by proteases released during inflammation by inhibiting cathepsin G and mast cell chymases [18]. Moreover, serpins have been also reported to participate in the regulation of complement system [19–21]. SerpinG1 (C1 inhibitor) is a plasma protein that inhibits various proteases of the complement system, contact system and fibrinolytic system in human [22]. Complement is an essential system for both innate and adaptive immunity against microbial infection within the vertebrate host [23–27]. In jawless vertebrates, the classical complement pathway is activated by variable lymphocyte receptors B (VLRB), which acts as an antibody and specifically binds to the antigens on pathogens [28]. By contrast, the innate immune system of lampreys presents great complexity. Although certain key complement components are identified, including C3, mannose-binding lectin (MBL), C1q, three types of MBL-associated serine proteases (MASP-1, MASP-A, MASP-B), factor B, short consensus repeats (SCR)-containing control protein, and factor I from lamprey at the protein and/or DNA levels [29,30]. The later components (C5, C6, C7, C8, and C9) involved in the lytic pathway have not been founded in lamprey. And L-C1q (Lamprey C1q) is complexed with MASP-A in a Ca^{2+}-dependent manner, then L-C1q-MASP cleaved C3 to generate C3b in the fluid phase [31], which differs from the C1q protein previously identified in mammals that C1q is associated with C1r and C1s and binds to immunoglobulins (IgG and IgM) with concomitant activation of C4 and C2 via the classical pathway [29]. Findings have shown that lamprey C1q binds to VLRB with concomitant activation of complement, similar to the classical pathway of jawed vertebrates [27]. At present, fewer than 10 serpins have been identified in lamprey, which are distributed into four groups [5]. Different with *Danio rerio*, there is no Ig (immunoglobulin-like) domain identified in lamprey [26]. Thus, lampreys may be the best model organism to research the origin of adaptive immune system not based on immunoglobulin (Ig) molecules.

This study reported a serpin from *L. Japonica* (designated L-serpin), with the objectives to (1) analyze its domain structure and motif characteristics, (2) investigate its expression in different tissues and the immune response after *Vibrio anguillarum, Staphylocccus aureus* and Poly I:C challenge, (3) insight into the interaction between L-serpin and LPS, (4) identify L-serpin involving in lamprey innate immunity system by inhibit the activity of lamprey C1q protein.

2. Materials and methods

2.1. Lamprey, challenge, and cell culture

Adult lamprey (*L. japonica)* (200–220 g in weight), including males and females, were obtained from the Tongjiang Valley of Songhua River, Heilongjiang Province, China. These lampreys were maintained in 200 L tanks of a recirculating system at 10 ± 3 °C at Liaoning Normal University for two weeks prior to challenge and RNA isolation. The animal experiments were performed in accordance with the regulations of the Animal Welfare and Research Ethics Committee of the Institute of Dalian Medical University's Animal Care protocol (Permit Number: SCXK2008-0002). Lampreys were assigned equally to four groups and separately immunized with 100 μL of 1×10^7 cells/ml gram-negative bacterium (*Vibrio anguillarum*), gram-positive bacterium (*Staphylococcus aureus*) or Poly I:C (1 μg/μL) via intraperitoneal injection for 0, 2, 8, 24, 48 and 72 h, respectively. Peripheral blood was collected from the caudal subcutaneous sinus of lampreys and diluted 1:1 with PBS, 30 mM EDTA. In our previous study, ficoll-Paque is a well-referenced media for density gradient centrifugation of blood, and buffy coat leukocytes were extracted after 10 min centrifugation at 160 × *g* by Ficoll-Paque gradient centrifugation using the Ficoll-Paque medium (1.092 g/mL) [32]. Total RNA was extracted from each lamprey tissue using TRIzol (Invitrogen, USA), and the RNA was treated with DNase I (TaKaRa, China). Reverse transcription was then performed as previously described [33,34]. Controls omitting the reverse transcriptase (No-RT) were prepared for each sample. The cDNA stored at −80 °C until used in quantitative real-time PCR.

Human cells used, HeLa cells, H293T cells, RAW264.7 cells, HUVEC cells and MCF-7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM, RPMI-1640 supplemented with 10% FBS (Gibco, Grand Island, NY) in a humidified atmosphere containing 5% CO_2 at 37 °C. These cell lines were purchased frozen and were freshly thawed before the experiments.

2.2. Cloning L-serpin genes and bioinformatics analysis

Based on the expressed sequence tag analysis of the cDNA library, which was constructed using lamprey leukocytes in our laboratory, a serpin homolog sequence was found using Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) database. Total RNA was isolated from lamprey leukocytes using TRIzol (Invitrogen, USA), and cDNA was synthesized using a High Fidelity PrimeScript™ RT-PCR Kit (TaKaRa Biotechnology, Dalian, China). Primers L-serpin F1 and R1 (Table 1) were designed based on the expression sequence tag (EST) of the most homologous serpin. The PCR product was purified and cloned into the pMD-19T vector using a DNA ligation kit (TaKaRa Biotechnology, Dalian, China), and transformed into *Escherichia coli* (*E. coli*) DH5α competent cells. Then the positive clones were sequenced by TaKaRa.

The amino acid sequence deduced from the L-serpin gene was analyzed using online tools available at ExPASy (http://www.expasy.org/tools/scanprosite). The putative ORF was analyzed for the presence of N-linked glycosylation sites with the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). The glycosylation sites prediction was conducted with CBS Prediction Servers (http://www.cbs.dtu.dk/services/). Total amino acid sequence alignments of serpin family members, including L-serpin, were performed using ClustalX 1.81 with the default settings. The obtained results were converted into MEGA format and imported into MEGA 5.05 to construct a phylogenetic tree using the neighbor joining (NJ) method and 1000 bootstrapped replicates. Multiple sequence alignments were performed using ClustalX 1.81 and BioEdit. Genomic data for serpins from vertebrates were extracted from the Ensembl genome browser (www.ensembl.org). The conserved motif analyses were performed on-line using MEME version 5.0.5 (http://meme-suite.org/) with the default settings. Functional domain analyses of L-serpin were conducted using SMART (http://smart.embl-heidelberg.de) online tools, and SWISS-MODEL (http://swissmodel.expasy.org/interactive) online was used to predict the 3D structure of L-serpin.

2.3. Expression and purification of the L-serpin protein

The open reading frame (ORF) of L-serpin, flanked by *BamH*I and *Hind*III restriction sites, was amplified and subcloned into the pCold I vector (TaKaRa, Japan) with a histidine (His) tag. The recombinant pCold I-L-serpin was expressed in Rosetta blue [35,36]. In brief, rL-serpin protein expression was induced with 0.1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) (Sangon Biotech, China) for 24 h at 16 °C. L-serpin purification was performed by Ni-NTA His-Bind resin column and the concentration was measured using a Bicinchoninic Acid (BCA) Protein Assay kit (Beyotime, China). The purified L-serpin protein was then examined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue (Sangon Biotech, China).

2.4. Preparation of the anti-L-serpin polyclonal antibody and western blot analysis

A polyclonal antibody against rL-serpin was generated by subcutaneously injecting the purified protein (200 μg, emulsified with Freund's adjuvant) into adult New Zealand White rabbits as described previously [33–35]. After 6 subsequent injections, increasing the immunizations at 1-week intervals, blood was drawn from the rabbit carotidartery, and the antibody was purified using Protein G MagBeads (GenScript, USA). For antiserum titer determination by ELISA, 200 ng of rL-serpin protein was diluted in 100 of polyclonal anti-L-serpin antibody at different dilutions (2000-fold–640,000-fold) in each well of a 96-well ELISA plate. Total proteins were extracted from lamprey tissues using cell lysis buffer (Beyotime, China) and the protein concentration was measured using Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime, China). For western blots, 50 μg of total protein from the different lamprey tissues was subjected to 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk and incubated with rabbit anti-L-serpin (1:1000 dilution) antibody overnight at 4 °C followed by incubation with HRP-conjugated goat anti-rabbit IgG (1:5000). The membrane was developed with enhanced chemiluminescence (ECL) substrate (Beyotime, China).

2.5. Quantitative real-time PCR (Q-PCR)

The template was L-serpin plasmid as previously described, PCR amplification L-serpin gene CDS area 1371 bp, removed termination codon of 3' race, and added a *Nhe*I restriction site and a *Sal*I site, was amplified and subcloned into the pIRES2-AcGFP1-Nuc vector (Clontech, USA) to produce pIRES2-AcGFP1-Nuc-L-serpin.

