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Immunosuppressive effects of sialostatin L1 and L2 isolated from the taiga tick *Ixodes persulcatus* Schulze

Yamato Sajiki[a], Satoru Konnai[a,*], Akie Ochi[a], Tomohiro Okagawa[a], Naftaly Githaka[a], Masayoshi Isezaki[a], Shinji Yamada[a], Takuya Ito[b], Shuji Ando[c], Hiroki Kawabata[c], Carlos Logullo[d], Itabajara da Silva Vaz Jr[e], Naoya Maekawa[a], Shiro Murata[a], Kazuhiko Ohashi[a]

[a] Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, 060-0818, Sapporo, Hokkaido, Japan
[b] Hokkaido Institute of Public Health, 060-0819, Sapporo, Hokkaido, Japan
[c] National Institute of Infectious Diseases, Toyama, Shinjuku-ku, 162-8640, Tokyo, Japan
[d] Laboratório Integrado de Bioquímica Hatisaburo Masuda and Laboratório Integrado de Morfologia, NUPEM-UFRJ, Macaé, RJ, Brazil
[e] Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Avenida Bento Goncalves, 9500, Prdio 43421, Porto Alegre 91501-970, RS, Brazil

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ABSTRACT

Tick saliva contains immunosuppressants which are important to obtain a blood meal and enhance the infectivity of tick-borne pathogens. In Japan, *Ixodes persulcatus* is a major vector for Lyme borreliosis pathogens, such as *Borrelia garinii*, as well as for those causing relapsing fever, such as *B. miyamotoi*. To date, little information is available on bioactive salivary molecules, produced by this tick. Thus, in this study, we identified two proteins, *I. persulcatus* derived sialostatin L1 (Ip-sL1) and sL2 (Ip-sL2), as orthologs of *I. scapularis* derived sL1 and sL2. cDNA clones of Ip-sL1 and Ip-sL2 shared a high identity with sequences of sL1 and sL2 isolated from the salivary glands of *I. scapularis*. Semi-quantitative PCR revealed that Ip-sL1 and Ip-sL2 were expressed in the salivary glands throughout the life of the tick. In addition, Ip-sL1 and Ip-sL2 were expressed even before the ticks started feeding, and their expression continued during blood feeding. Recombinant Ip-sL1 and Ip-sL2 were developed to characterize the proteins via biological and immunological analyses. These analyses revealed that both Ip-sL1 and Ip-sL2 had inhibitory effects on cathepsins L and S. Ip-sL1 and Ip-sL2 inhibited the production of IP-10, TNFα, and IL-6 by LPS-stimulated bone-marrow-derived dendritic cells (BMDCs). Additionally, Ip-sL1 significantly impaired BMDC maturation. Taken together, these results suggest that Ip-sL1 and Ip-sL2 confer immunosuppressive functions and appear to be involved in the transmission of pathogens by suppressing host immune responses, such as cytokine production and dendritic cell maturation. Therefore, further studies are warranted to investigate the immunosuppressive functions of Ip-sL1 and Ip-sL2 in detail to clarify their involvement in pathogen transmission via *I. persulcatus*.

1. Introduction

Tick saliva plays an important role to obtain a blood meal, and enhances pathogen transmission. Tick saliva contains several bioactive factors, such as anti-coagulants, vasodilators, and immunosuppressants (Brossard and Wikel, 2004). Among the varied functions of tick salivary proteins, suppression of host immunity via reduction of cytokine production or inhibition of activation and proliferation of immune cells influences anti-tick defenses of the host to the greatest extent (Kazimírová and Štibrániová, 2013).

Cystatins, which are cysteine protease inhibitors, are potential vaccine antigens, related to tick blood digestion, embryogenesis, and evasion of host immune response (Parizi et al., 2018). BrBmcys2b and BrBmcys2c are cystatins identified from *Rhipicephalus microplus*, and are involved in blood digestion and evasion of host immune responses (Parizi et al., 2013, 2015). Rhcyst-1 is a cystatin identified in *Rhipicephalus haemaphysaloides*, and the disruption of the *RHcyst-1* gene induces a significant decrease in hatching rate (Wang et al., 2015). In addition, sialostatin L1 (sL1) and sialostatin L2 (sL2) are tick-derived cystatins isolated from the salivary glands of *Ixodes scapularis*. Both sL1 and sL2 are present in the tick saliva of *I. scapularis* and are injected into hosts during blood feeding. These proteins inhibit the activity of cathepsins L and S (Kotsyfakis et al., 2006, 2007), which play important roles in antigen processing, dendritic cell maturation, and cytotoxic T

* Corresponding author: Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, 060-0818, Sapporo, Hokkaido, Japan.
E-mail addresses: konnai@vetmed.hokudai.ac.jp (S. Konnai), carlos.logullo@bioqmed.ufrj.br (C. Logullo).

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cell (CTL) proliferation (Honey and Rudensky, 2003; Lombardi et al., 2005; Lieskovská et al., 2015a). Indeed, these proteins have been reported to inhibit CTL proliferation, reduce inflammatory cytokine production by dendritic cells, and suppress dendritic cell responses to interferon by inhibiting the JAK–STAT pathway (Kotsyfakis et al., 2006; Sá-Nunes et al., 2009; Lieskovská et al., 2015a, b). Additionally, several reports have indicated that sL2 is an important factor during blood feeding because the number of ticks attached to hosts and extent of blood feeding were significantly reduced by sL2 immunization (Kotsyfakis et al., 2007, 2008). More interestingly, sL2 enhances the transmission of *Borrelia burgdorferi* sensu lato (Kotsyfakis et al., 2010). Additionally, in *I. scapularis*, sL1 has been shown to exhibit the potential to prevent autoimmune diseases in a murine model, suggesting that this protein can be used as a novel therapeutic agent (Sá-Nunes et al., 2009). Thus, sL1 and sL2 have been proposed as vaccinating antigens that interact with both host immunity and hemostasis.

We have previously identified tick molecules, such as defensin (Saito et al., 2009), Salp15 (Mori et al., 2010), lipocalins (Konnai et al., 2011), TROSPA (Konnai et al., 2012), Salp16 (Hidano et al., 2014), metalloproteases (Ali et al., 2014), and Ipis (Toyomane et al., 2016) in *I. persulcatus*. *I. persulcatus* is a vector for pathogens of Lyme borreliosis agent (Nakao and Miyamoto, 1994; Masuzawa, 2004), human granulocytic anaplasmosis (Ohashi et al., 2005; Murase et al., 2011), and tick-borne relapsing fever (Taylor et al., 2013; Takano et al., 2014) in Japan. However, unlike the abundant data available for *I. scapularis* or *I. ricinus*, information regarding bioactive molecules in *I. persulcatus* remains limited. In addition, no candidate vaccinating antigens against *I. persulcatus* have been reported to date.

Thus, in this study we sought to determine whether the sL1- and sL2-like molecules isolated from *I. persulcatus*—named Ip-sL1 and Ip-sL2, respectively—can inhibit the host immune response. For this purpose, we cloned and sequenced Ip-sL1 and Ip-sL2 transcripts and subsequently generated recombinant proteins for biological and immunological assays.

2. Materials and methods

2.1. Tick RNA samples

Samples derived from *I. persulcatus* were obtained from laboratory colonies maintained using Syrian hamsters as hosts (Japan SLC Inc., Shizuoka, Japan) in an isolated experimental animal facility under a strict acaricidal control regimen (Experiment No. 11-0093) (Konnai et al., 2008). All experiments conformed to the guidelines of the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University, Japan, as described previously (Konnai et al., 2008).

