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Identification and characterization of ecto-nucleoside triphosphate diphosphohydrolase 1 (CD39) involved in regulating extracellular ATP-mediated innate immune responses in Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

Extracellular adenosine triphosphate (eATP), released following inflammatory stimulation or infection, is a potent signaling molecule in activating innate immune responses in fish. However, the regulation of eATP-mediated innate immunity in fish remains unknown. Ecto-nucleoside triphosphate diphosphohydrolase 1 (CD39) is a critical molecular switch for controlling the ATP levels in the extracellular space. CD39 plays a key role in regulating eATP-activated innate immune responses through the phosphohydrolysis of pro-inflammatory eATP to inactive AMP. Here, we identified and characterized a *CD39* homolog (namely, *poCD39*) in the Japanese flounder *Paralichthys olivaceus* and analyzed its regulatory role in eATP-mediated innate immunity. Real-time quantitative PCR analysis revealed that *poCD39* is ubiquitously present in all tested normal tissues with dominant expression in enriched Japanese flounder head kidney macrophages (HKMs). Immune challenge experiments demonstrated that *poCD39* expression was upregulated by inflammatory stimulation and *Edwardsiella tarda* infection. Biochemical and immunofluorescence analysis revealed that poCD39 is a functional glycosylated membrane protein for the hydrolysis of eATP. Inhibition of poCD939 activity with the ecto-NTPDase inhibitor ARL 67156 resulted in increased *IL-1beta* gene expression and ROS production in Japanese flounder HKMs. In contrast, overexpression of poCD39 in Japanese flounder FG-9307 cells reduced eATP-induced pro-inflammatory cytokine *IL-1beta* gene expression. Finally, *poCD39* expression was significantly induced by eATP stimulation in the HKMs, suggesting that eATP may provide a feedback mechanism for transcriptional regulation of fish *CD39*. Taken together, we identified and characterized a functional fish CD39 protein involved in regulating eATP-mediated innate immune responses in fish.

1. Introduction

Extracellular ATP (eATP), released from host cells under conditions of stress, inflammatory stimulation or infection, is an important endogenous signaling molecule with pro-inflammatory properties through action on ATP-gated P2 receptors. Our previous studies revealed that inflammatory challenge or pathogen infections can elicit significant ATP release into the extracellular space through pannexin 1 and/or connexin 43 channels in fish (Li et al., 2018d, a; Li et al., 2016c). In addition, several ATP-gated purinergic receptor subtypes, including P2X2, P2X4, P2X7 and P2Y$_2$, were detected in various Japanese flounder (*Paralichthys olivaceus*) tissues and immune cells (Li et al., 2016b, 2015b, 2018b). Furthermore, we also showed that eATP plays critical roles in regulating pro-inflammatory cytokine gene expression (Li et al., 2014b), NOD-like receptor family CARD domain containing 3 (NLRC3) inflammasome immune signaling (Li et al., 2016a), and the production of inflammatory mediators including ROS and NO in Japanese flounder head kidney macrophages (Li et al., 2018a). Moreover, we recently found that eATP broadly modulated the expression and enzyme activities of several caspases including caspase 1, 2, 3, 6 and 8 in Japanese flounder immune cells (Li et al., 2019, 2017), suggesting the involvement of eATP in fish apoptosis. These findings indicate that eATP is a potent signaling molecule that plays important roles in *P. olivaceus* innate immunity.

Despite the positive roles of eATP in triggering host innate immune responses, the contents of eATP must be tightly controlled as excessive

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Table 1
Sequence of primers used in this study.

Primers	sequence(5'→3')
F1	GSGATTGTYRYCHTGGTRWC
R1	AARAYMCCRTTGAADGARCA
GSP	CTGCCAGTGTTGTTATCTTT
UPM-L	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UPM-S	CTAATACGACTCACTATAGGGC
NUP	GGCCACGCGTCGACTAGTAC
F2	CCAGGGAGCTCCTGCCACCT
PAGE	TCGAATTCGGATCCGAGCTC
F3	GCTGCTGAGAAAGAACCCATG
R2	TTCCCTTCACCAACTTTACT
CD39-FLAG-f	CCCAAGCTT TCCGCACAAAGAGAGATGA
CD39-FLAG-r	CGGAATTC TTATATGATCTGTGGTTGT
qβ-actin-f	AGGTTCCGTTGTCCCG
qβ-actin-r	TGGTTCCTCCAGATAGCAC
qCD39-f	CCTTCCGACTCTACGGTAATGAC
qCD39-r	GGATCTGATATGGATACTGGACCT
qIL-1β-f	CCTGTCGTTCTGGGCATCAA
qIL-1β-r	CACCCCGCTGTCCTGCTT

q: quantitative real-time PCR; f: forward; r: reverse.

accumulation of eATP may lead to excessive inflammatory responses and result in detrimental effects or damage to the immune system. To restore homeostasis and avoid excessive inflammation-induced tissue damage, timely attenuation or termination of eATP-induced inflammatory responses is thus critically required. The metabolism of eATP is one of the critical mechanisms that attenuate eATP-stimulated pro-inflammatory responses in innate immunity.

Ecto-nucleoside triphosphate diphosphohydrolase, namely, NTPDase, is an enzyme family that catalyzes the hydrolysis of γ- and β-phosphate residues of nucleotides, albeit to different degrees of effectiveness (Zimmermann, 2001). NTPDase 1 (EC 3.6.1.5), also known as CD39 or ecto-apyrase, was initially described as an activation marker of lymphoid cells (Maliszewski et al., 1994). A growing number of studies, however, have demonstrated that CD39 is a dominant NTPDase in immune tissues/cells and plays an important role in regulating eATP-mediated innate immunity by reducing the levels of pro-inflammatory ATP in the extracellular space (Mizumoto et al., 2002). CD39 inhibits inflammation by phosphohydrolyzing ATP to AMP, and the latter is further metabolized by another ecto-5′-nucleotidase (CD73) to anti-inflammatory adenosine (Colgan et al., 2006). CD39 is thus tightly linked with purinergic signaling in inflammatory responses (Dwyer et al., 2007; Mizumoto et al., 2002). For instance, through the hydrolysis of eATP, CD39 can terminate P2 receptor activation and plays a key role in the control of P2X7R-dependent macrophage responses (Levesque et al., 2010) and P2X7R-mediated inflammatory cell death in mast cells (Kuhny et al., 2014). In addition, it has been found that CD39 controls IL-8 production *via* regulation of $P2Y_2$ receptor activation in human neutrophils (Kukulski et al., 2011). Furthermore, CD39 can protect against excessive organ inflammation and damage, enhancing the survival of septic mice (Csoka et al., 2015). In contrast, suppression of constitutive CD39 expression may result in elevated concentrations of ATP and ADP at the vascular interface that could lead to a predisposition to thrombosis and inflammation (Imai et al., 1999). Finally, CD39 has been suggested to play a role in dampening the general immune activation in T cells (Schulze zur Wiesch et al., 2011).