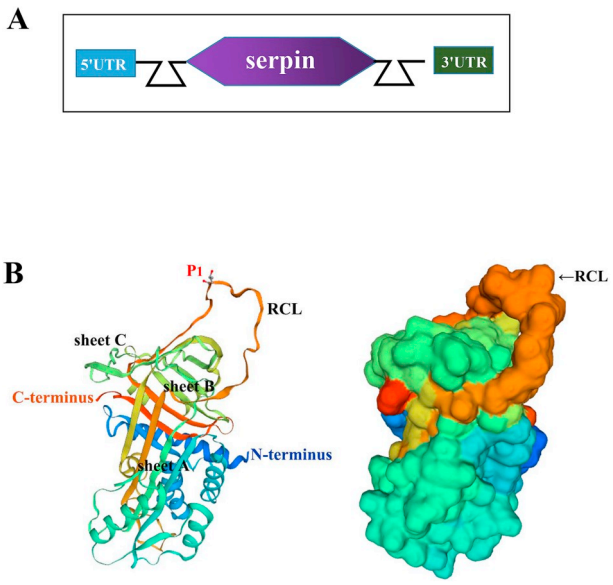


Fig. 1. Sequences analysis and the predicted structure of the L-serpin from *L. japonica*. (A) The functional domains of L-serpin are according to http://smart.embl-heidelberg.de. The L-serpin protein includes a serpin domain in the N-terminus. The two loop-like regions indicate the omitted amino acids. (B) Schematic representation of L-serpin fold. Blue, helical region; and the three β-sheets are distinguished by different color. The exposed RCL is located near the carboxyl-terminus, and is colored in orange. (C) Phylogenetic tree for L-serpin among different species. A NJ tree was constructed using the amino acid sequences of L-serpin proteins (see C; right) and nucleotide sequences (left) of the complete genome of serpins isolated from different regions. Sequences are identified by the species and name abbreviations, followed by the accession numbers of sequences from GenBank or Ensembl (http://www.ensembl.org). The bar (0.1) indicates genetic distance. The triangle indicates L-serpin from lamprey. (D) Multiple alignments of L-serpin with other known clade F serpins of vertebrates. Completely conserved amino acids are indicted by "*", and conservative substitutions by "; ". The P1–P1′ residues are indicated. Sequences are identified by species and name abbreviations, followed by the accession numbers of the amino acid sequences extracted from the NCBI protein database in brackets. Species abbreviations: (Hs) *Homo sapiens*; (Mm) *Mus musculus*; (Am) *Alligator mississippiensis*; (Xl) *Xenopus laevis*; (Dr) *Danio rerio*; (Gg) *Gallus gallus*; (*Pm*) *Petromyzon marinus*; (Lf) *Lampetra fluviatilis*; (Ga) *Gasterosteus aculeatus*; (Ga) *Gasterosteus aculeatus*; (Oa) *Ovis aries*; (Pa) *Parambassis ranga*; (As) *Alligator sinensis*; (Tr) *Takifugu rubripes*; (Pr) *Parambassis ranga*; (Bt) *Bos taurus*; (Rn) *Rattus norvegicus*; (Ci) *Ctenopharyngodon idella*; (Tn) *Tetraodon nigroviridis*; (Sp) *Strongylocentrotus purpuratus*; (Xt) *Xenopus tropicalis*. Serpin name abbreviations: (C1INH) C1 inhibitor; (MENT) myeloid and erythroid nuclear termination; (PEDF) pigment epithelium-derived factor; (HC2 or HCII) heparin cofactor 2; (AGT) angiotensinogen; (AT) antithrombin; (HSP47) Heat shock protein 47 kDa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

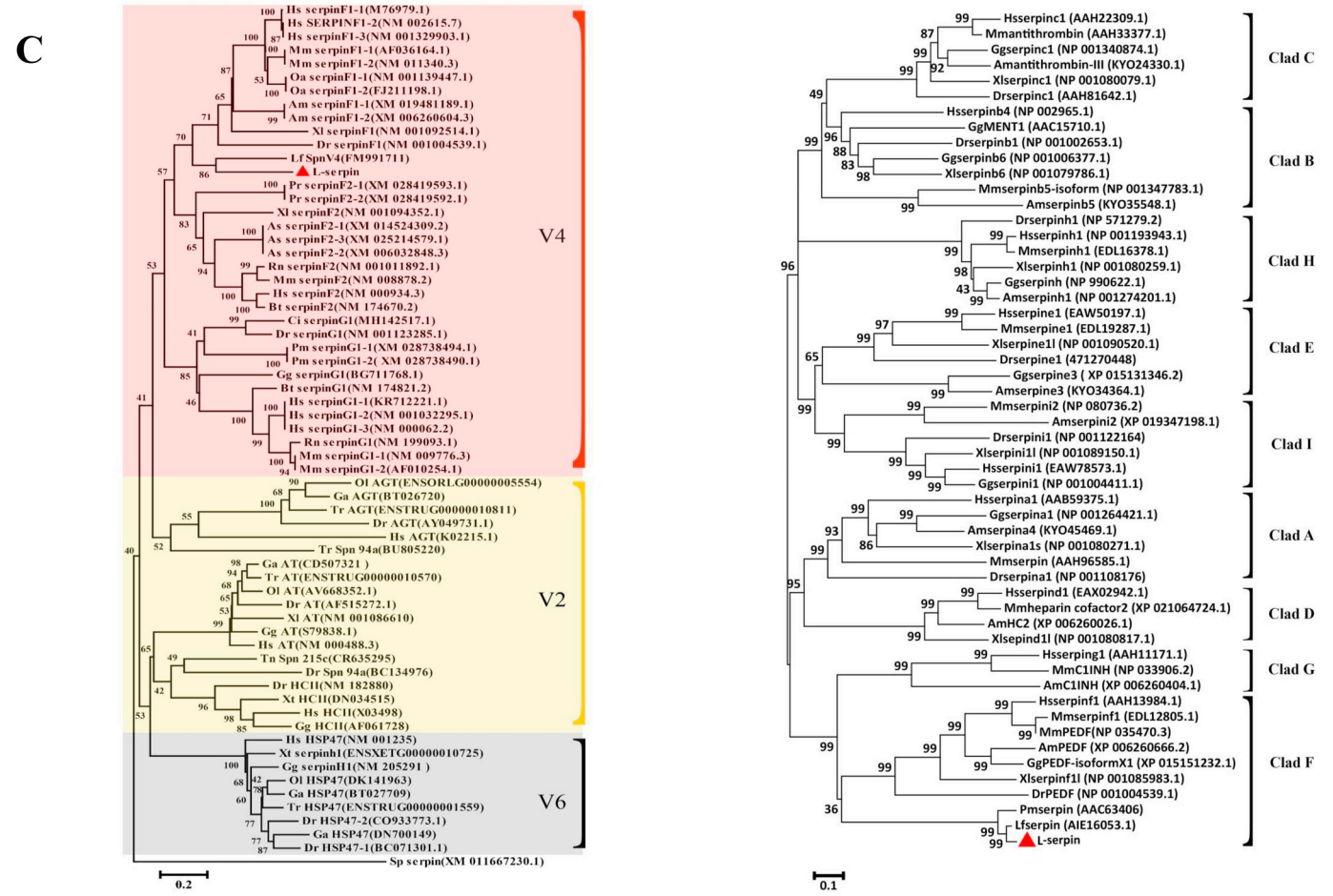


Fig. 1. (*continued*)

The pIRES2-AcGFP1-Nuc-L-serpin or pIRES2-AcGFP1-Nuc were transfected into H293T cells for 48 h. Then, treated with LPS (2.5 μg/mL) or PBS for 4 h. The cells were collected and extracted total RNA using TRIzol, and the RNA was treated with DNase I (TaKaRa, China). Reverse transcription was performed as previously described [33–35]. Controls omitting the reverse transcriptase (No-RT) were prepared for each sample. Q-PCR was conducted with the TaKaRa SYBR® Prime-Script™ RT-PCR Kit according to the manufacturer's protocol. The level of expressed protein in each sample was normalized relative to the *gapdh* gene (GenBank accession no. KU041137.1). The details of the primer sequences for Q-PCR are listed in Table 1. The data are shown as the mean ± SD from three independent experiments, and p values were calculated using Student's t-test (*$p < 0.05$, **$p < 0.01$).