2.2. Identification of full-length nucleotide sequences of sL1 and sL2 isolated from I. persulcatus

cDNA synthesis and RT-PCR were performed as described previously (Konnai et al., 2008). Specific primers were designed based on the sequence of *I. scapularis* sL2 reported in the GenBank database (AF483724). An aliquot (1 μl) of cDNA obtained from the salivary glands of fed ticks was used as a template in a 20 μL PCR reaction mixture containing primers specific to the open reading frame of sL2 (sense primer: 5′-ATG ACT GCT TCC TTC GCT TTG G-3′, anti-sense primer: 5′-TTA TGC GGC CGC ACA CTC GAA-3′). PCR conditions using Taq polymerase (Takara Bio, Otsu, Japan) were as follows: initial 5-min incubation at 94 °C, followed by 45 cycles of incubation at 94 °C for 30 s, 60 °C for 60 s, and 72 °C for 60 s and final extension at 72 °C for 7 min. The PCR product was purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) and cloned into a pGEM T Easy vector (Promega, Madison, WI, USA) for sequencing. The purified plasmids were sequenced using an 8-capillary Beckman CEQ 2000 automated sequencer using a Quick Start kit (Beckman Coulter Inc., Fullerton, CA, USA), according to the manufacturer's instructions. The 3′ and 5′ RACE system (Invitrogen, Carlsbad, CA, USA) for rapid amplification of cDNA ends was used according to the manufacturer's protocol to obtain the full-length sequences of the cDNAs encoding transcripts of Ip-sL1 and Ip-sL2. The following primers were designed and used based on the obtained sequences. For 5′ RACE: Ip-sL1 5′ GSP1, 5′-CTG AGG AAC TTG TCT C-3′; Ip-sL1 5′ GSP2, 5′-TGC GAC CTT GAG CGT CAG TC-3′; Ip-sL1 5′ GSP3, 5′-ACC AGC AAC CGT CTG CGT CT-3′; Ip-sL2, 5′ GSP1, 5′-CGT CAG TCT GTA GTT AGT-3′; Ip-sL2 5′ GSP2, 5′-CGT TTC GAC CTT CAG CAC TT-3′; and Ip-sL2 5′ GSP3, 5′-ACG GTG TCG AAG TGA GTC TTG-3′. For 3′ RACE: Ip-sL1 3′ GSP1, 5′-GAT ACA GCG AGA AGG CGA AC-3′ and Ip-sL1 3′ GSP2, 5′-CAC GTC CAC GTA CAA CAA GG-3′. After obtaining the 5′- and 3′-end sequences, the following primers were designed for cloning the full-length nucleotide sequences: Ip-sL1-ORF-5′, 5′-ATG ACT GCT TCC TTC GCT TTG G-3′ and Ip-sL1-ORF-3′, 5′-TTA TGC GGC CGC ACA CTC GAA-3′ for Ip-sL1; and Ip-sL2-ORF-5′, 5′-ATG ACT TCC TCC CTC GCT TTG G-3′ and Ip-sL1-ORF-3′ for Ip-sL2. Products were sequenced as described above.

2.3. Phylogenetic analysis

A phylogenetic analysis was conducted as described previously, with slight modifications (Mori et al., 2010; Rangel et al., 2017). Briefly, we performed the neighbor-joining method using MEGA software version 7. Evolutionary distances were calculated using the Poisson correction method. Bootstrap support was assessed using 1000 replicates. The accession numbers of analyzed have been provided previously (Rangel et al., 2017).

2.4. Expression analysis using semi-quantitative PCR

Synthesis of cDNA from several tissues and stages and under different blood feeding conditions was performed as described previously (Mori et al., 2010). PCR was conducted as described earlier except that the following specific primer sets were used: for Ip-sL1 (Ip-sL1-ORF-5′ and Ip-sL1-ORF-3′ as above), for Ip-sL2 (Ip-sL2-ORF-5′ and Ip-sL1-ORF-3′) and for the tick actin transcript (tick Act-for-108: 5′-TGG ATC GGC GGC TCC ATC CT-3′, tick Act-rev-A: 5′-GAA GCA CTT GCG GTG GAC AAT G-3′). Amplicons were separated by electrophoresis on a 2 % agarose gel.

2.5. Preparation of recombinant proteins, Ip-sL1-his and Ip-sL2-his

Oligonucleotide primers for the construction of Ip-sL1-his and Ip-sL2-his were designed to contain restriction enzyme recognition sites. The open reading frame of Ip-sL1 or Ip-sL2 was amplified using following primers; forward for Ip-sL1-his: 5′-CGG GAT ATC GCC GCC ACC ATG ACT GCT TCC TTC GCT TT-3′ (containing EcoRV restriction site); forward for Ip-sL2-his: 5′-CGG GAT ATC GCC GCC ACC ATG ACT TCC TCC CTC GCT TT-3′ (containing EcoRV restriction site); reverse for Ip-sL1-his and Ip-sL2-his: 5′-CGG CTC GAG TTA ATG GTG ATG GTG ATG GTG TGC GGC CGC ACA CTC GAA-3′ [containing one XhoI restriction site and six histidines (his)]. After digestion with EcoRV (New England Biolabs, Ipswich, MA, USA) and XhoI (New England Biolabs), the fragments were inserted into the cloning site of a pCXN2.1(+) vector. Purified vector (30 μg) was transfected into 7.5×10^7 of Expi293 F cells (Life Technologies, Carlsbad, CA) using ExpiFectamine 293 Reagent, ExpiFectamine 293 Transfection Enhancer 1 and ExpiFectamine 293 Transfection Enhancer 2 (Life Technologies). Transfectants were cultured in Expi293 Expression Medium (Life Technologies) for 7 days at 37 °C in an atmosphere containing 8 % CO_2; the culture supernatants were collected by centrifugal separation. The supernatants were then filtered through Acrodisc syringe filters (0.2 μm, Pall Corporation, Port Washington, NY, USA), and the proteins were purified using TALON Metal Affinity Resin (Clontech, Mountain View, CA, USA). The protein