Although intensive investigations in mammals have been conducted, the properties and immune relevance of CD39 in fish have not been investigated. In this report, we identified and characterized a *CD39* gene, namely, *poCD39*, in the Japanese flounder (*Paralichthys olivaceus*) and analyzed its regulatory role in eATP-mediated innate immune responses. We demonstrated for the first time that poCD39 is a functional membrane glycoprotein involved in regulating eATP-mediated innate immune responses in fish.

2. Materials and methods

2.1. Experimental animals

Japanese flounder *P. olivaceus* obtained from a local fish farm in Tianjin, China, were cultured in an aerated recirculating seawater system at 21 °C in laboratory for two weeks before experimentation commenced. Fish were handled and clinically examined as described in our previous study (Li et al., 2014a); only healthy animals without any pathological signs were used in experiments. All experiments were carried out in accordance with the NIH guidelines for the care and use of experimental animals.

2.2. Chemicals and antibodies

ATP, ARL 67156, LPS, zymosan, anti-Flag monoclonal antibody and anti-alpha tubulin monoclonal antibody were ordered from Sigma-Aldrich. HRP-labeled goat anti-mouse secondary antibody and goat anti-mouse secondary IgG labeled with AlexaFluor 488 were purchased from Invitrogen. Unless stated otherwise, all other chemicals were obtained from Sigma-Aldrich.

2.3. RNA purification, cDNA preparation and gene cloning

Total RNA from *P. olivaceus* tissues was purified by TRIzol reagent (Invitrogen). RNA from cells was isolated with an RNeasy plus mini kit (Qiagen). RNA quality was examined by electrophoresis on a 1.2% formaldehyde-denatured agarose gel stained with ethidium bromide. The purified RNA was quantified by measuring OD_{260} with a NanoDrop 2000 UV/Vis spectrophotometer (ThermoFisher Scientific) and treated with DNase I (amplification grade, Invitrogen) before reverse transcription according to the manufacturer's recommendations. Aliquots of total RNA (2 μg) were transcribed into cDNAs using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

To clone the *poCD39* gene, an internal fragment of *poCD39* cDNA was initially amplified from Japanese flounder liver tissue by RT-PCR using a degenerate primer pair F1/R1 (Table 1), which was designed based on the conserved region of the *CD39* gene across different fish species. Negative controls, using templates from cDNA synthesis reactions without reverse transcriptase, amplified no PCR products (data not shown). The obtained sequence was then used to design gene-specific primers to amplify the 5′- and 3′-untranslated regions of *poCD39* cDNA. To amplify the 3′-terminal end of the *poCD39* cDNA sequence, nested PCR was performed with a gene-specific forward primer F2 and a universal reverse adaptor primer PAGE (Table 1). Two rounds of nested PCR were applied to amplify the 5′-terminal end of the *poCD39* cDNA sequence using a SMARTer™ RACE amplification kit (Clontech) according to the manufacturers' instructions. Briefly, the first round of PCR analysis was performed with a forward primer UPM (a mixture of primers UPM-L and UPM-S, Table 1) and a reverse gene specific primer GSP (Table 1); the PCR products were then diluted 100 times, and a nested amplification with primer pair NUP/GSP (Table 1) was performed in a MyCycler™ gradient thermocycler (Bio-Rad). Based on the sequences of RACE PCR, the gene-specific primer pair F3 and R2 (Table 1) corresponding to the 5′- and 3′-untranslated regions of *poCD39* cDNA, respectively, was designed; RT-PCR was then performed to obtain the complete sequence of Japanese flounder *poCD39* cDNA. PCR products were separated by 1.5% agarose gel electrophoresis, visualized by ethidium bromide staining and recorded with a BioRad Gel Doc 1000 system. PCR bands with the expected size were cloned into pGEM-T vector (Promega) and sequenced. Three independent clones were picked and sequenced on both strands. *In silico* analysis indicated that they matched exactly with sequences derived from RACE PCR.

2.4. Sequence analysis, domain predication and phylogenetic analysis

Gene identities were obtained by searching against the GenBank database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) using the BlastX algorithm. The transmembrane domain and conserved protein domain of poCD39 were analyzed by the TMHMM Server version 2.0 (www.cbs.dtu.dk/services/TMHMM) and the SMART tool (http://smart.embl-heidelberg.de/), respectively. Potential glycosylation and phosphorylation sites in poCD39 protein were predicted by the NetNGlyc program (http://www.cbs.dtu.dk/services/NetNGlyc/) and the NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/), respectively. Multiple alignments of the amino acid sequences of selected CD39 proteins were conducted using the ClustalW program (http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis was performed using MEGA software version 5.0, and a phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replications.

2.5. Cell culture, plasmid preparation and cell transfection

Japanese flounder head kidney macrophages (HKMs) and peripheral blood leukocytes (PBLs) were prepared by discontinuous Percoll density gradient centrifugation as previously described (Li et al., 2018c). HKMs and PBLs were cultured in RPMI1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin liquid (Invitrogen) at 21 °C in 2.5% CO_2. The Japanese flounder gill epithelial cell line FG-9307 was cultured with MEM medium (Invitrogen) supplemented with 10% FBS, 2.5 μg/ml fungizone and 1% penicillin-streptomycin liquid at 21 °C in 2.5% CO_2. HeLa cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in the presence of 5% CO_2.