2.6. Fluorescence-activated cell sorting (FACS) analysis

Lamprey were injected intraperitoneally with 100 μL the gram-negative bacterium (*V. anguillarum*, 8×10^7 CFU) for 0, 12 and 24 h, respectively. Isolate the leukocytes of the lamprey and preparation for further analysis.

L.*japonica* leukocytes cells were plated in tubes and fixed for 20 min in 90% methanol in PBS at room temperature. Then, the cells were washed three times with PBS, blocked with normal goat serum for 30 min, and incubated with anti-L-serpin rabbit antibody (200-fold) in PBS for 1 h at room temperature. After being washed three times, the cells were incubated with FITC-conjugated donkey anti-rabbit IgG (500-fold) for 45 min at room temperature in the dark followed by three washes with PBS. The cells were resuspended with PBS and analyzed on a FACS by flow cytometer (BD Biosciences). Cells incubated with FITC-conjugated goat anti-rabbit IgG were used as isotype controls. Data analysis was performed using Flowjo software (Tree Star).

2.7. Laser scanning confocal microscopy

MCF-7 cells were plated on glass coverslips (Fisher Scientific, Pittsburgh, PA), treated with LPS (175 ng/mL) or LPS (350 ng/mL) in the presence of 1640 with L-serpin (2.0 μg/mL, 2 h, 37 °C), the rabbit IgG (2.0 μg/mL) as the negative control. And then cells washed with PBS, and fixed with 4% formaldehyde. MCF-7 were blocked with normal goat serum for 30 min, and incubated with rabbit anti-L-serpin antibody (200-fold) at 4 °C overnight. After the overnight incubation, the cells were washed twice with PBS and then incubated with Alexa Fluor 488 (400-fold) goat anti-rabbit IgG. Following two more washes with PBS, the cells were stained with DAPI (200-fold). After two washes with PBS, the coverslips were mounted on glass slides with one drop of antifade solution. The immunofluorescence was visualized and captured with a Zeiss LSM 780 inverted microscope (Carl Zeiss, Inc) and analyzed using Zeiss ZEN LE software.

2.8. High content imaging assay

The exponentially-growing H293T cells were seeded into 96-well plates at a density of 1×10^5 cells/ml in a final volume of 100 μL medium for 24 h. Then, the pIRES2-AcGFP1-Nuc-L-serpin plasmids were transfected into H293T cells for 48 h using a Lipo3000 kit (Invitrogen, USA). Subsequently, after treated with LPS for 4 h, the cells were applied to a fluorescent dye cocktail, which contained Propidium Iodide (PI) and Hoechst to determine the cell number and nuclear

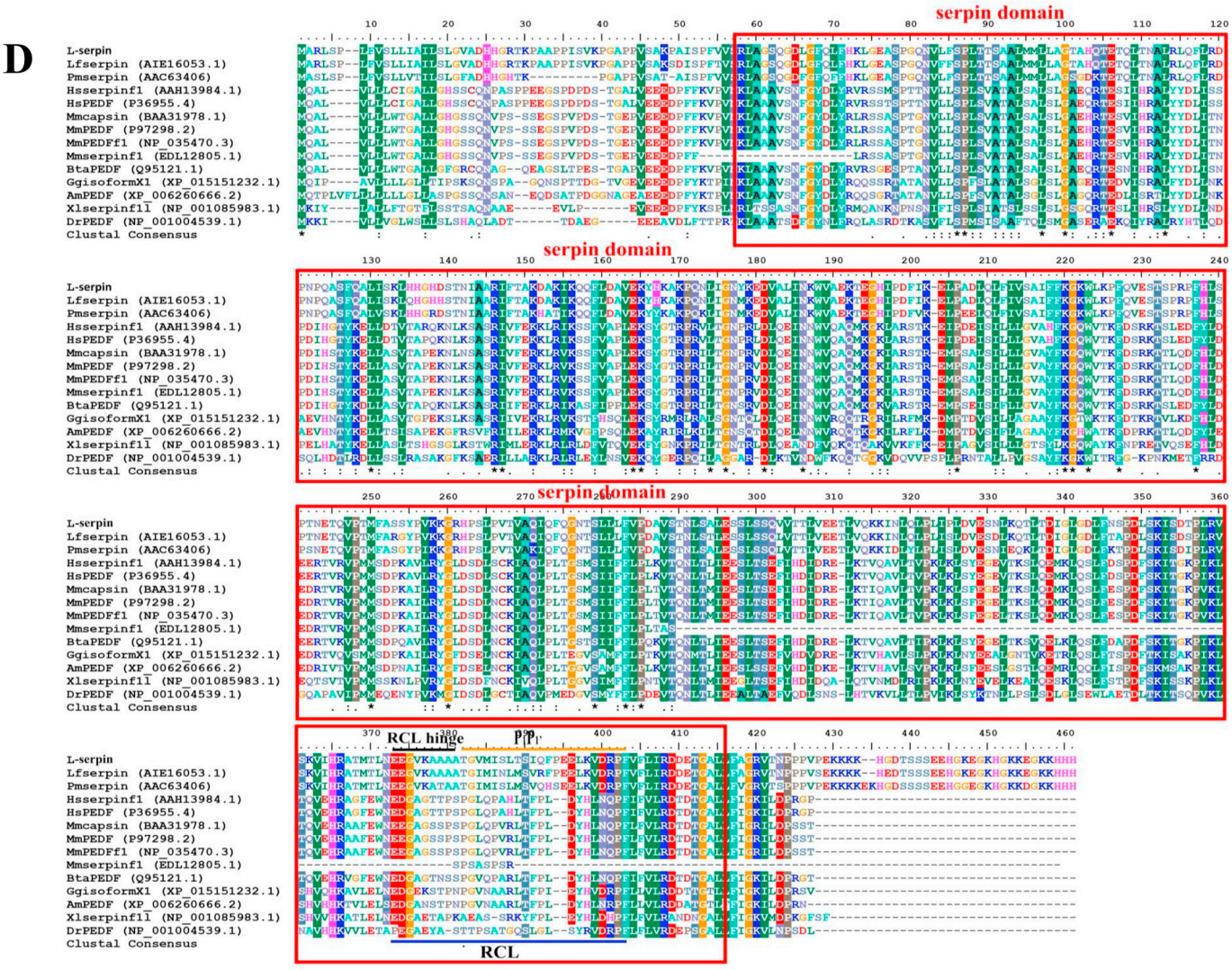


Fig. 1. (*continued*)

intensity. The excitation and emission wavelengths were 346 nm and 460 nm, respectively. The signal of the cell membrane permeability dye was acquired with 504 nm excitation and 523 nm emission wavelengths. The images were observed and examined using 20 × objective The empty pIRES2-AcGFP1-Nuc vector was used as a control.

2.9. LPS and microbial binding assay

Analysis of ligand binding kinetics was performed at 25 °C on a BIAcore T200 Surface Plasmon Resonance (SPR) instrument (GE Healthcare, USA) [36]. The running buffer was HBS-EP (GE Healthcare,

Table 2
Conserved motifs discovered in vertebrates using the MEME syestem.