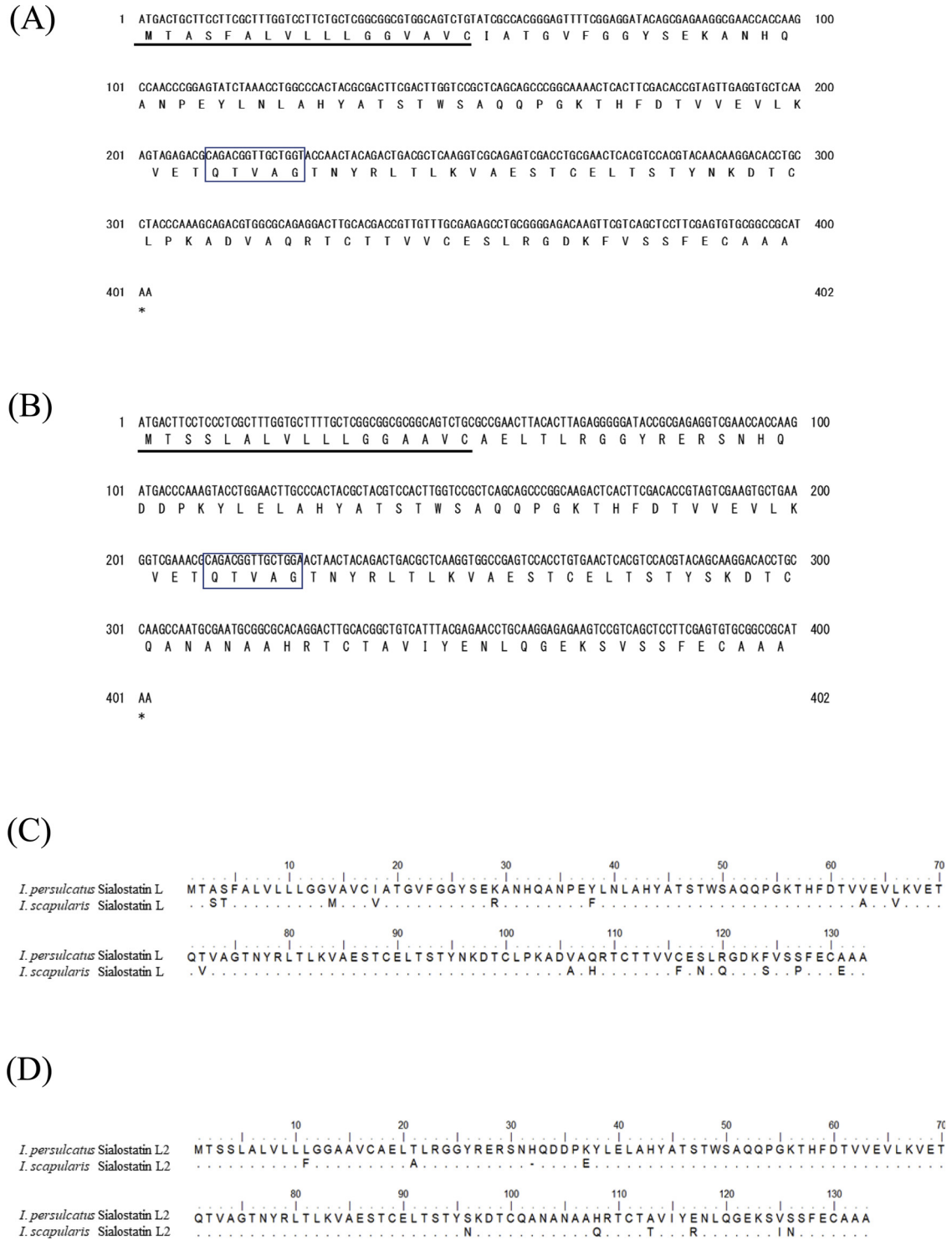

Fig. 1. Cloning of sL1 and sL2 from *I. persulcatus*. (A and B) Nucleotide and deduced amino sequences of cDNAs encoding sL1 (A) and sL2 (B) from *I. persulcatus*. Putative signal peptide sites are underlined. Cysteine protease-binding motif is boxed. (C and D) Alignment of deduced amino acid sequences of sialostatins L1 (C) and L2 (D) from *I. persulcatus* and *I. scapularis*.

buffer was exchanged with phosphate-buffered saline (PBS) by Amicon ultrafiltration (10 kDa, Merck Millipore, Burlington, MA, USA). To confirm protein expression, the supernatant samples were subjected to SDS–PAGE under reducing conditions, as described previously (Toyomane et al., 2016).

2.6. Enzymatic activity

Inhibitory effects of Ip-sL1 and Ip-sL2 on cathepsins L and S were evaluated using SensoLyte Rh110 Cathepsin L Assay Kit (ANASPEC, Fremont, CA, USA) and SensoLyte 520 Cathepsin S Assay Kit (ANASPEC) according to the manufacturer's instructions. Y-axis shows the percentage of remaining enzymatic activity of cathepsins L and S. The group, which was incubated without Ip-sL1-his and Ip-sL2-his,

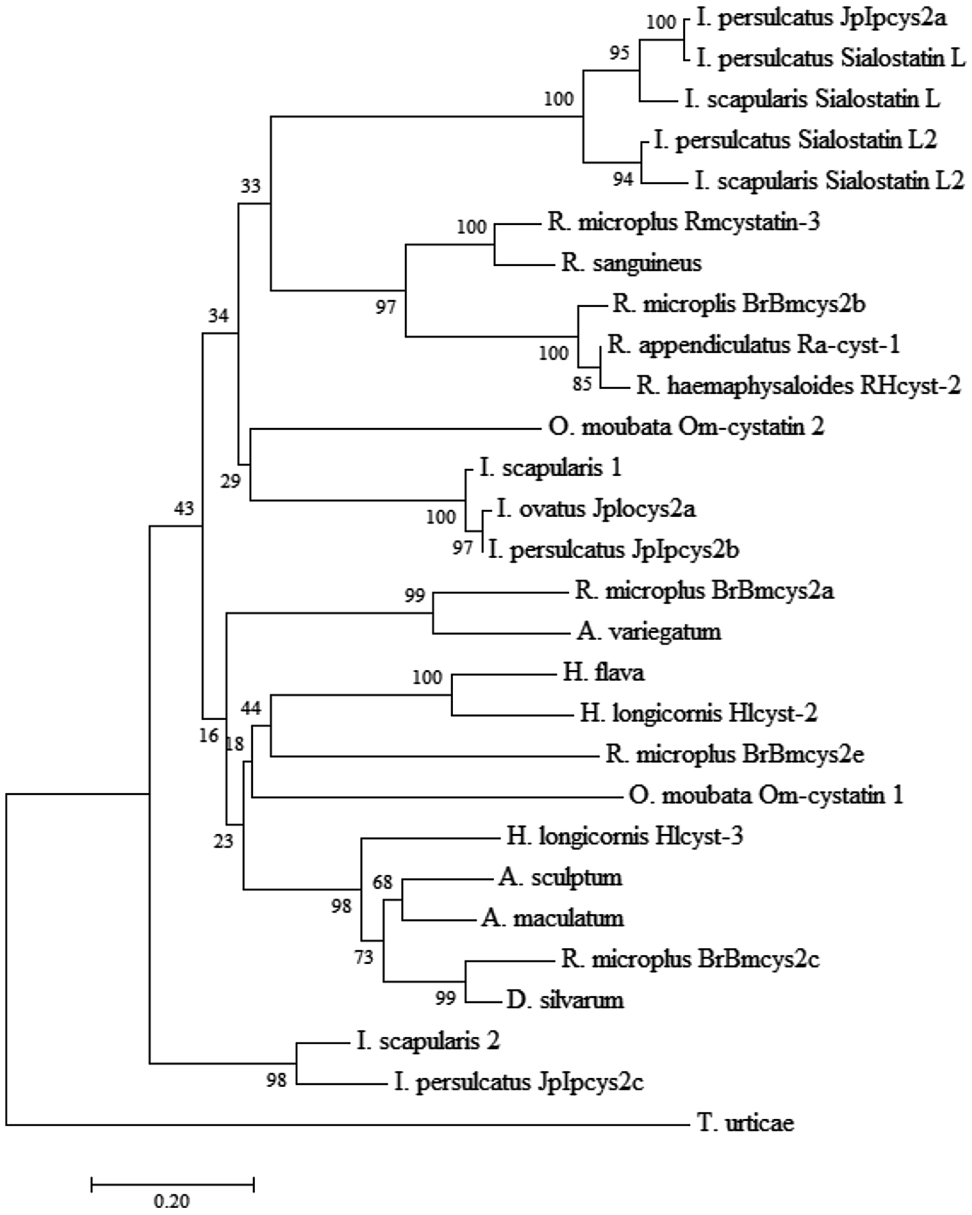

Fig. 2. Phylogenic tree constructed using amino acid sequences of Ip-sL1 and Ip-sL2. Scale bar indicates branch lengths.

represents 100 % enzymatic activity of cathepsins.

2.7. Generation of bone-marrow-derived dendritic cells (BMDCs)

BMDCs were prepared according to previous reports with slight modifications (Lutz et al., 1999; Lieskovská et al., 2015a, 2015b). Briefly, mice (BALB/c, female, 8 weeks of age) were sacrificed by isoflurane inhalation and cervical dislocation, and their femurs and tibias were removed. Bone marrow cells were collected by repeated flushing with PBS including 2 % heat-inactivated fetal calf serum (FCS; Thermo Fisher Scientific, Waltham, MA, USA). Bone marrow cells (4×10^5 cells/mL, 5 mL/well) were cultured for 7 days using a 6-well plate (Corning Inc., Corning, NY, USA) in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) including 10 % heat-inactivated FCS, 50 μM 2-mercaptoethanol (Sigma-Aldrich), 100 U/mL penicillin (Thermo Fisher Scientific), 100 μg/mL streptomycin (Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), 20 ng/mL murine GM-CSF (Sigma-Aldrich), and 20 ng/mL murine IL-4 (Sigma-Aldrich). Non-adherent cells were removed on days 3 and 5, and 70 % of medium was replaced with the fresh medium. Cells were harvested on day 7, and their purity was checked with FACS Verse (BD Biosciences, Franklin Lakes, NJ, USA) using anti-mouse/human CD11b (M1/70, Sony Biotechnology Inc., San Jose, CA, USA) and anti-mouse CD11c (N418, Sony Biotechnology Inc.).