To ectopically express poCD39 in different cell lines, the coding region of *poCD39* cDNA was amplified with the primer pair CD39-FLAG-f/CD39-FLAG-r (Table 1) using PfuUltra II fusion HS DNA polymerase (Stratagene). The PCR products were purified, digested by EcoR I/Hind III, and cloned into the p3xFLAG-CMV-7.1 expression vector (Sigma) containing a FLAG epitope at the N-terminus. Plasmid DNA was prepared using a QIAfilter Plasmid Maxi kit (Qiagen). The recombinant plasmid was sequenced to confirm that no mutation was introduced and the reading frame was correct. FG-9307 and HeLa cells were transfected with Flag-tagged poCD39 plasmid using Lipofectamine™ LTX and Plus transfection reagent (Invitrogen) following the manufacturer's instructions. The expression of Flag-tagged poCD39 protein was confirmed by western blot analysis using anti-Flag monoclonal antibody (Sigma) with beta-tubulin serving as a loading control. Protein functional assays were performed 48 h post-transfection.

2.6. Gene expression profile analysis of poCD39 in Japanese flounder tissues and immune cells

The relative basal gene expression levels of *poCD39* in *P. olivaceus* tissues and immune cells were analyzed by real-time quantitative PCR (RT-qPCR). Tissues including the brain, blood, gill, head kidney, trunk kidney, heart, liver, skin, gonad, muscle, intestine and spleen were separated and collected from five Japanese flounder (average 500 ± 20 g). Each kind of tissue was equally pooled to decease individual variation. Total RNA from Japanese flounder tissues, HKMs and PBLs were purified, followed by DNase I treatment and reverse transcription as described previously. The relative gene expression level of *poCD39* was determined by RT-qPCR.

2.7. Glycosylation modification analysis, immunofluorescence microscopy and western blot

To study the post-translational glycosylation modification of poCD39 proteins, Flag-tagged poCD39 plasmid was transfected into FG-9307 cells for 48 h. The transfected cells were washed twice with cold PBS and extracted with radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 1 mM EDTA) supplemented with a protease inhibitor cocktail (Roche). Cell lysates were then centrifuged at 13,000 rpm for 15 min at 4 °C and the supernatants were collected. A de-glycosylation assay was performed by incubating 10 μg protein samples for 8 h at 37 °C with 250 units of glycosidase Endo H or PNGase F (New England Biolabs) according to the manufacturer's instructions. Glycosylation modification of poCD39 proteins was then analyzed by western blotting using anti-FLAG antibody (see below). The *N*-linked glycosylated modification of poCD39 proteins was further analyzed by LC–MS/MS analysis. To this aim, Flag-tagged Japanese flounder poCD39 was overexpressed in FG-9307 cells. The recombinant poCD39 proteins were enriched by co-immunoprecipitation using anti-Flag antibody as described in the previous study (Li et al., 2015b). The obtained poCD39 proteins were concentrated by ultrafiltration. The glycosylation modification of poCD39 was analyzed by Shanghai Applied Protein Technology Co. Ltd with a protocol described previously (Zhang et al., 2018).

The cellular localization of poCD39 protein was analyzed by immunofluorescence microscopy. For this purpose, Japanese flounder FG-9307 cells at a density of 0.5×10^5 were seeded onto 0.01% poly-L-lysine-coated (Sigma) 25 mm coverslips. Overnight cultured cells were then transfected with FLAG-tagged poCD39 plasmid. Forty-eight hours after transfection, cells were washed with cold PBS, fixed with 4% paraformaldehyde for 20 min, washed again with PBS, and permeabilized with 0.1% Triton X-100 containing PBS for 15 min at room temperature. Coverslips were washed three times each for 5 min with PBS, blocked with PBS containing 1% BSA for 45 min at room temperature and incubated with 1:300 diluted anti-FLAG M2 monoclonal antibody (Sigma) at 4 °C overnight. After three washes with PBS, cells were incubated with 1:600 diluted goat anti-mouse secondary IgG labeled with Alexa Fluor 488 (Invitrogen) for 2 h at room temperature. Finally, cover slips were washed with PBS three times and mounted with anti-fading mounting medium (Invitrogen) for visualization with a fluorescence microscope (Nikon) using a 40x oil immersion objective. The p3XFLAG-CMV-7.1 vector-only transfected cells did not show any obvious fluorescence (data not shown).

For western blot analysis, the extracted supernatants were separated by electrophoresis through a 12.5% SDS-polyacrylamide gel and subsequently transferred to polyvinylidene fluoride membranes (Millipore). The membranes were incubated with anti-FLAG monoclonal antibody (1:3000, Sigma), followed by goat anti-mouse secondary antibody conjugated to horseradish peroxidase (1:10 000, Pierce). Target proteins were visualized using SuperSignal West Pico detection systems (Pierce) and Kodak BioMax MR Film according to the manufacturer's directions.

2.8. Immune challenge and extracellular ATP treatment

To study the responses of *poCD39* to immune stimuli, *in vitro* inflammatory challenge experiments and *in vivo* bacterial infection experiments were performed. The protocols for the immune challenge experiments were described in our previous study (Li et al., 2015a). Briefly, for *in vitro* experiments, Japanese flounder HKMs were treated with LPS or zymosan for the indicated times. In addition, *in vivo* bacterial challenge experiments were also performed by intraperitoneal injection with *Edwardsiella tarda* or an equal volume of sterilized physiological saline as control.

To test the effect of eATP stimulation on *poCD39* expression, Japanese flounder HKMs (5×10^6 cells/well) were stimulated with 200 μM ATP (cell culture grade, Sigma) for the indicated durations. *poCD39* gene expression responses to immune challenges or eATP stimulation were determined by RT-qPCR.

2.9. ATP hydrolysis assay

To test whether poCD39 is a functional E-NTPDase, HeLa cells (1.2×10^4 cell/well) were seeded into a 96-well plate and transfected with Flag-tagged poCD39 plasmid as a source of enzyme or with empty vector as a control. The expression of poCD39 was confirmed by western blot analysis using anti-Flag antibody. The hydrolysis of ATP in the culture medium by poCD39 ectopically expressed HeLa cells was assessed by a bioluminescent assay. Briefly, after 48 h of transfection, the transfected cells were incubated with 50 μM ATP in culture medium without serum, and the changes in eATP concentrations after different incubation time points were measured with an ATP bioluminescent assay kit CLS II (Roche) in a Tecan Infinite® M200 PRO multimode microplate reader (Switzerland). The enzyme activity of the recombinant poCD39 proteins was indicated by the decreased ATP content (bioluminescence intensity expressed in arbitrary units) in the culture medium.