Motif	Width	Best possible match
1	29	YHVBRPFLFVJRDDETGALLFIGKVLBPN
2	29	PDLSKISDKPLKVSSVQHRATLELNEEGV
3	33	KELPTDVSJLLLGAIHFKGKWLTKFDPRKTSPR
4	30	VAQLPFKGNMSLLFFLPDKVTQNLSLIEES
5	37	LAAAVSBFGYDLFRKLAEESPTPNVJLSPLSVATALS
6	30	RIYLEKGLRIKEDFLEQVEKYYGAKPQILT
7	50	MLSLGAGZRTESQITRALYYDLLRDPEVHATYKDLLSSLTAPEKSLKSAA
8	21	DLAEINKWVKEQTEGKIPRFL
9	22	FHLDEDRTVQVPMMSAPKYPLR
10	29	AVLKLPKJKLDYESELKQTLTELGLQELF
11	27	KLALGATNSTEERLKEGLHAKSEPCLH
12	14	EEEDPFYKTPVNK
13	14	EAAAATGVMISRMS
14	41	MARLSPLFVSLLIAILSLGVADHHGRTKPAAPPISVKPGAP
15	15	SEFVHDIDRELKTVH
16	12	KHGDSSSSEEHGGEGKKEGKKHHH

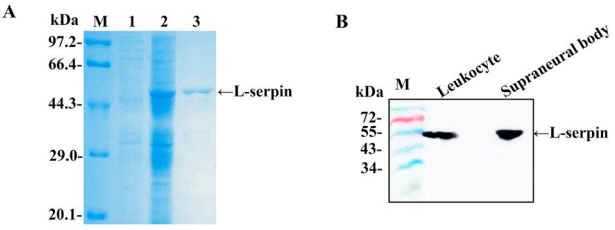


Fig. 2. Titer and specificity analysis of a polyclonal antibody against the rL-serpin protein. (A) SDS-PAGE analysis of rL-serpin protein expressed in Rosetta blue bacteria. M: high molecular weight protein marker; Lane 1: non-induced expression of Rosetta blue/pColdI-rL-seroin; Lane 2: induced expression of Rosetta blue/pCold I-rL-serpin; Lane 3: purified recombinant protein. (B) Western blotting confirmed the specificity of the anti-L-serpin polyclonal antibody. M: pre-stained protein ladder, Lane 1: lamprey leukocytes; Lane 2: lamprey supraneural body tissues. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

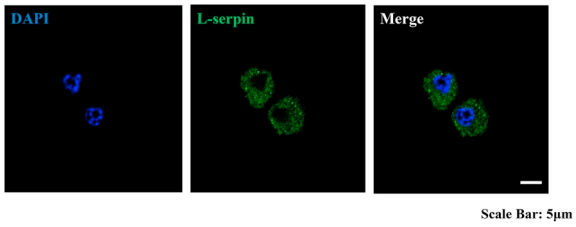


Fig. 3. The subcellular location of L-serpin. The localization of endogenous L-serpin in leukocytes via Immunofluorescence. Binding of antibody to L-serpin was visualized by Alexa Fluor 488 goat anti-rabbit antibody (green), and the nucleus was stained with DAPI (blue), bar = 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

USA) containing 10 mM HEPES (pH 7.5), 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) Surfactant P20. Firstly, rL-serpin was immobilized onto the CM5 sensor chip surface according to the instructions of Amine Coupling Kit (GE Healthcare, USA). The LPS is injected into the wells and the flow was controlled at a flow rate of 30 μL/min. Lastly, the LPS were washed with 10 mM glycine-HCl (pH 1.5) at a flow rate of 20 mL/min. The resulting date after subtracting the control values were analyzed by fitting to a 1:1 Langmuir binding model using the BIAcore T200 evaluation software.

Microbial-binding activity of rL-serpin was measured according to previous report with some modification [37]. Gram-negative bacteria (*Vibrio anguillarum* and *Vibrio splendidus*), Gram-positive bacteria (*Micrococcus lysodeikticus, Bacillus cereus*) were employed to detect the microbial-binding ability of rL-serpin. The microbes were suspended with TBS, and incubated with rL-serpin (500 μL, 0.30 mg/mL) under slight rotation at 4 °C overnight. After rinsed by TBS for five times, the bound proteins were dissociated from the microorganisms by loading buffer and analyzed by SDS-PAGE and Western blot. The rTrx protein was employed as negative control.

2.10. Site-directed mutagenesis and detection of cytotoxicity of lamprey serum

For functional analysis, the L-serpin sequences were systematically mutated in the predicted variable region [clones M1 (T386A) and M2 (S387A)] using oligonucleotide-based site-directed mutagenesis. The mutant molecules were expressed, purified as specified above for the wild type molecules.

To detection of cytotoxicity of lamprey serum. Addition of L-serpin-WT protein (8 μg), L-serpin-M1 protein (8 μg), L-serpin-M2 protein (8 μg), rabbit anti-L-serpin antibody (10 μg) or rabbit IgG antibody (10 μg) to the native lamprey serum, then slight rotation at 4 °C overnight. Subsequently, after pre-incubation of two antibody groups with 50 μL protein G at 37 °C for 6 h. All groups centrifugation at 3000 × *g*, 4 °C for 50 min, the supernatant (10 μL) were added to HeLa cells or MCF-7 cells. The HeLa cells and MCF-7 cells were seeded in a 96-well plate with the density of 5×10^4 cells/well in the incubator. The cells were treated with above mentioned serum groups for 24 h, respectively. PBS as the negative control. After the addition of 10 μL CCK8 solution/well, they were incubated for 2 h. A microplate reader (BioTek, USA) was used to measure the absorbance at a wavelength of 450 nm.

2.11. Statistical analysis

All statistical analyses were done using GraphPad Prism 5.0 software. Differences between treatment groups were determined by Student's t-test. $P < 0.05$ was set as the threshold for significance (*$P < 0.05$, **$P < 0.01$). Bar charts show the means ± SDs of three independent experiments.

3. Results

3.1. Characterization of L-serpin and phylogeny analysis

The ORF sequence of L-serpin encoded a polypeptide of 456 amino acids with a predicted molecular mass of 49.6 kDa, as shown in Fig. S1A. The L-serpin contained an N-terminal serpin (serine protease inhibitor) domain (Fig. 1A). The tertiary structures of L-serpin containing the canonical α-helices and β-sheets, are conserved with other known serpins. The exposed RCL was located near the carboxyl-terminus (Fig. 1B).

As the previous study demonstrated that vertebrate serpins are divided into six groups (namely V1–V6) among serpin genes [4–6]. And a BLAST search of the NCBI database showed that the amino acid sequence deduced from L-serpin shares 33.51%–87.6% overall sequence identities with the group V4 serpins of the *Latimeria chalumnae*, *Perca flavescens*, *Numida meleagris*, *Gallus gallus*, *Petromyzon marinus* and *Lampetra fluviatilis* (Fig. S1C). Phylogenetic analysis based on the nucleotide sequences indicated that L-serpin is indeed the member belong to group V4 (Fig. 1C left). To identify the gene structure of the L-serpin and other group V4 serpin, this study demonstrated that the L-serpin gene consists of nine exons and eight introns, the automatic annotation by the Ensembl database reveals only eight exons in the serpin gene of *P. marinus*. In addition, the numbers of exons encoding the evolutionarily conserved serpin domain are reduced (Fig. S1D). In the phylogenetic tree, serpins from vertebrates include L-serpin were clustered together and formed nine sub-clusters from clade A to clade I. As shown in Fig. 1C (right), the L-serpin placed outside of the vertebrate clade F and belong to the clade F in the serpins superfamily. Multiple sequence alignment of the L-serpin and clade F serpins from vertebrates based on the phylogenetic tree, indicating that L-serpin has strongly conserved serpin domain and high sequence homology with other vertebrates. The P1–P1′ scissile bond of the core feature RCL from lamprey serpin is represented for Threonine-Serine (TS), in contrast to other vertebrates, the P1–P1' scissile bond almost is the Methionine-Serine or Leucine-Threonine (Fig. 1D). The comparison of clade F serpins of vertebrates using the MEME system indicated a high level of conservation in the motif composition (Fig. S1E and Table 2). The vertebrate serpins all contain nine motifs (motifs 5, 6, 8, 3, 9, 4, 10, 2, and 1), whereas certain other motifs (motifs 14 and 16) only exist in L-serpin and *Pm*serpin.