2.8. Functional analysis of Ip-sL1-his and Ip-sL2-his in the presence of LPS

BMDCs (2×10^5 cells) were seeded into a 96 well plate (Corning Inc.) and cultured for 20 h. Following 2 h incubation with Ip-sL1-his or Ip-sL2-his (final concentration of 3 μM each), BMDCs were stimulated with LPS (3 ng/mL, Sigma-Aldrich) for 6 h. The culture supernatant was then collected, and the concentration of IP-10, TNFα, and IL-6 was determined using Mouse IP-10 Matched Antibody Pair (abcam, Cambridge, UK), Mouse TNF alpha Matched Antibody Pair (abcam), and Mouse IL-6 ELISA (BD Biosciences) kits, respectively. After incubation, BMDCs were collected and subjected to flow cytometric analysis of CD80, CD86, and MHCII expression as well as to PCR array analysis of inflammation cytokines and receptors. Flow cytometry was performed using FACS Verse and following antibodies; anti-mouse/human CD11b (M1/70), anti-mouse CD11c (N418), anti-mouse CD80

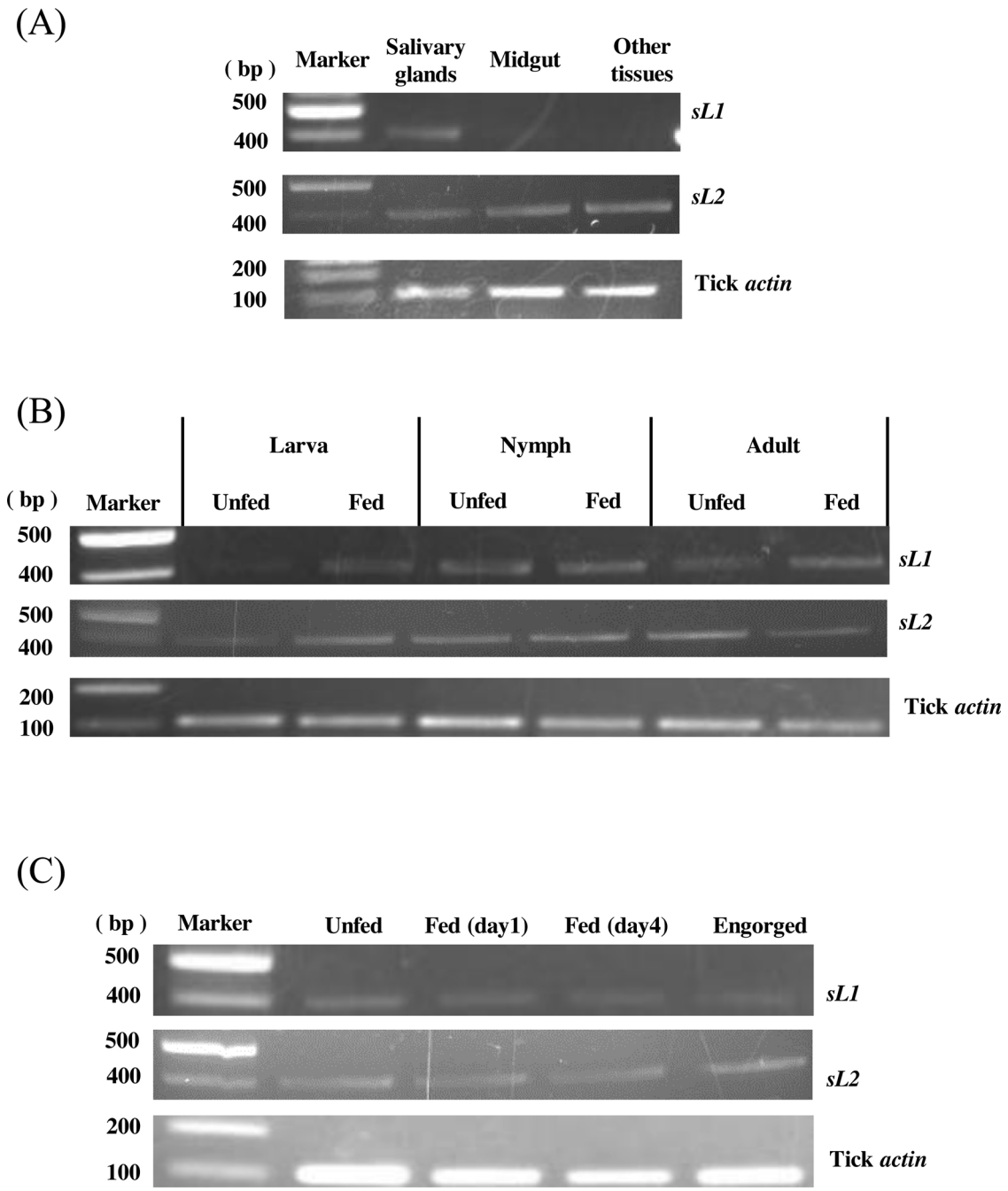

Fig. 3. Expression analysis of Ip-sL1 and Ip-sL2 genes. (A) Gene expression in different tissues of *I. persulcatus*. Tissues were collected from adult female ticks 4 days after attachment to hamsters. (B) Gene expression at different tick life stages. (C) Gene expression at different feeding stages. RNA samples were extracted from adult female ticks on days 1 and 4 after blood feeding as well as from the salivary glands of unfed and engorged ticks. Transcripts of tick actin were used as internal controls.

(16-10A1, Sony Biotechnology Inc.), anti-mouse CD86 (GL-1, Sony Biotechnology Inc.) and anti-mouse I-A/I-E (M5/114.15.2, Sony Biotechnology Inc.). PCR-array analysis was performed using an Inflammatory Cytokines and Receptors RT^2 Profiler PCR Array (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.9. Functional analysis of Ip-sL1-his and Ip-sL2-his in the maturation of BMDCs

Bone marrow cells (4×10^5 cells/mL, 1 mL/well) were cultured for 7 days in a 24-well plate (Corning Inc.) as described above. Non-adherent cells were removed on day 3, and 70 % of the medium was replaced with the fresh medium containing Ip-sL1-his or Ip-sL2-his (final concentration of 3 μM each). Non-adherent cells were removed on day 5, and 70 % of the medium was replaced with fresh medium. Cells were harvested on day 7, and the expression of CD80, CD86, and MHCII was assessed using FACS Verse and the antibodies described above.

2.10. Statistics

Differences were assessed using Steel-dwass test. A p value of < 0.05 was considered statistically significant.

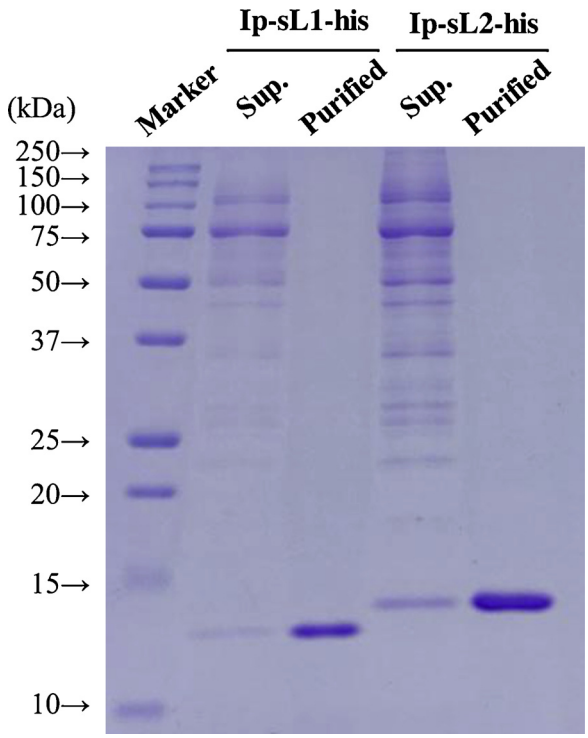

Fig. 4. Generation of Ip-sL1-his and Ip-sL2-his. Purified Ip-sL1-his and Ip-sL2-his proteins were visualized by SDS–PAGE. Sup., supernatant; Purified, purified protein.