2.10. ROS production measurement

To test the potential role of poCD39 in eATP-induced reactive oxygen species (ROS) production, overnight-cultured Japanese flounder HKMs in 24-well plates (2.65×10^6 cells/well) were pre-incubated with or without ecto-NTPDase inhibitor ARL 67156 at a final concentration of 100 μM for 2 h to downregulate poCD39 enzyme activity. The HKMs were then stimulated with different concentrations of ATP in the presence of 5 μM carboxymethyl-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) at the indicated amounts of time. The ROS production induced by ATP administration was quantified using a commercial kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) using a protocol described in our previous study (Li et al., 2018a). Triplicate experiments were performed, and the data are presented as the means ± standard deviation.

2.11. Extracellular ATP-induced IL-1beta expression

To investigate the regulatory role of poCD39 in eATP-induced *IL-1beta* gene expression, HKMs (5×10^6 cells/well) were pretreated with or without ecto-NTPDase pharmacological inhibitor ARL 67156 at a final concentration of 100 μM for 2 h to inhibit the endogenous enzymatic activity of poCD39. This was followed by stimulation with 200 or 1000 μM of ATP for 8 h in the presence or absence of ARL 67156. Changes in *IL-1beta* gene expression in response to ARL 67156 treatment were measured by RT-qPCR.

In addition, Japanese flounder FG-9307 cells were mock-transfected or co-transfected with the following plasmid combinations: Flag-tagged poCD39/P2X7R or P2X7R/empty vector in a 1:1 ratio for 48 h. the cells were then stimulated with 1000 μM ATP for 2 h. After treatment, total RNA was extracted, and eATP-induced *IL-1beta* gene expression levels were evaluated by RT-qPCR and normalized to the expression in the mock-transfected controls (set to 1) with *β-actin* serving as an internal reference gene.

2.12. Statistics

The results are expressed as the mean ± standard deviation across triplicate experiments. Comparison of the two groups was carried out using a two tailed unpaired t-test, with a *P* value less than 0.05 indicating significance. Comparison of more than two groups was carried out with one-way ANOVA followed by a Tukey test.

3. Results

3.1. Cloning and sequence analysis of CD39 from Japanese flounder P. olivaceus

Using a combined approach of RT-PCR and RACE technology, the *CD39* gene (namely, *poCD39*) was successfully cloned from Japanese flounder liver tissue. The complete Japanese flounder *poCD39* cDNA sequence comprised a 97 bp 5′-untranslated region, a 1523 bp open reading frame and a 48 bp 3′-untranslated region with a poly(A) tail (Supplementary Fig. 1). This cDNA sequence was deposited into the GenBank database with accession number KP347442. The deduced Japanese flounder poCD39 protein is composed of 497 amino acids with an estimated molecular mass of 55.3 kDa and an isoelectric point of 8.58. A BLAST search against the GenBank database revealed that the poCD39 protein shares approximately 42–89% sequence identity with other teleost CD39 proteins currently available in the NCBI database.

Alignment of the amino acid sequences of Japanese flounder CD39 protein and its counterparts revealed that the apyrase conserved regions (I—IV) in the amino terminus are highly conserved among the compared CD39 proteins in teleost and mammalian species (Fig. 1). In silico sequence analysis further revealed that poCD39 possesses two hydrophobic transmembrane domains, collected by a large extracellular loop and two short 12- and 13-amino acid intracellular domains at the N- and C-terminus, respectively. In the extracellular loop, 10 conserved cysteine residues that may form intra-loop disulfide bonds, 5 potential *N*-glycosylation sites and 21 putative phosphorylation sites were found.

Phylogenetic analysis demonstrated that poCD39 together with other vertebrate CD39 proteins are clustered into the E-NTPDase 1 subfamily, which is separated from the clades formed by E-NTPDase 2, 3, 4, 5, 6, 7 and 8 subfamily proteins (Supplementary Fig. 2). These data suggest that poCD39 is a newly identified member of the E-NTPDase 1 subfamily proteins.

3.2. poCD39 is widely expressed in Japanese flounder tissues and immune cells

The relative basal gene expression levels of *poCD39* in Japanese flounder tissues and immune cells were investigated by RT-qPCR. As shown in Fig. 2, the predominant expression of *poCD39* was found in HKMs, and the lowest expression was found in gonad tissue and FG-9307 cells. Relatively higher expression of *poCD39* was found in the liver, muscle, intestine and heart. An intermediate expression level of *poCD39* was observed in the brain, PBLs, skin and head kidney. Lower expression of *poCD39* was observed in the blood, spleen, gill and trunk kidney tissues.

3.3. poCD39 is a membrane protein with glycosylation modification

The cellular localization of poCD39 proteins was investigated by immunofluorescence microscopy. Because commercially available