3.2. Purification of recombinant L-serpin and the subcellular location of L-serpin

The recombinant plasmid (pCold I-*L-serpin*) was transformed into Rosetta blue. After IPTG induction at 16 °C 100 rpm for 24 h, the whole cell lysate was analyzed by SDS-PAGE, and a distinct band was revealed with a molecular mass of 49.6 kDa (Fig. 2A), which was in accordance

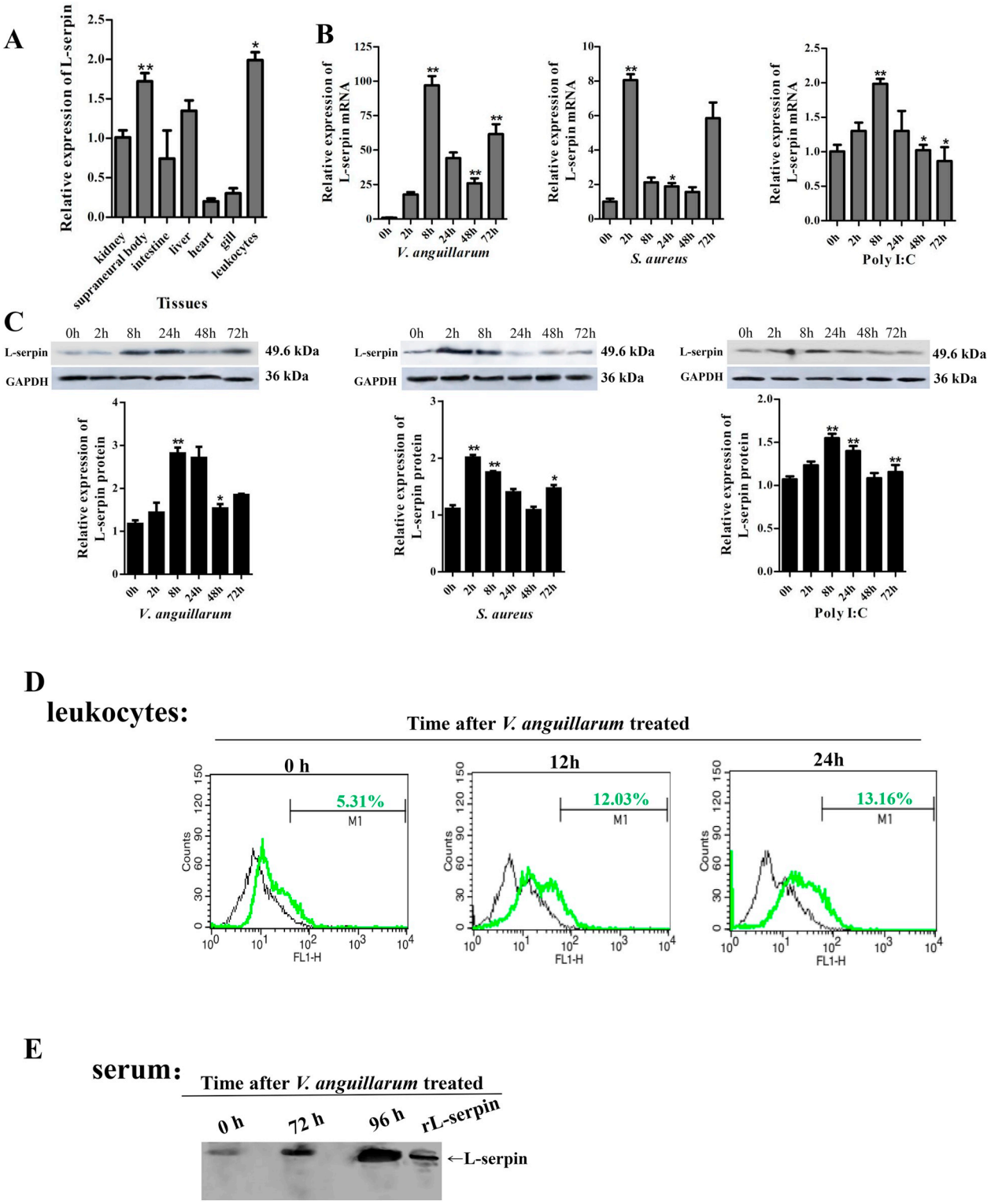
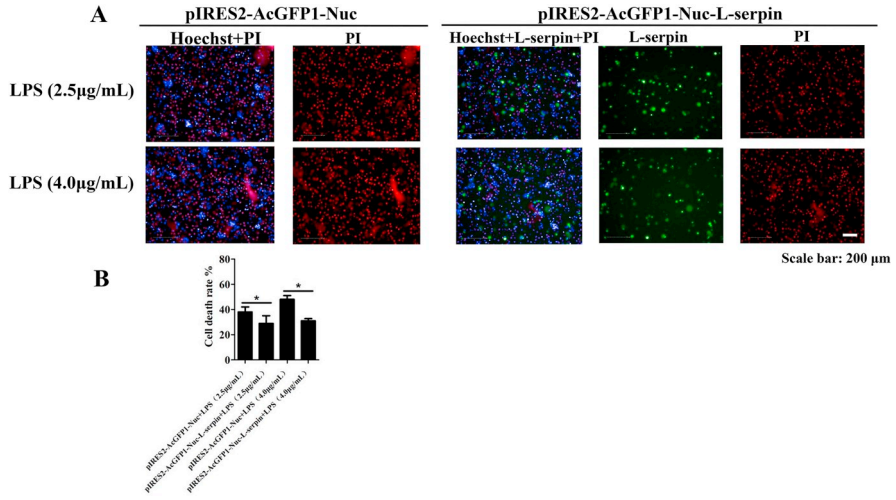
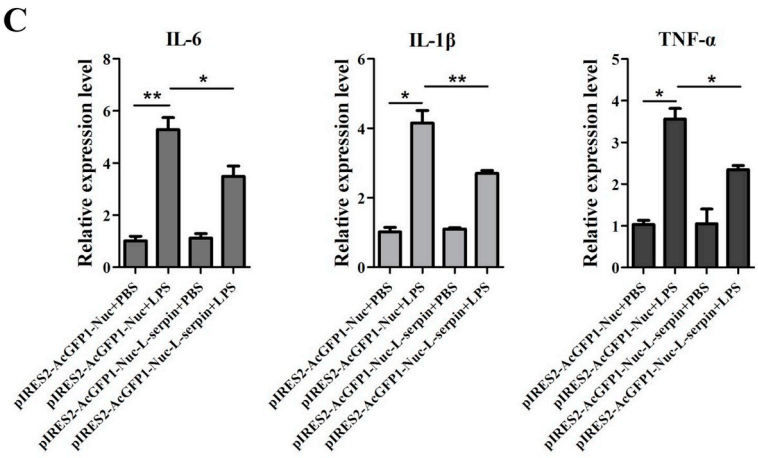


Fig. 4. Expression of the L-serpin in *L. japonica*. (A) Q-PCR analysis of L-serpin mRNA expression in adult lamprey tissues. Immune response to bacteria. (B) Q-PCR and (C) Western blot analysis of L-serpin in lamprey to characterize L-serpin mRNA and protein expression levels in leukocytes at 0, 2, 8, 24, 48 and 72 h after treated with *V. anguillarum*, *S. aureus* and Poly I:C. Histogram showing statistics for the above results. (D) The detection of the L-serpin protein expression in leukocytes from lampreys after *V. anguillarum* treated by flow cytometric analysis. Representative contour plots gated on serpin positive leukocytes (green line). The black line represents cells with the IgG isotype control. (E) Western blot analysis of L-serpin protein expression in lamprey serum at 0, 72 and 96 h after treated with *V. anguillarum*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

with the predicted molecular mass of L-serpin.

Next, a rabbit anti-L-serpin polyclonal antibody was generated and antibody titer was detected by ELISA. Plasma L-serpin antibody concentrations were increased by 640,000-fold over preimmunization levels, and pre-immunized rabbit IgG was used as a negative control (Fig. S2). The specificity of the Abs, as determined by western blotting, showed that the anti-L-serpin antibody recognized native serpin protein from lamprey leukocytes and supraneural body tissues (Fig. 2B). No