3. Results

3.1. Cloning and sequence analysis of sL1 and sL2 of I. persulcatus

Two cDNA clones encoding sL1 and sL2 (402 bp) were isolated from the salivary gland of a fed female *I. persulcatus* tick and designated Ip-sL1 and Ip-sL2, respectively. Nucleotide sequences of Ip-sL1 and Ip-sL2 have been deposited in the EMBL/GenBank database (accession numbers: Ip-sL1: LC311727, Ip-sL2: MK524726). The deduced amino acid sequences of Ip-sL1 and Ip-sL2 contained 133 amino acids and included a putative signal peptide of 17 amino acids, a cystatin-like domain, and a cysteine protease-binding motif (QXVXG) similar to that in *I. scapularis* sL1 (Figs. 1A and 1B). The amino acid sequence of Ip-sL1 shared 87.2 % identity with the published sequence of *I. scapularis* sL1, while Ip-sL2 shared 93.2 % identity with the corresponding *I. scapularis* sequence (Figs. 1C and 1D). Phylogenetic analysis was performed to clarify the evolutionary relationship among tick cystatins using the neighbor-joining method as reported previously (Mori et al., 2010; Rangel et al., 2017). The results showed that Ip-sL1 and Ip-sL2 did not form a distinct cluster and instead clustered together with *I. scapularis* sL1 and sL2 (Fig. 2).

3.2. Expression analysis of Ip-sL1 and Ip-sL2

Expression analysis was conducted to determine expression profiles of Ip-sL1 and Ip-sL2. The mRNA transcript of Ip-sL1 was specifically detected in the salivary glands but not in the midgut or other tissues. In contrast, Ip-sL2 transcript was detected in all samples tested, including salivary glands (Fig. 3A). Additionally, both Ip-sL1 and Ip-sL2 mRNA transcripts were detected at all tick life stages (Fig. 3B). Ip-sL1 and Ip-sL2 transcripts were expressed at the same levels in salivary glands in unfed, blood-feeding, and engorged ticks (Fig. 3C). Collectively, these data suggest that both Ip-sL1 and Ip-sL2 are present during and after blood-feeding and expressed independent of the life-stage.

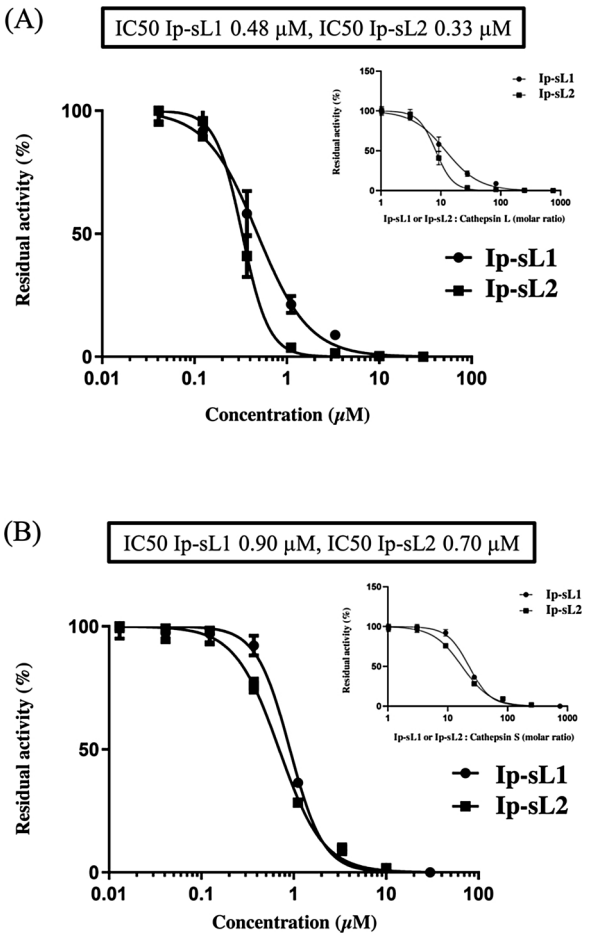

Fig. 5. Inhibitory effects of Ip-sL1-his and Ip-sL2-his on cathepsins. Cathepsins L (A) and S (B) were incubated with fluorimetric substrates in the presence of different concentrations of Ip-sL1-his and Ip-sL2-his. The X-axis shows inhibitor concentrations; the Y-axis shows percentage of remaining enzymatic activity. Incubation of cathepsins L and S without Ip-sL1-his and Ip-sL2-his represents 100 % of enzymatic activity. In the insert, the X-axis shows molar ratio of enzyme/inhibitor. IC50, 50 % inhibitory concentration.

3.3. Enzymatic activity of Ip-sL1 and Ip-sL2

Ip-sL1-his and Ip-sL2-his were produced in a mammalian expression system. The purified Ip-sL1-his and Ip-sL2-his migrated as bands of approximately 14 kDa on 15 % polyacrylamide gels (Fig. 4). A previous study has demonstrated that sL1 and sL2, derived from *I. scapularis*, reversibly inhibit the activity of papain-like cysteine proteases, such as cathepsins L and S (Kotsyfakis et al., 2010). The inhibition assays of cathepsins L and S were performed to confirm whether Ip-sL1 and Ip-sL2 have the inhibitory effects on cathepsins L and S. As shown in Fig. 5, both Ip-sL1-his and Ip-sL2-his inhibited the activity of cathepsins L and S in a dose-dependent manner. Additionally, the 50 % inhibitory concentrations of Ip-sL1 and Ip-sL2 for cathepsin L were 0.48 μM and 0.33 μM, respectively. The 50 % inhibitory concentrations of Ip-sL1 and Ip-sL2 for cathepsin S were 0.90 μM and 0.70 μM, respectively.

3.4. Functional analysis of Ip-sL1 and Ip-sL2 in the presence of LPS stimulation

To investigate the effect of Ip-sL1-his and Ip-sL2-his on cytokine production and dendritic cell activation, BMDCs were cultivated with Ip-sL1-his or Ip-sL2-his in the presence of LPS stimulation. Both Ip-sL1-his and Ip-sL2-his significantly inhibited the production of IP-10, TNFα, and IL-6 from BMDCs (Fig. 6). In addition, the expression of CD80,