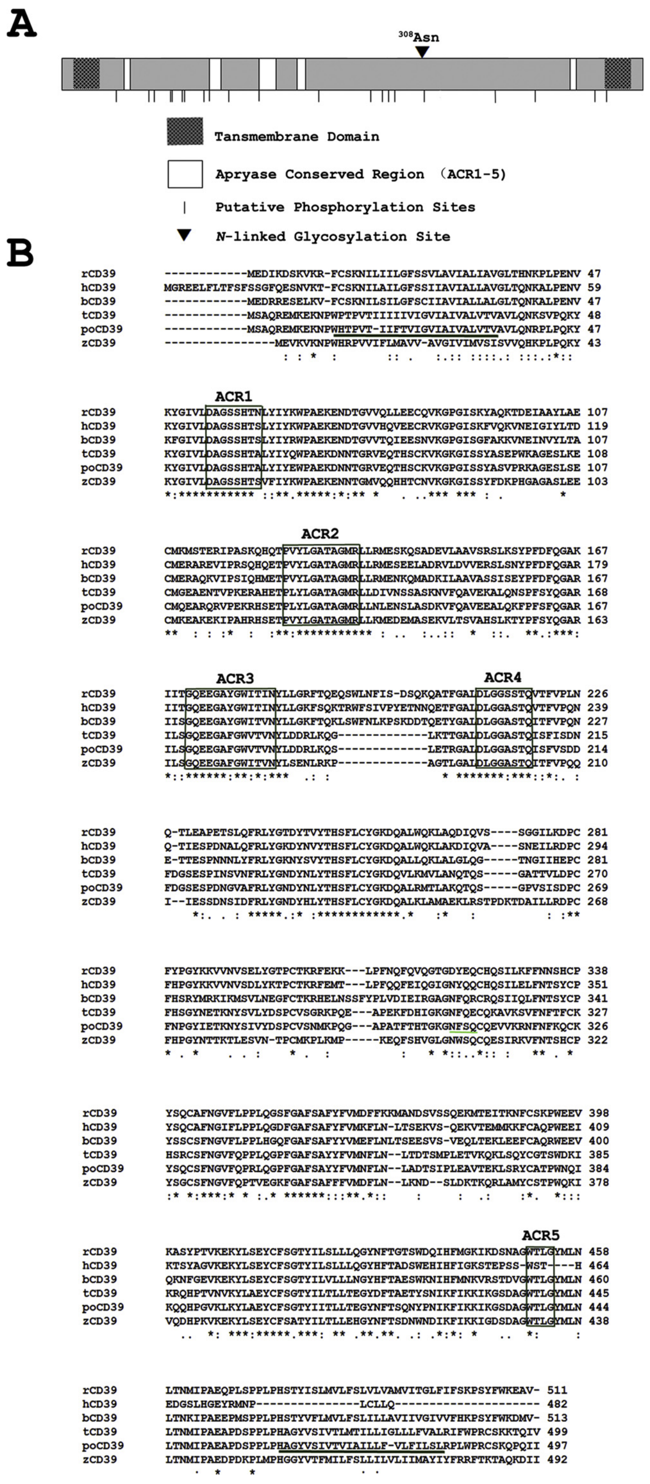

Fig. 1. Schematic representation of Japanese flounder CD39 protein and sequences alignment of selected mammalian and teleost CD39 proteins. (A) Schematic domain structures of poCD39 protein. The *N*-glycosylation site is indicated by black invert arrows on top of the rectangle; the putative phosphorylation residues are indicated by lines under the rectangle. The transmembrane domains are shown as lined boxes. The apyrase conserved regions (ACR 1–5) are shown as empty boxes. (B) Sequence alignment of Japanese flounder CD39 and its homologous proteins from other vertebrate species using the ClustalW program. The amino acid sequences of CD39 proteins with GenBank accession numbers from selected vertebrate species are *Rattus norvegicus* CD39: (rCD39, NP_072109.1); *Homo sapiens* CD39 (hCD39, XP_006718139.1); *Bos taurus* CD39 (bCD39, NP_776961.1); *Oreochromis niloticus* CD39 (tCD39, XP_003441815.2); *Danio rerio* CD39 (zCD39, NP_001003545.1) and *Paralichthys olivaceus* CD39 (poCD39, KP347442). Highly conserved (:), less conserved (.) and identical (*) amino acid residues identified in all proteins are indicated. The five apyrase conserved regions (ACR 1–5) in all CD39 proteins were boxed. The two transmembrane domains and the consensus sequence for *N*-glycosylation in poCD39 protein were underlined in black and green, respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

CD39 antibodies can not specifically recognize the endogenously expressed poCD39 proteins (data not shown), we exogenously expressed Flag-tagged poCD39 protein in Japanese flounder FG-9307 cells. As shown in Fig. 3A, the exogenously expressed poCD39 in FG-9307 cells is clearly targeted on the cell surface, indicating that poCD39 may serve as a membrane-bound enzyme.

Because glycosylation modification is required for cell surface appearance and enzymatic activity of CD39 (Wu et al., 2005), we investigated the glycosylation status of poCD39 protein by digestion with Endo H and PNGase F, which cleave high-mannose *N*-linked glycosylation and all carbohydrate modifications, respectively, from *N*-linked glycoproteins. As shown in Fig. 3B, ectopically expressed poCD39 protein in FG-9307 cells shows two bands. Endo H, which cleaves high-mannose *N*-linked glycosylation modifications, can only remove the lower band, while enzymatic digestion with PNGase F led to disappearance of both the upper and lower bands and caused a faster electrophoretic migration with decreased apparent molecular weight at ˜55 kDa. The glycosylation modification was further validated by LC–MS/MS analysis, showing that poCD39 possesses an *N*-linked glycosylation site at the position of ^{308}Asn (Fig. 3C). Taken together, these findings demonstrated that poCD39 is heterogeneously *N*-glycosylated by high-mannose and complex type glycans at ^{308}Asn.

3.4. poCD39 gene expression induced by immune challenges

To examine the response of poCD39 to inflammatory stimulation in Japanese flounder immune cells, HKMs were stimulated with LPS or zymosan and inflammatory challenge-induced *poCD39* gene expression was measured by RT-qPCR. We found that *poCD39* gene expression was significantly induced by both LPS and zymosan treatment in HKMs (Fig. 4), suggesting that poCD39 may play a role in the response to bacterial and fungal infections. This hypothesis was supported by the upregulated *poCD39* gene expression in Japanese flounder head kidney, spleen and gill tissues following *Edwardsiella tarda* infection (Fig. 5).

3.5. poCD39 is a functional enzyme for hydrolysis of eATP

Our above findings demonstrated that poCD39 is a membrane-bound protein. To test whether poCD39 is a functional enzyme in the hydrolysis of eATP, Flag-tagged poCD39 proteins were exogenously expressed in HeLa cells and the transfected cells were incubated with 50 μM ATP. The changes in eATP levels in the culture medium during the incubation process were measured. In general, in association with the extended incubation durations, ATP concentrations in the culture media from both empty vector- and poCD39 plasmids-transfected cells progressively decreased (Fig. 6). However, compared with the empty vector-transfected control cells, poCD39-transfected cells showed reduced eATP levels after 3 min of incubation. Specifically, after 30 min of incubation, the eATP levels in poCD39-transfected cells were much lower compared with the empty vector-transfected cells, as indicated by a markedly decreased bioluminescence intensity, demonstrating that poCD39 is a functional enzyme for hydrolyzing eATP.