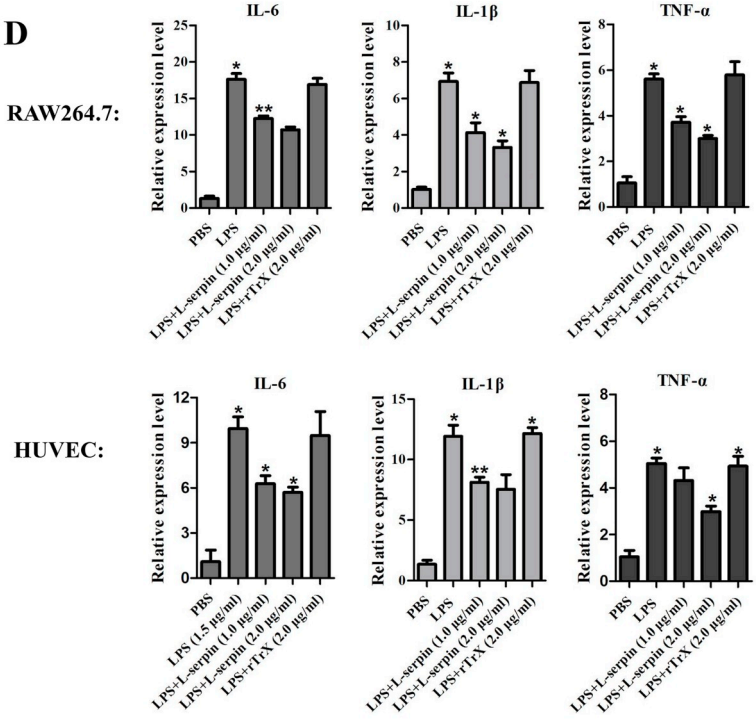


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Fig. 5. L-serpin inhibits LPS-induced cell death and inflammatory factors secretion in vitro. (A–C) H293T cells were transfected with or without L-serpin plasmid at 37 °C for 48 h, then treated by LPS or PBS for 4 h. High content screening (A–B) and Q-PCR (C) were used to observe the cell death rate and the inflammatory factors expression levels of H293T cells. (D) Cultured RAW246.7 cells and HUVEC cells were grown on 6-well plates and then incubated with 2.5 μg/mL LPS in the absence or presence of L-serpin for 4 h. Q-PCR analysis the levels of IL-6、IL-1β and TNF-α. *P < 0.05, **P < 0.01. (E) L-IL-17, L-IL-8, L-HMGB1 and L-LECT-2 mRNA levels were determined via Q-PCR in lamprey supraneural body cells or leukocytes after stimulation with LPS (2.5 μg/mL) for 4 h in the absence or presence of L-serpin. (F–G) The effect of LPS on the binding of L-serpin to MCF-7 cells. MCF-7 cells were incubated with L-serpin (2.0 μg/mL) in the absence or presence of LPS for 4 h, then were examined using laser scanning confocal microscopy. The rabbit IgG (2.0 μg/mL) as the negative control. Here are a representative of three independent experiments with similar results.

visible reaction band was found in the negative control (data not shown).

The endogenous localization of L-serpin in leukocyte was detected by immunofluorescence and confocal laser-scanning microscopy. The nucleus was stained with DAPI and observed in blue, while the positive signals of L-serpin in green were distributed in the cytoplasm (Fig. 3).

3.3. Bacterial infection induces L-serpin expression in leukocytes and serum

In the present study, the mRNA expression of L-serpin in kidney, gill, intestine, heart, leukocytes, supraneural body and liver were analyzed by the Q-PCR method, the *gapdh* gene was used as an internal control. As the results showed that the highest expression of L-serpin was detected in leukocytes, and moderate expression was examined in supraneural body, liver, and kidney (Fig. 4A).

To further investigate the immune response of L-serpin to pathogenic infection, lampreys were challenged with *Vibrio anguillarum, Staphylocccus aureus* and ploy I: C, respectively. After infection, the expression of leukocyte-enriched L-serpin was detected by Q-PCR (Fig. 4B) and western blotting (Fig. 4C). As shown in Fig. 4B, a significant increase mRNA level of L-serpin at 2 h post *S. aureus* injection (hpi) was detected ($P < 0.01$). Later on, with time passing, at 8 hpi, the expression of L-serpin at both ploy I: C and *V. anguillarum* challenged groups were remarkably elevated ($P < 0.01$), especially *V. anguillarum* stimulation. The expression of L-serpin was decreased at *S. aureus* (24 hpi) and other two groups (48 hpi). As for L-serpin protein level, the results of Western blot were basically consistent with those of Q-PCR analysis, and the most potent effect of L-serpin was observed at *V. anguillarum* after the injection (Fig. 4C). Furthermore, the expression levels of L-serpin protein in the leukocytes by FACS analysis after lampreys are challenged with *V. anguillarum* for 0, 12 and 24 h (Fig. 4D). The results showed that the expression of L-serpin increased after challenged with *V. anguillarum* in the adult lamprey leukocytes, when compared with 0 h normal controls. To investigate whether L-serpin protein is expressed in lamprey serum, we detected L-serpin protein expression levels in lamprey serum after treated with *V. anguillarum* for 0, 72 and 96 h by Western blot (Fig. 4E). Our data revealed that the expression of L-serpin significantly upregulated at 72 h and 96 h in lamprey serum in response to *V. anguillarum*, when compared with 0 h normal controls.

3.4. L-serpin protects cells against LPS-mediated cell death and pro-inflammatory cytokine expression

To determine the effect of L-serpin on cell death rate and pro-inflammatory cytokine expression in human or lamprey cells induced by LPS in vitro. H293T cells were treated with LPS for 4 h, after pIRES2-AcGFP1-Nuc-serpin was transfected into the H293T cells for 48 h, the pIRES2-AcGFP1-Nuc-transfected H293T cells were used as negative controls (Fig. 5A and Fig. S3). The results shown that L-serpin prevented LPS-induced apoptosis in H293T cells using high content screening (HCS) (Fig. 5B). In addition, the mRNA expression of IL-1β, IL-6 and TNF-α were measured by Q-PCR whether H293T cells or H293 cells overexpression L-serpin. The over-expression of L-serpin dramatically decreased pro-inflammatory cytokine levels as compared with the vector alone (Fig. 5C). In order to detect anti-inflammatory effect of L-serpin, RAW246.7 cells and HUVEC cells were treated with LPS in the absence or presence of recombinant L-serpin for 4 h. The results showed that the IL-1β, IL-6 and TNF-α levels in the cells were significantly decreased by addition of L-serpin as compared with the negative control (Fig. 5D). In order to further identify the role of L-serpin in anti-inflammatory activity for lamprey cells. Lamprey leukocytes and supraneural body cells were treated with LPS in the absence or presence of L-serpin for 4 h and the expression of L-IL-17, L-IL-8, L-LECT-2 and L-HMGB1 were tested. The results of Q-PCR demonstrated that the mRNA expression of L-IL-17, L-IL-8, L-HMGB-1 and L-LECT2 was significantly increased in response to LPS stimulation. However, the expression of L-IL-17, L-IL-8, L-HMGB-1 and L-LECT2 was also reduced with LPS in combination with rL-serpin treatment (Fig. 5E). Based on these findings, L-serpin protects cells against LPS-mediated cell death and pro-inflammatory cytokine expression.

To identify the relationship between LPS and L-serpin, MCF-7 cells were cultured in the presence of L-serpin that had been incubated with or without LPS. Then were imaged using laser scanning confocal microscopy. The fluorescent signals on the surface of the cells were significantly increased at a concentration of 175 ng/mL LPS and 350 ng/mL LPS, when compared with the control (Fig. 5F and G). This suggests that the L-serpin protein protect cells from effect of LPS by binding to the cell surface.

3.5. The binding activity of L-serpin towards gram-negative bacteria or LPS

To further investigate whether L-serpin prevented LPS-induced inflammatory factors by binding directly to Gram-negative endotoxin LPS, SPR experiments was performed to explore the binding affinity of L-serpin (0.30 mg/mL) towards LPS with gradient concentrations (0.24 μM, 0.48 μM, 0.97 μM, 1.95 μM, 3.9 μM, 7.8125 μM and 15.625 μM). The KD value of L-serpin towards LPS was 6.14×10^{-7} M (Fig. 6A). This shown that L-serpin displayed a strong LPS binding activity.

Lipopolysaccharide is a major constituent of the outer membrane of Gram-negative bacteria and is a key molecule in the pathogenesis of Gram-negative endotoxemia, sepsis, and septic shock [39]. The microbe binding assay was carried out to analyze the ability of rL-serpin to bind microbes. A clear band was detected for *V. anguillarum, V. plendidus*, *M. Lysodeikticus, B. cereus* respectively, while no band was observed for TBS rinsed water. The binding activity of rL-serpin towards Gram-negative bacteria was stronger than that towards Gram-positive bacteria microbes (Fig. 6B). No visible band was observed in negative control. The results of Western blot were basically consistent with SPR. These results suggested that the L-serpin displayed LPS binding activities.