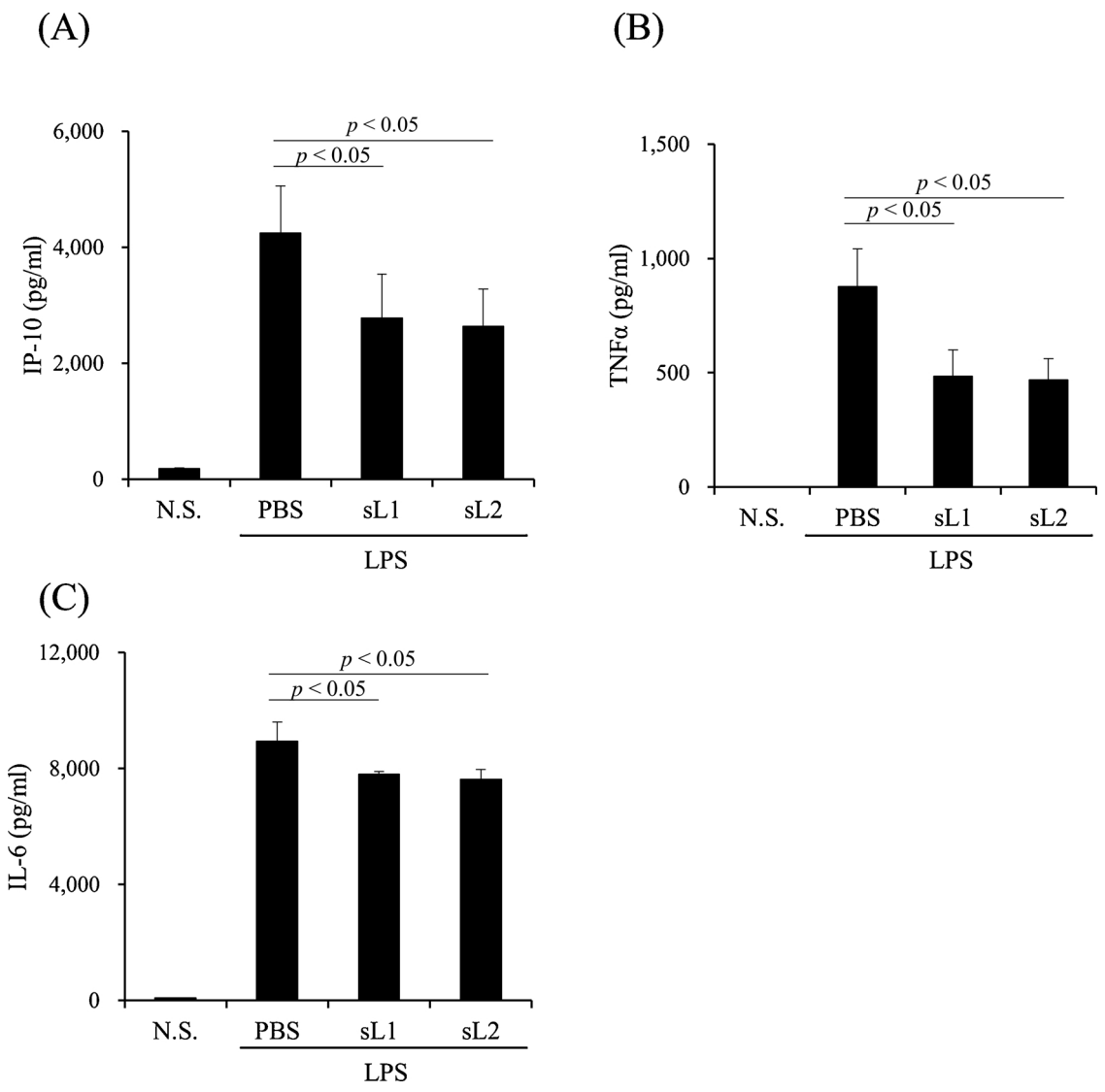

Fig. 6. Suppression of cytokine production by Ip-sL1 and Ip-sL2 in the presence of LPS stimulation. (A–C) BMDCs were cultured with Ip-sL1-his or Ip-sL2-his in the presence of LPS stimulation. The concentration of IP-10 (A), TNFα (B), and IL-6 (C) in culture supernatants were determined by ELISA. PBS was used as a negative control. Statistical significance was determined using Steel–Dwass test. N.S., no stimulation; sL1, Ip-sL1-his; sL2, Ip-sL2-his.

CD86 and MHCII in BMDCs was inhibited in the presence of Ip-sL1-his (Fig. 7). The treatment of Ip-sL2-his tended to downregulate the expression of these molecules (Fig. 7). Furthermore, we produced cDNA from these BMDCs and performed PCR-array analysis to evaluate the effect of these proteins on the gene expression of inflammatory cytokines and chemokines. Ip-sL1-his downregulated the expression of *Cxcl9*, *Ccl12*, *Cxcl11*, *Ccr10*, *Fasl*, *Ccl12*, *Ccl7*, *Ifng*, *Il1b*, *Il3*, *Ccl12*, *Tnfsf10*, *Ccl1*, *Tnfsf11*, *Cxcl10*, *Ccr2*, and *Ccl9* (Table 1), whereas Ip-sL2-his downregulated the expression of *Ifng*, *Cxcl13*, *Il11*, *Tnfsf11*, *IL17b*, *Cxcl10*, *Tnfsf10*, and *Ccl22* (Table 2). Interestingly, both Ip-sL1-his and Ip-sL2-his induced the expression of Th2-related genes, such as *Il4* and *Il5ra* (Tables 1 and 2).

3.5. Inhibition of BMDC maturation by Ip-sL1 and Ip-sL2

To determine whether Ip-sL1 and Ip-sL2 inhibit the maturation of BMDCs, bone marrow cells differentiated into dendritic cells in the presence of Ip-sL1-his or Ip-sL2-his. Flow cytometric analysis revealed that Ip-sL1-his significantly inhibited the expression of CD80, CD86 and MHCII, whereas Ip-sL2 reduced the levels of these proteins, although the changes were not statistically significant (Fig. 8).

4. Discussion and conclusion

Previous studies have described immunosuppressive roles of the salivary gland proteins sL1 and sL2 isolated from *I. scapularis*, a major vector for the Lyme borreliosis pathogen in North America (Kotsyfakis et al., 2006, 2007). sL1 and sL2 belong to cystatin family 2, and they reversibly inhibit the activity of papain-like cysteine proteases, such as cathepsins L and S (Kotsyfakis et al., 2010). Although cathepsins in lysosome are important for the removal of cellular waste products, their overexpression or hyperactivation causes histological damage. Indeed, overexpression of cathepsin is one of the mechanisms underlying various diseases, such as rheumatoid arthritis, cardiovascular disease, cancers, and autoimmune diseases (Otto and Schirmeister, 1997; Sudhan and Siemann, 2015; Wilkinson et al., 2015). Thus, cystatins contribute to homeostasis by regulating cathepsins. Interestingly, sL2 enhances blood meal acquisition and facilitates the transmission of *B. burgdorferi* s.l. by interfering with blood coagulation and immunity via cathepsin inhibition (Kotsyfakis et al., 2006, 2010). Therefore, cystatins may be important factors for tick-borne pathogen transmission. However, little information is available on cystatins produced by the taiga tick *I. persulcatus*. Therefore, in this study, we sought to identify the sL1