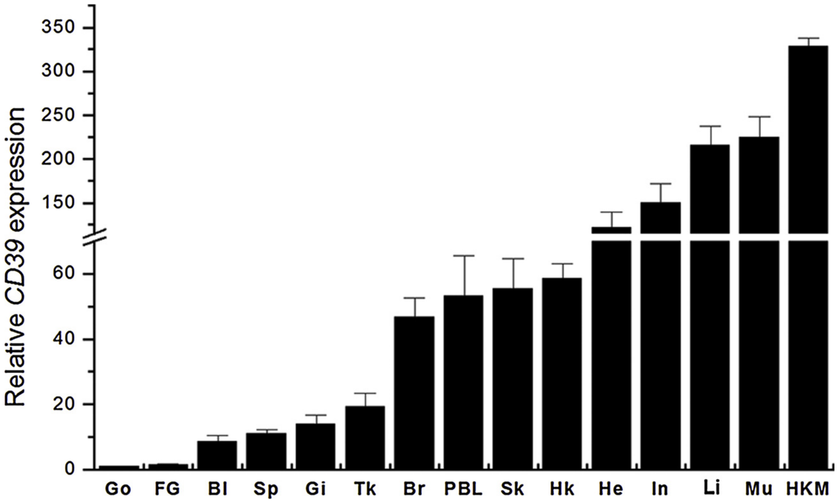

Fig. 2. RT-qPCR analysis of the relative basal expression level of *poCD39* mRNA transcripts in Japanese flounder tissues and immune cells. Individual tissues from five healthy Japanese flounders were equally pooled for evaluation of the relative gene expression level of *poCD39* with *β-actin* serving as an internal reference gene. Go: gonad; FG: Japanese flounder FG-9307 cell line; Bl: blood; Sp: spleen; Gi: gill; TK: trunk kidney; Br: brain; PBL: peripheral blood leukocytes; Sk: skin; HK: head kidney; He: heart; In: intestine; Li: liver; Mu: muscle; HKM: head kidney macrophages. The identities of all PCR products were confirmed by DNA sequencing. Error bars indicate standard deviation (n = 3).

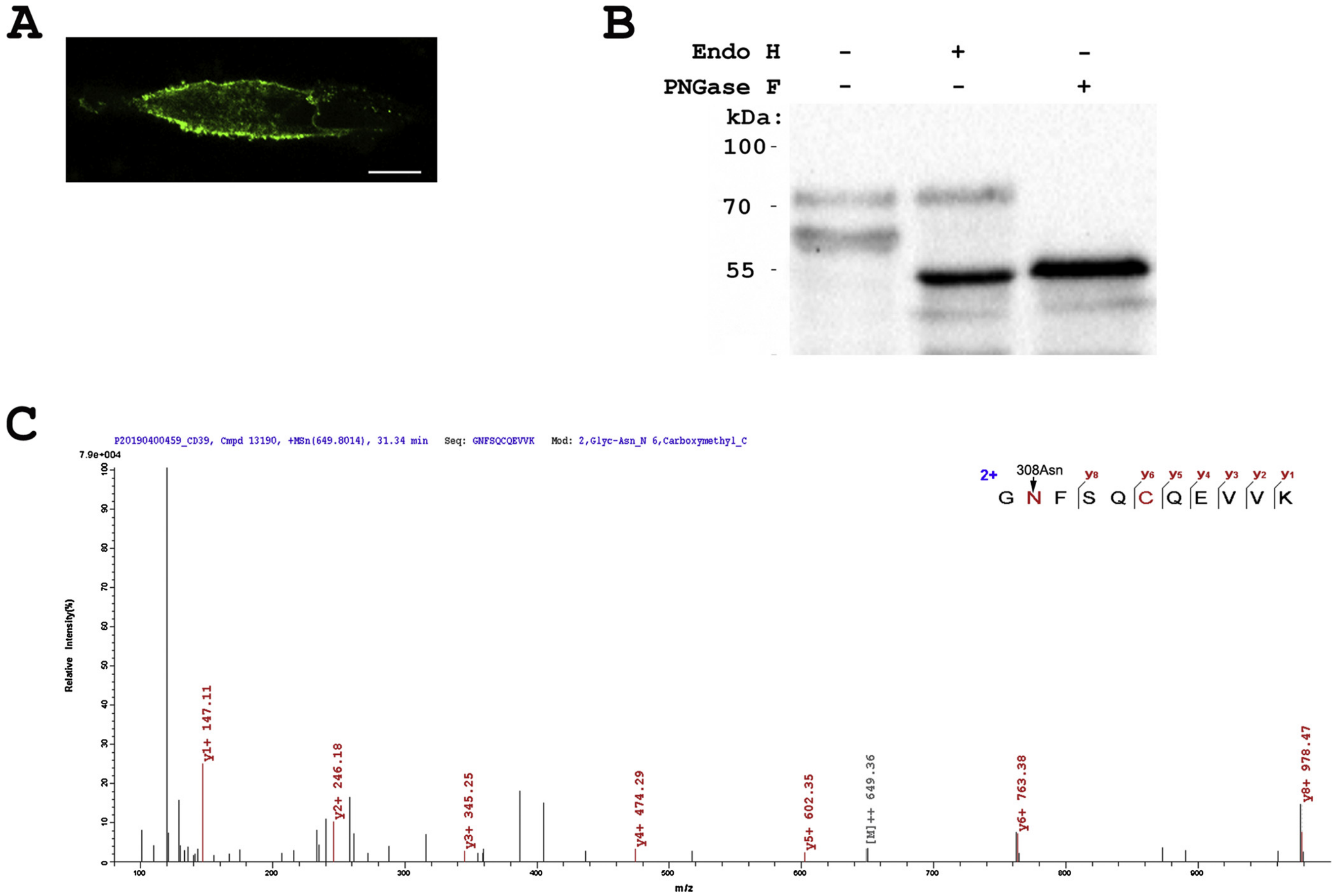

Fig. 3. Cellular distribution and glycosylation modification analysis of poCD39 proteins. (A). Immunofluorescence microscopy using anti-Flag antibody revealed the membrane distribution of exogenously expressed poCD39 proteins in Japanese flounder FG-9307 cells. (B). Glycosylation modification of poCD39 proteins. Cell lysates from FG-9307 cells exogenously expressed Flag-tagged poCD39 proteins was denatured and treated with *N*-glycosidase F or Endo H for 8 h at 37 °C according to the manufacturer's instructions. The proteins were resolved on 12.5% SDS-PAGE and immunoblotted by anti-FLAG antibody. Two major bands (˜80 and ˜65 kDa) proteins represent the glycosylated forms of poCD39 proteins while the band ˜55 kDa represents the de-glycosylated form of poCD39. The data are representative of three experiments. (C). LC−MS/MS analysis of the *N*-linked glycosylation site of poCD39 proteins. Flag-tagged Japanese flounder poCD39 was transfected in FG-9307 cells. The recombinant poCD39 proteins were enriched by co-immunoprecipitation using anti-Flag antibody. The *N*-glycan profiling of poCD39 was determined by LC−MS/MS identification using a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer.