3.6. L-serpin inhibit the complement-dependent cytotoxicity of lamprey serum

As a previous study had shown that P1 and P1' residues play the primary determinants of serpin specificity by contributing most of the interactions [40]. Thus, we substituted these two sites with single alanine residues, respectively (Fig. 7A). And expression and purification of the two L-serpin mutant proteins (Fig. 7B). Then SPR analysis was performed using a Biacore T200 to identify the interacting and binding affinity of L-C1qDC-1 (0.35 mg/mL) towards L-serpins with gradient

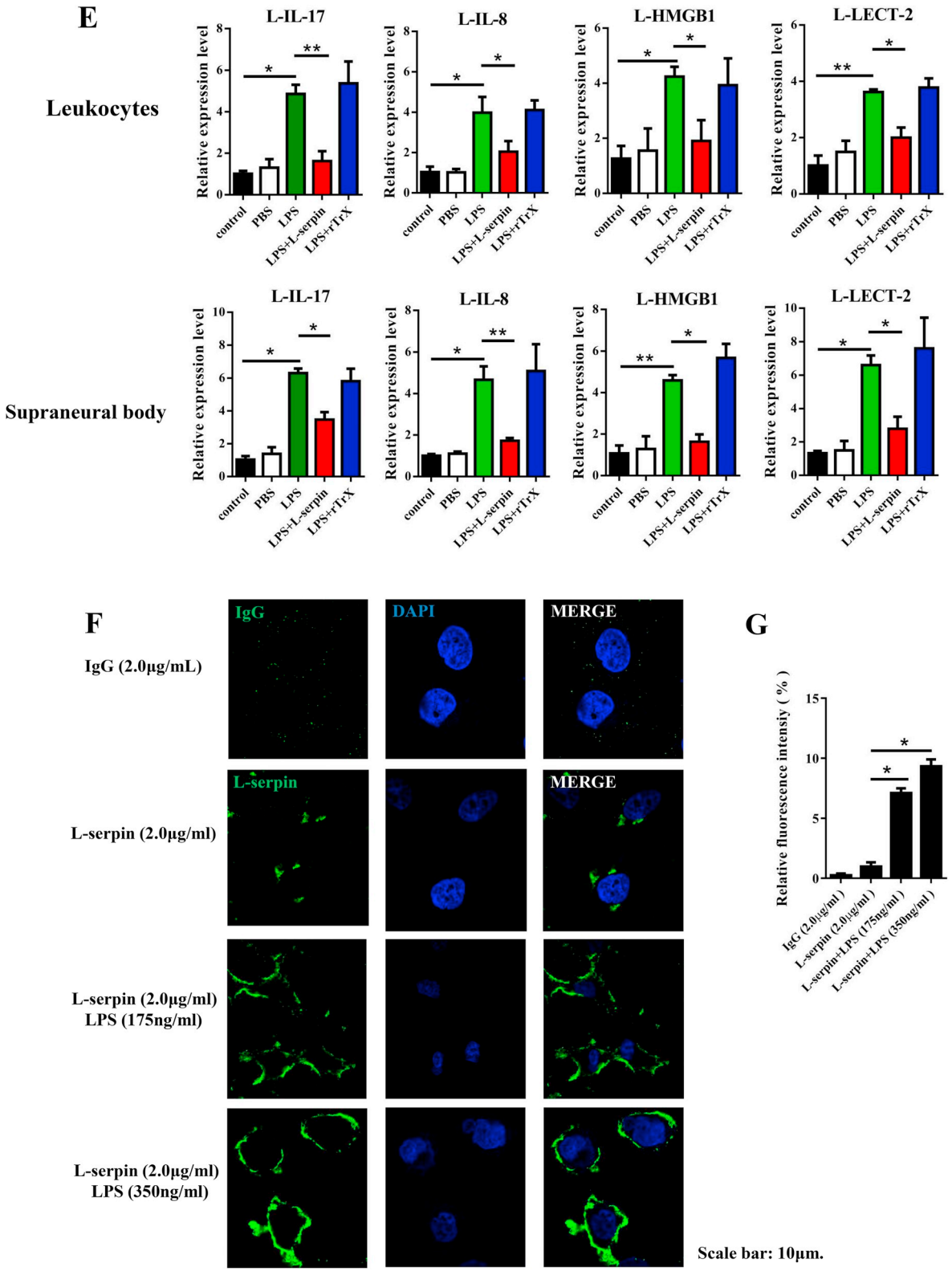

Fig. 5. (*continued*)

concentrations (0.134685 μM, 0.296375 μM, 1.0775 μM, 2.155 μM, 4.310 μM and 8.620 μM). The KD value of L-serpin towards L-C1qDC-1 was 2.06×10^{-6} M, while no binding activity was observed for other L-serpin mutant proteins (Fig. 7C).

Our previous study demonstrated that L-C1q protein mediated complement-dependent cytotoxicity in the killing of bacteria and tumor cells in previous study [41]. To investigate whether L-serpin inhibit the tumor cells killing activity of lamprey serum, HeLa cells and MCF-7 cells were treatment with serum plus rL-serpin or L-serpin-depleted serum, and the cell viability was calculated by CCK8 assay, the native serum treated group as the negative control. The results showed that the lamprey serum depleted of native L-serpin increase the cytolytic activity to MCF-7 cells and HeLa cells compared with naive serum. Adding the wild type L-serpin (L-serpin-WT) to the serum reduced their

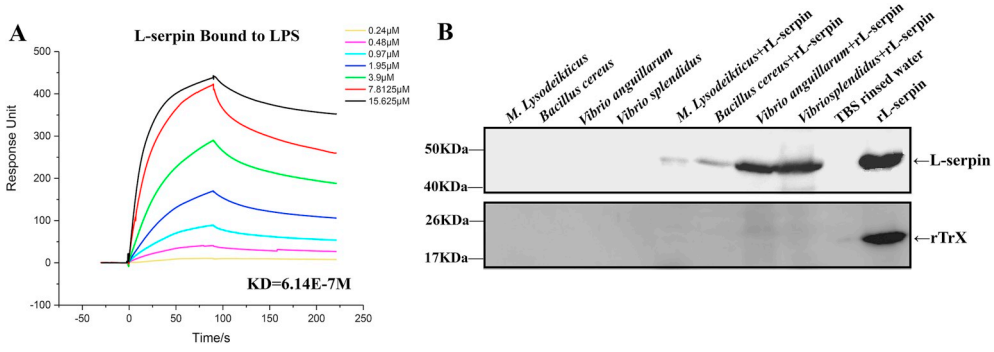

Fig. 6. Interaction of L-serpin with LPS. (A) Response units were plotted against protein concentrations. BIAcore diagram of L-serpin protein bound to the LPS. The KD values were calculated by the BIAcore T200 analysis software (BIAevaluation Version 3.1). The data presented here are a representative of three independent experiments with similar results. (B) The microbe binding activity of rL-serpin revealed by western blotting. Recombinant L-serpin (rL-serpin) was used as the positive control. PBST rinsed water for the last time was to confirm there was no free recombinant protein in sediment. The recombinant trx protein was used as negative control.