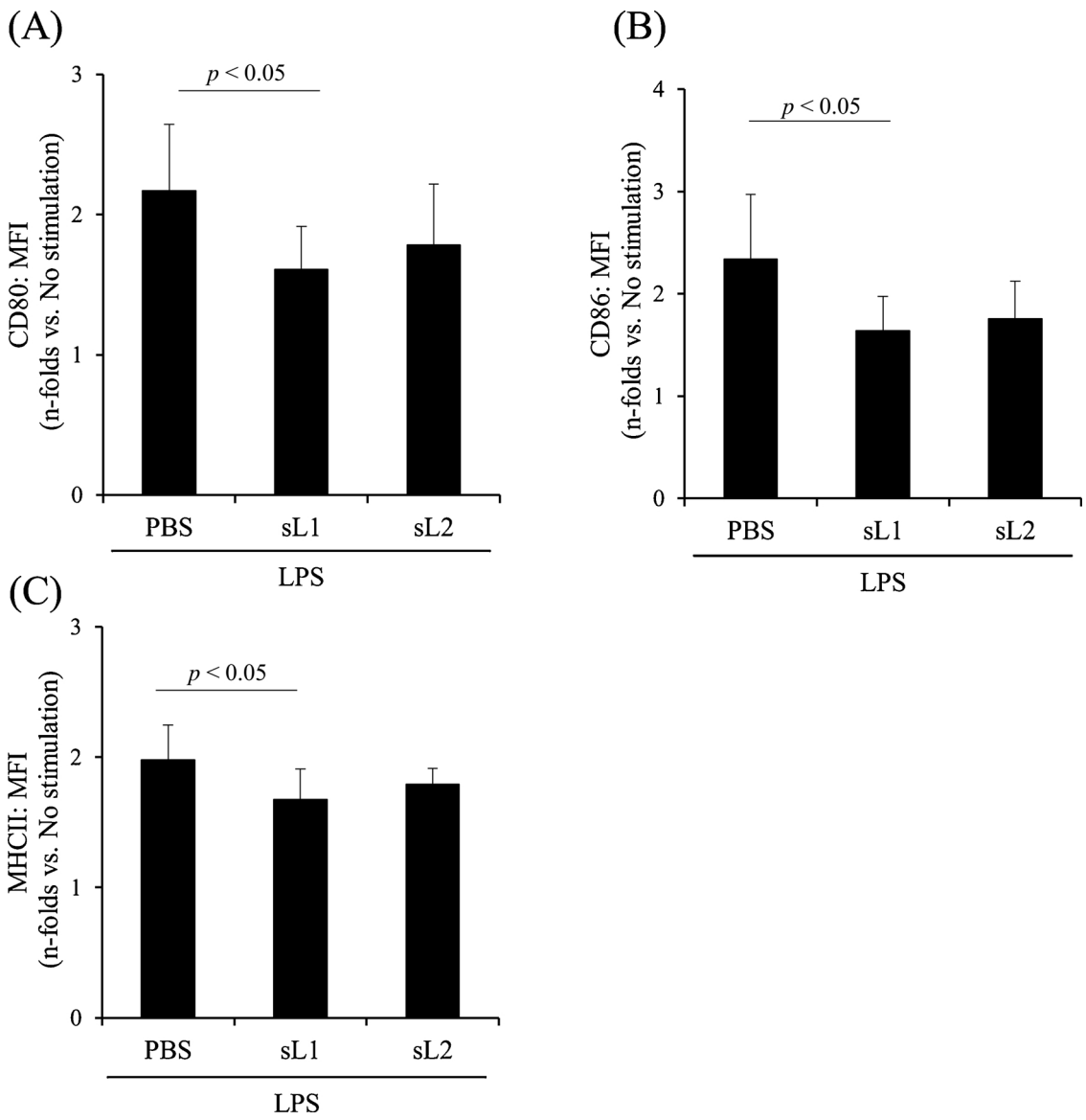

Fig. 7. Downregulation of CD80, CD86, and MHCII expression by Ip-sL1 and Ip-sL2 in the presence of LPS stimulation. (A–C) BMDCs were cultured with Ip-sL1-his or Ip-sL2-his in the presence of LPS stimulation. The expression of CD80 (A), CD86 (B), and MHCII (C) in BMDCs was analyzed by flow cytometry. PBS was used as a negative control. Statistical significance was determined using the Steel–Dwass test. MFI, mean fluorescence intensity.

and sL2 orthologs of *I. persulcatus* and to study their biological and immunological functions *in vitro*.

We isolated the cDNA clones of Ip-sL1 and Ip-sL2 from *I. persulcatus* and demonstrated that they share a high identity with sequences of sL1 and sL2 isolated from the salivary glands of *I. scapularis*. Expression analysis revealed that Ip-sL1 and Ip-sL2 transcripts were expressed in the salivary glands of fed ticks, suggesting that Ip-sL1 and Ip-sL2 were expressed in *I. persulcatus* during blood feeding. In addition, Ip-sL2 was detected in unfed ticks and in the midgut of fed ticks. Previous reports have demonstrated that cystatins derived from *R. microplus* are involved in tick blood digestion (Parizi et al., 2015), and a cystatin derived from *R. haemaphysaloides* is involved in embryogenesis (Wang et al., 2015). Thus, Ip-sL2, which is expressed in the salivary glands and in other tissues, may be involved in blood digestion and embryogenesis. Further studies are warranted to elucidate functions of Ip-sL1 and Ip-sL2 in tick physiology.

I. persulcatus is the vector of the causative agents of Lyme borreliosis (Masuzawa, 2004). Typically, dendritic cells play pivotal roles in host defenses against Lyme borreliosis (Mason et al., 2014). However, dendritic cell functions are suppressed by components of tick saliva (Cavassani et al., 2005; Skallová et al., 2008). In addition, previous studies reported that *I. scapularis* sL1 inhibited LPS-induced production of TNFα by DCs, and *I. scapularis* sL2 decreased PIK3/Akt and NF-kB pathways in response to *Borrelia* spirochetes (Sá-Nunes et al., 2009; Lieskovská et al., 2015a). Based on these previous observations, in this study, we measured the concentrations of TNFα and IL-6, as representative inflammatory cytokines regulated via PIK3/Akt and NF-kB pathways, produced from LPS-stimulated BMDCs. As shown in Fig. 6, both Ip-sL1 and Ip-sL2 impaired the production of these cytokines from BMBCs. *I. scapularis* sL2 inhibited LPS-induced production IP-10 (CXCL10) from BMDCs (Lieskovská et al., 2015b). Our result also confirmed downregulation of this chemokine by treatment of Ip-sL1 and Ip-sL2 in transcription and protein levels. These results suggest a similarity with sL1 and sL2 isolated from *I. scapularis*. In other words, Ip-sL1 and Ip-sL2 exerted anti-inflammatory effects by suppressing dendritic cell function. In addition, Ip-sL1 significantly inhibited the expression of CD80, CD86, and MHCII in BMDCs. Ip-sL2 also downregulated their expression, albeit not significantly. These results suggest that these proteins inhibited the maturation and function of BMDCs. Reportedly, cathepsin S mediated the degradation of the MHCII

Table 1
Change of gene expressions in the presence of Ip-sL1-his.

Accession number	Gene symbol	Gene description	Relative value	Fold change	p value
NM_008599	*Cxcl9*	Chemokine (C-X-C motif) ligand 9	−6.25	0.16	0.045
NM_011331	*Ccl12*	Chemokine (C-C motif) ligand 12	−4.62	0.22	0.276
NM_019494	*Cxcl11*	Chemokine (C-X-C motif) ligand 11	−4.15	0.25	0.168
NM_007721	*Ccr10*	Chemokine (C-C motif) receptor 10	−3.05	0.33	0.403
NM_010177	*Fasl*	Fas ligand (TNF superfamily, member 6)	−2.84	0.35	0.178
NM_011331	*Ccl12*	Chemokine (C-C motif) ligand 12	−2.75	0.36	0.209
NM_013654	*Ccl7*	Chemokine (C-C motif) ligand 7	−2.70	0.37	0.226
NM_008337	*Ifng*	Interferon gamma	−2.66	0.38	0.032
NM_008361	*Il1b*	Interleukin 1 beta	−2.35	0.43	0.147
NM_010556	*Il3*	Interleukin 3	−2.24	0.45	0.301
NM_011333	*Ccl2*	Chemokine (C-C motif) ligand 2	−2.20	0.45	0.214
NM_009425	*Tnfsf10*	Tumor necrosis factor (ligand) superfamily, member 10	−2.19	0.46	0.219
NM_011329	*Ccl1*	Chemokine (C-C motif) ligand 1	−2.14	0.47	0.262
NM_011613	*Tnfsf11*	Tumor necrosis factor (ligand) superfamily, member 11	−2.09	0.48	0.289
NM_021274	*Cxcl10*	Chemokine (C-X-C motif) ligand 10	−2.04	0.49	0.136
NM_009915	*Ccr2*	Chemokine (C-C motif) receptor 2	−1.97	0.50	0.047
NM_011338	*Ccl9*	Chemokine (C-C motif) ligand 9	−1.78	0.56	0.035
NM_008370	*Il5ra*	Interleukin 5 receptor, alpha	2.28	2.28	0.329
NM_010558	*Il5*	Interleukin 5	3.07	3.07	0.154
NM_021283	*Il4*	Interleukin 4	5.11	5.11	0.082

Table 2
Change of gene expressions in the presence of Ip-sL2-his.

Accession number	Gene symbol	Gene description	Relative value	Fold change	p value
NM_008337	*Ifng*	Interferon gamma	−6.30	0.16	0.010
NM_018866	*Cxcl13*	Chemokine (C-X-C motif) ligand 13	−4.02	0.25	0.665
NM_008350	*Il11*	Interleukin 11	−2.94	0.34	0.714
NM_011613	*Tnfsf11*	Tumor necrosis factor (ligand) superfamily, member 11	−2.78	0.36	0.158
NM_019508	*Il17b*	Interleukin 17B	−2.40	0.41	0.228
NM_021274	*Cxcl10*	Chemokine (C-X-C motif) ligand 10	−2.18	0.46	0.143
NM_009425	*Tnfsf10*	Tumor necrosis factor (ligand) superfamily, member 10	−2.08	0.48	0.216
NM_009137	*Ccl22*	Chemokine (C-C motif) ligand 22	−1.33	0.75	0.020
NM_021283	*Il4*	Interleukin 4	3.09	3.09	0.787
NM_008370	*Il5ra*	Interleukin 5 receptor, alpha	4.87	4.87	0.001

invariant chain Ii in mouse antigen-presenting cells (Saegusa et al., 2002). As shown in Fig. 5, both Ip-sL1 and Ip-sL2 inhibited the enzymatic activity of cathepsin S. Thus, the effects of Ip-sL1 and Ip-sL2 are presumably due to cathepsin S inhibition. A previous study demonstrated that *I. scapularis* sL1 strongly inhibited cathepsin S, whereas *I. scapularis* sL2 is only weakly inhibited this enzyme (Kotsyfakis et al., 2010). Thus, this is one of the functional differences between sialostatins of *I. persulcatus* and *I. scapularis*.