3.6. eATP upregulates poCD39 expression in Japanese flounder HKMs

CD39 is an important immunological switch that controls eATP-driven pro-inflammatory immune cell activities (Antonioli et al., 2013; Boeynaems and Communi, 2006). The upregulated expression of CD39 is often associated with numerous pathological conditions that facilitate eATP hydrolysis (Brisevac et al., 2015); therefore, we examined whether eATP stimulation can alter *poCD39* gene expression in Japanese flounder HKMs. As shown in Fig. 7, *poCD39* was significantly upregulated (up to approximately 7-fold compared with untreated control

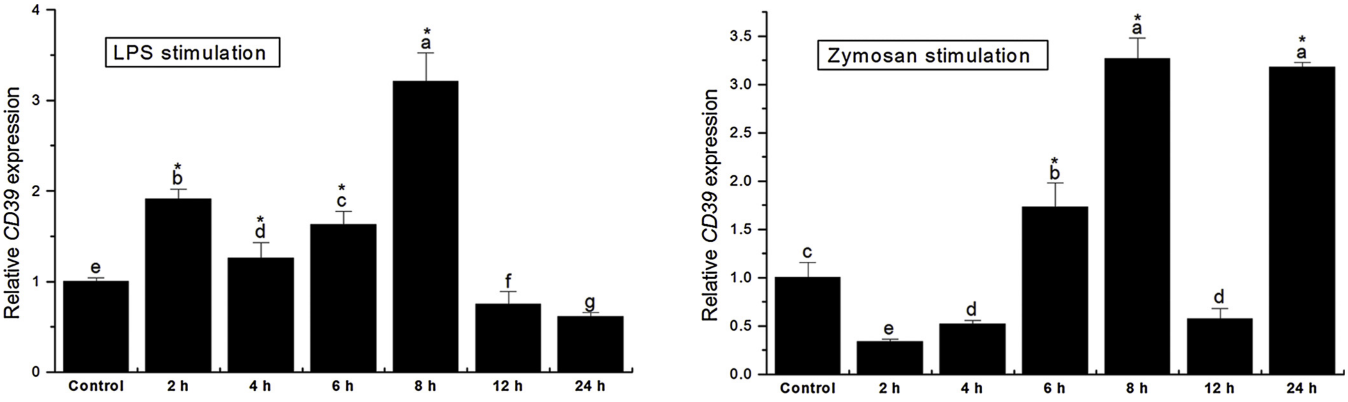

Fig. 4. *poCD39* gene expression in response to inflammatory challenges in Japanese flounder HKMs. The relative gene expression levels of *poCD39* after LPS (A) and zymosan (B) stimulations in Japanese flounder HKMs was quantified by RT-qPCR with *β-actin* serving as an internal reference gene. The expression of *poCD39* gene in response to inflammatory challenges was normalized to untreated control cells (set to 1). Data are presented as means ± standard deviations (n = 3). Groups marked by different *lowercase* letters above each bar represent a significant difference at $p < 0.05$. The same letters indicate no significant difference between groups. Asterisks (*) mark the significant increase of *poCD39* gene expression compared with the untreated controls ($p < 0.05$).

cells) 2 h following ATP treatment, and this upregulated expression lasted 24 h after ATP stimulation.

3.7. poCD39 is involved in regulation of eATP-induced pro-inflammatory cytokine IL-1beta gene expression and nitric oxide production

To gain insight into the regulatory role of poCD39 in Japanese flounder innate immunity, we used the ecto-ATPase inhibitor ARL 67156, to inhibit the enzyme activity of poCD39. We then examined the resultant eATP-induced pro-inflammatory cytokine *IL-1beta* gene expression and ROS production in the HKMs. As shown in Fig. 8A, *IL-1beta* gene expression was induced by ATP treatment in the HKMs and was augmented by inhibition of poCD39 activities with ARL 67156. Since ARL 67156 can inhibit the enzyme activities of both CD39 and other NTPDases (Lévesque et al., 2007), we attempted to silence the endogenous expression of *poCD39* in the HKMs by small interfering RNA (siRNA). Unfortunately, all sets of siRNA targeted at different regions of the *poCD39* gene failed to downregulate the endogenous expression of *poCD39* in the HKMs. To overcome this limitation, we exogenously expressed poCD39 in Japanese flounder FG-9307 cells and examined the resultant *IL-1beta* gene expression changes. As shown in Fig. 8B, after ATP treatment, co-expression of poCD39 with P2X7R led to decreased *IL-beta* gene expression compared to co-expression of P2X7R with empty vector-transfected cells. These data indicate that poCD39 plays an important role in downregulating eATP/P2X7R-induced *IL-1beta* gene expression.

We next examined the regulatory role of poCD39 in eATP-induced ROS production in the HKMs. Fig. 8C shows that inhibition of poCD39 enzyme activity with ARL 67156 significantly increased eATP-induced ROS production at 10 min after 1000 μM ATP treatment. However, the impact of ARL 67156 on eATP-induced ROS production was only associated with the administration of higher ATP concentrations (1 mM) as there was no significant difference in ROS production when 200 μm ATP was applied (data not shown).

4. Discussion

Our previous studies demonstrated that ATP released from fish cells under conditions of inflammatory stimulation or infection, is a critical extracellular signaling molecule that plays important roles in the activation of fish innate immune responses through activation of purinergic signaling cascades (Li et al., 2018a, 2014b). Although proper inflammation is essential for the repair and resolution of infection and damage, excessive inflammation is deleterious and may cause serious diseases or other negative outcomes. In contrast to the pro-inflammatory functions driven by eATP, ATP metabolites can exert anti-inflammatory roles (Antonioli et al., 2013). The delicate control of eATP concentrations is thus very important to maintain moderate and efficient immune response levels in response to microbial insults or tissue damage. eATP metabolism provides a critical means to control the eATP-initiated innate immune response. CD39 is a membrane-bound ectonucleotidase that hydrolyzes extracellular nucleoside tri- or diphosphates to monophosphate. CD39 plays important roles in regulating eATP-mediated inflammatory responses by tightly controlling the ATP level in the extracellular space (Dwyer et al., 2007; Mizumoto et al., 2002). Recent evidence indicates that CD39 is a negative regulator of P2X7-mediated inflammatory cell death in mast cells (Kuhny et al., 2014). However, the immune significance of CD39 in lower vertebrates, especially in teleosts, is still unknown. In this study, we identified and characterized a *CD39* gene from *P. olivaceus* and investigated its potential role in regulating the eATP-induced innate immune responses in fish.