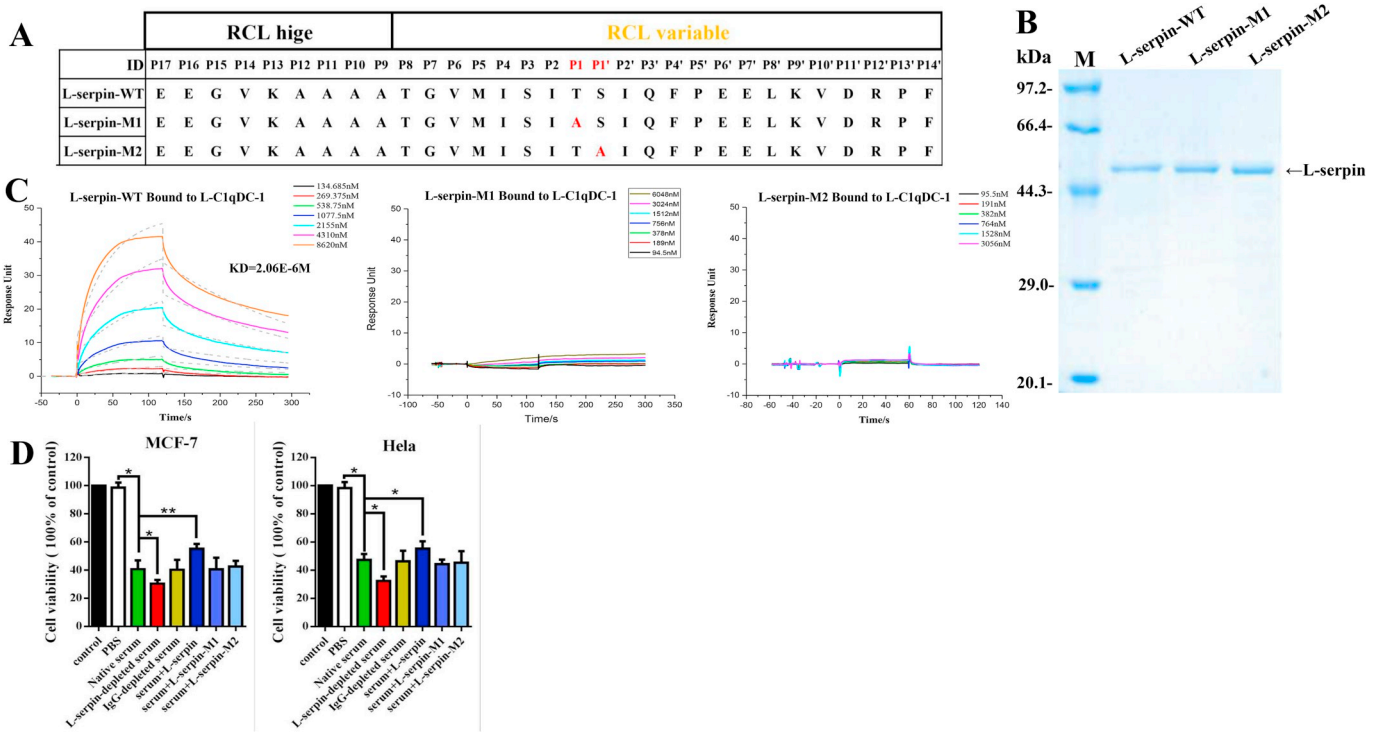

Fig. 7. L-serpin inhibit the complement-dependent cytotoxicity of lamprey serum. (A–B) Sequence details and recombinant expression of L-serpin and mutants. (A) Shown are the sequence changes in the L-serpin mutants compared to wild type sequences. Point mutations were introduced in the variable region of the RCL domain that resembles the protease substrate and is attacked by a target protease. (B) Purification of L-serpin and mutants. Shown are the purified recombinant proteins by SDS-PAGE and Coomassie staining. Both L-serpins revealed the expected bands of 49.6 kDa. M: pre-stained protein ladder. (C) Response units were plotted against protein concentrations. BIAcore diagram of L-serpin-WT, L-serpin-M1, L-serpin-M2 protein, bound to the L-C1qDC-1 protein. The KD values were calculated by the BIAcore T200 analysis software (BIAevaluation Version 3.1). The data presented here are a representative of three independent experiments with similar results. (D) Cytotoxic effects of *Lampetra japonica* serum as determined by a CCK-8 assay in the panel of MCF-7 cells or HeLa cells after treatment with serum plus L-serpin or L-serpin-depleted serum compared to native serum.

cytolytic activity compared with the naive serum, while the two mutants showed distinct changes in inhibition, completely abolished its ability to inhibit the tumor cells killing activity (Fig. 7D). These results indicated that L-serpin inhibited cytotoxicity of lamprey serum via binding to L-C1qDC-1.

4. Discussion

Serpins are a family of serine protease inhibitors that play significant roles in numerous physiological processes and are known to regulate innate immunity pathways. Serpin is structurally conserved in the core serpin domain from *Petromyzon marinus* to mammalian. In this study, a new serine protease inhibitor (L-serpin) with a typical serpin domain was identified from lamprey (*L. japonica*). L-serpin shares the common primary amino acid sequence with typical serpins, while the P1–P1' scissile bond of the core feature RCL is represented for Threonine-Serine (TS) not the Methionine-Serine or Leucine-Threonine in other serpins, speculating that the special amino acid substituents may affect the recognition for proteases. Furthermore, P17 within the hinge region is typically a glutamic acid residue. This P17 has been identified as glutamic acid for 39 serpins analyzed [38].

In order to gain a better understanding of the tissues distribution of L-serpin in the lamprey, the transcription and protein profiles of L-Serpin were investigated in lamprey by quantitative real-time PCR and western blotting, respectively. The mRNA of L-serpin was expressed in all the examined tissues of Lamprey with a high level in liver, kidney, supraneural body and leukocytes (Fig. 4A). The result suggested that liver was the major organ for the expression of serpin in normal

conditions, in good agreement with other species, such as human, mouse, rock bream and rainbow trout [42–45]. In human, hepatic parenchymal cells are the major serpin synthesis site [46]. In mice, Q-PCR analyses revealed higher mRNA levels in liver, lung and heart [47].

Regardless of the nature role, we aimed to confirm whether the L-serpin was involved in the immune response against foreign pathogens. The mRNA and protein expression of L-serpin were up-regulated upon challenging by pathogenic bacteria in leukocytes and serum (Fig. 4B–E). After challenges with *V. anguillarum*, the highly significant up-regulation in expression of L-serpin gene was appeared early at 2 h post-infection (p.i.) ($P < 0.05$). However, following the *V. anguillarum* challenge, a down-regulation occurred late during 8–48 hpi. in leukocytes (Fig. 4B). Similar to the results previously observed in black rockfish and grass carp, the serpin mRNA expression was strongly up-regulated in a time-dependent increase manner throughout the challenge period in black rockfish and grass carp [48,49]. In addition, the expression of *Oreochromis niloticus* serpin (C1 inhibitor) apparently increased in monocytes/macrophages against *S. agalactiae* and *A. hydrophila* [20]. In light of these results, L-serpin as an immune molecular participates in the immune response of host against pathogen bacteria.

Complement system plays an important role in host defense by mediating acute inflammatory reactions and killing pathogenic microorganisms [50]. In the present study, L-serpin was found to display a specific binding to L-C1qDC-1 while its mutant protein not. However, the complement system is nonspecific and thus capable of attacking pathogens as well as host cells, if without proper regulatory mechanisms to restrict its activity, which will cause detrimental effects on healthy host cells. Since serpin is the well-known complement regulator of the classical and lectin pathways, the investigation of expression profiles of this regulatory mediator in response of pathogen challenge will provide potential clues on how L-serpin regulate the response in lamprey.

Our data presented here suggest that in addition to protection via inhibition of complement and contact system activation, L-serpin may also protect from endotoxin shock by directly interacting with endotoxin. Importantly, we demonstrated that L-serpin prevented LPS-induced inflammatory factors by binding directly to Gram-negative endotoxin LPS. Carbohydrates that can be recognized by the corresponding domains in PRRs. The lectins with multiple domains display a much wider recognition spectrum of microbes and stronger binding affinity to the carbohydrates on the surface of pathogens [51,52]. In addition, serpin inhibited LPS-triggered macrophage expression of TNF-α mRNA by interacting directly with LPS in mice [39]. CgCaspase-1 and CgCaspase-3 could also directly recognize LPS, involving in the innate immune response of oyster [53,54]. As expected, interaction of L-serpin and LPS was determined via surface plasmon resonance experiments (SPR), and microbial binding assay confirmed that L-serpin bound and deposited on Gram-negative bacteria, while recombinant thioredoxin did not display binding activity as negative control (Fig. 6B). However, we also found that L-serpin weakly bound and deposited on Gram-positive bacteria, speculating that the interaction of L-serpin with lipoteichoic acid (LTA) can be another potential interesting target to further research.

All these evidences collectively might imply that L-serpin plays a role on the regulation of complement system in response of pathogen infection. Moreover, L-serpin was a key molecule to balance the immune response of host against pathogen bacteria as anti-inflammation factor. Our previous study demonstrated that lamprey LECT-2 gene (L-LECT-2) was a chemotactic factor in lamprey, and the release of lamprey HMGB1 gene (L-HMGB1) can induce cell inflammation [55,56]. The expression of L-IL-17, L-IL-8, L-HMGB-1 and L-LECT2 was also reduced with recombinant L-serpin treatment (Fig. 5E). Taken together, the current study provided the evidence for the possible involvement of L-serpin in lamprey against pathogenic bacteria, and the difference among three levels (molecular, protein and cellular) of L-serpin in response to different stimuli. The findings suggest potentially productive and intriguing avenues for future research. Herein, the regulatory mechanism of L-serpin remains to be fully elucidated.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.062.

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