In this study, Ip-sL1 and Ip-sL2 showed low affinity against commercial human cathepsins used in the inhibitory tests. Nevertheless, their inhibitory activities confirmed similar biochemical characteristics of other tick cystatins (Zavašnik-Bergant et al., 2017; Schwarz et al., 2012). Our results did not permit to determine the native target in the host or the tick tissues for Ip-sL1 and Ip-sL2, however indicate that they could be involved in the host immunosuppressive activity (Porter et al., 2017; Parizi et al., 2018). Additional studies are needed to determine the native target for Ip-sL1 and Ip-sL2.

Several reports have shown that *I. scapularis* sL2 facilitates the transmission of *B. burgdorferi* s.l. (Kotsyfakis et al., 2010; Lieskovská et al., 2015a). However, the effects of sialostatins on tick-borne pathogen transmission are not fully elucidated. In this study, our results demonstrated that both Ip-sL1-his and Ip-sL2-his induced the expression of Th2-related genes, *IL-4* and *IL-5*. These cytokines have a role to downregulate the production of Th1 cytokines and the differentiation of Th1 cells (Wurtz et al., 2004). In fact, our data clearly showed that the expression of *IFN-γ*, one of the Th1 cytokines, was significantly inhibited by the treatment of Ip-sL1 and Ip-sL2 (Table 1 and 2). Additionally, the production of TNFα and IP-10 from BMDCs was decreased by the treatment of Ip-sL1 and Ip-sL2. TNFα is known as one of the Th1 cytokines, and IP-10 has important roles for effector T-cell generation and trafficking (Dufour et al., 2002). Collectively, these results suggest that these proteins activate Th2 responses, whereas suppress Th1 responses, via dendritic cells. In the murine model of *B. burgdorferi* s.l. infection, Th2 cytokines play important roles for the transmission (Zeidner et al., 2008). In contrast, Th1 cytokines, such as IFN-γ, IL-2, and TNFα, have protective roles against *B. burgdorferi* s.l. infection (Zeidner et al., 1996). Thus, Ip-sL1 and Ip-sL2 might mediate the transmission of tick-borne pathogens via the change of T-cell function and polarization. This finding related to Th2 cytokines could be a novel insight into tick sialostatins provided by this study.

In conclusion, in the present study, cDNA clones of Ip-sL1 and Ip-sL2 were isolated from the salivary glands of *I. persulcatus*. Immunological analyses revealed that these proteins suppressed cytokine production as well as gene expression of inflammatory cytokines and chemokines in BMDCs. Additionally, Ip-sL1 and Ip-sL2 impaired maturation and function of BMDCs. Thus, Ip-sL1 and Ip-sL2 serve immunosuppressive functions. To our best knowledge, this is the first report which indicates immunosuppressive function of salivary gland proteins from *I. persulcatus* using murine immune cells. *I. persulcatus* is one of the most important vectors of tick-borne diseases, such as Lyme borreliosis, and *B. miyamotoi* infection (Nakao and Miyamoto, 1994; Taylor et al., 2013). Additionally, rodents including mice are considered as the reservoirs of these diseases (Levine et al., 1985; Krause et al., 2015). Therefore, this paper might have a potential to accelerate demonstrating of the role of salivary gland proteins from *I. persulcatus* in pathogen transmission using murine models. Further analyses of mechanistic details of these immunosuppressive effects as well as their association with the transmission of tick-borne pathogens are warranted to develop effective

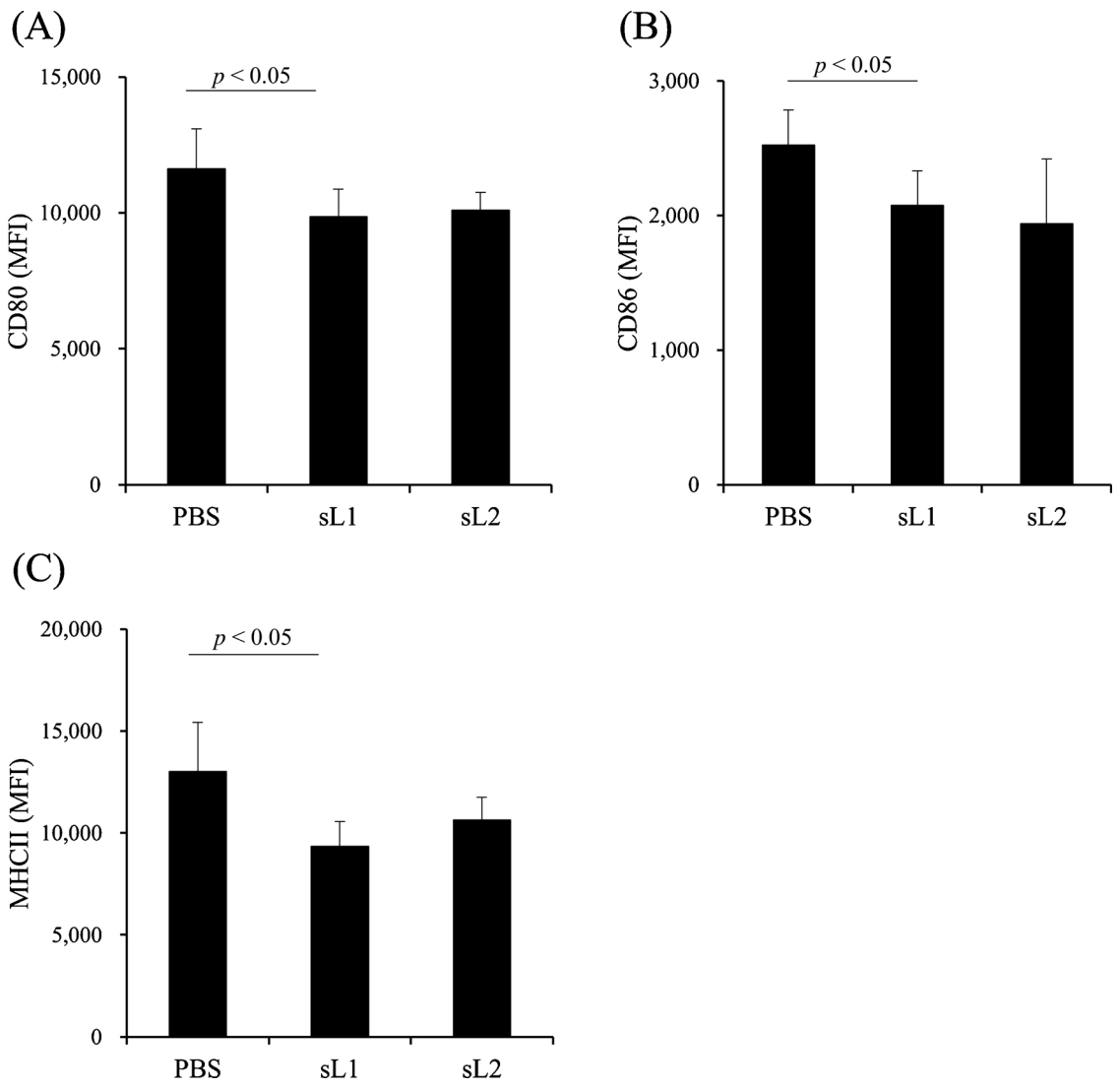

Fig. 8. Inhibition of BMDC maturation by Ip-sL1 and Ip-sL2. (A–C) BMDCs were differentiated in the presence of Ip-sL1-his or Ip-sL2-his. The expression of CD80 (A), CD86 (B), and MHCII (C) in BMDCs was analyzed by flow cytometry. PBS was used as a negative control. Statistical significance was determined using the Steel–Dwass test.

control strategies against *I. persulcatus*.

Author contributions

SK, SM, and KO: designed the work; YS, AO, NG, SY, TI, SA and HK: generated the biological samples; YS, AO, TO, MI, SY and NM: performed the experiments; SK, CL, ISVJ: acquired, analyzed, and interpreted the data; YS and SK: wrote the manuscript; SY, SA, HK, CL, ISVJ, NM, SM and KO: revised and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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