Similar to its human counterpart (Maliszewski et al., 1994), Japanese flounder CD39 protein harbors several potential *N*-linked glycosylation sites, which are crucial for correct protein folding, membrane targeting, and enzyme activity (Smith and Kirley, 1998). In the extracellular hydrophobic domain, *P. olivaceus* CD39 possesses five highly conserved domains, known as apyrase conserved regions (ACRs) 1–5, which are pivotal for the catabolic activity of the enzyme (Heine et al., 2001). In addition, there is a conserved phosphate-binding motif in both ACR 1 and ACR 5 domains in Japanese flounder CD39 protein, which has been shown to be critical for stabilizing the interaction between the enzyme and its nucleotide substrate during phosphate cleavage (Antonioli et al., 2013). Given that poCD39 processes potential glycosylation sites and glycosylation modification is important for cell surface appearance and enzyme activity of CD39 (Wu et al., 2005), we ectopically expressed poCD39 in Japanese flounder FG-9307 cells and investigated its glycosylation entities. We found that, similar to mammalian CD39 proteins (Papanikolaou et al., 2011), poCD39 shows two bands that could be removed by PNGase F digestion, suggesting that poCD39 is a glycosylated protein. The glycosylation modification profiling was further confirmed by LC–MS/MS analysis. Because poCD39 possesses the critical glycosylation residue that has been confirmed to be principally required for cell surface appearance of enzymatically active CD39 (Wu et al., 2005), the glycosylated residue may contribute importantly to poCD39 enzymatic activities. In addition, phosphorylation of proteins plays a cardinal role in regulation of many cellular processes (Hunter, 1995), and mammalian CD39 proteins have been demonstrated to be phosphorylated proteins with clear implications for the regulation of the enzyme activity (Wink et al., 2000). In this study,

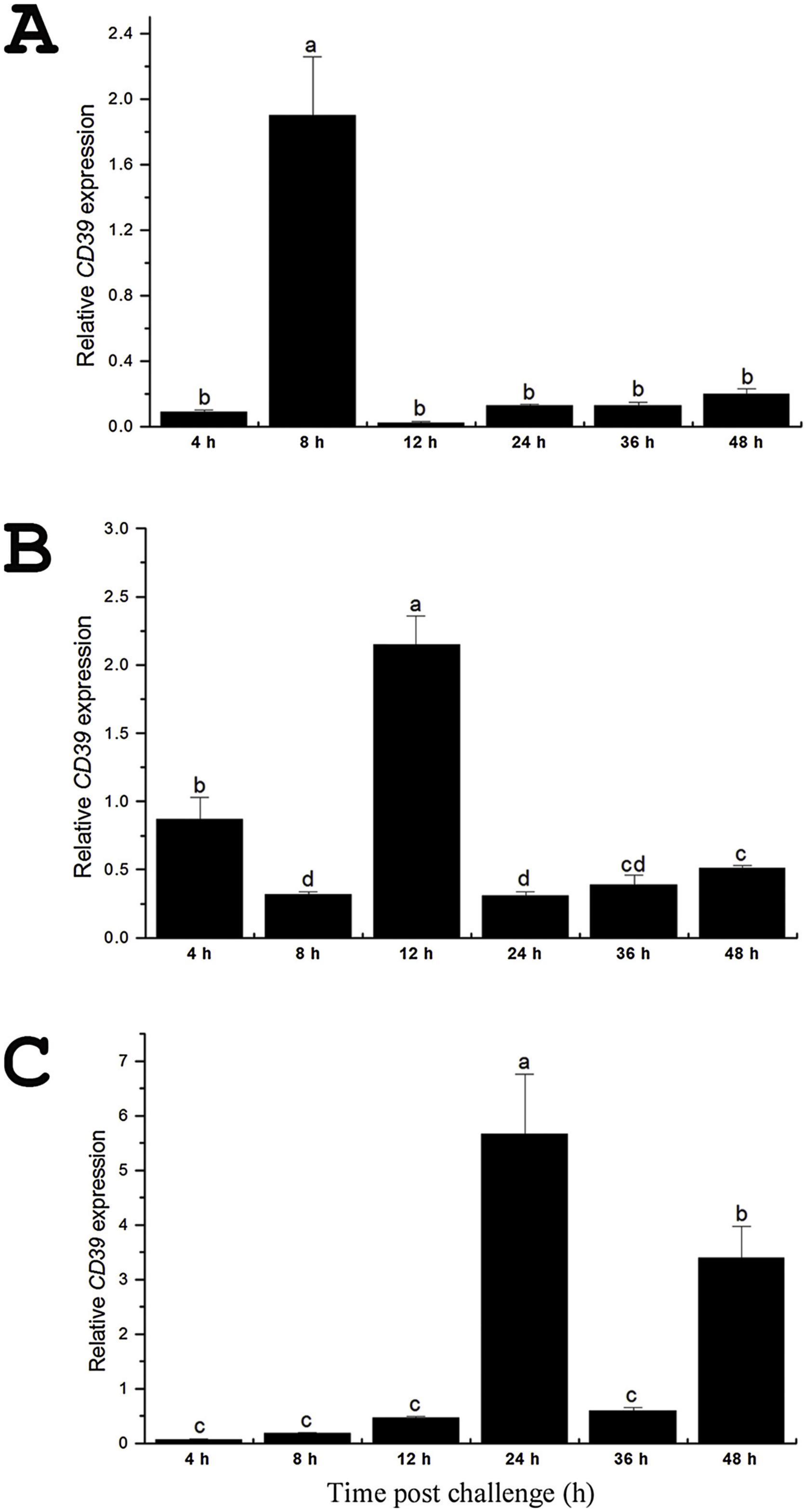

Fig. 5. *poCD39* gene expression in response to bacterial infections. RT-qPCR was applied to analyze the bacterial challenge-induced *poCD39* gene expression. The expression levels of *poCD39* gene after *E. tarda* challenges in spleen (A), head kidney (B) and gill (C) were relative to its expression in control experiments of each time point (normalized to 1) with *β-actin* serving as an internal reference gene. Data are presented as means ± standard deviation (n = 3). Different lowercase letters indicate significant difference ($p < 0.05$) among treatments.

poCD39 also processes several potential phosphorylation sites, indicating that the enzyme activity of poCD39 may be regulated by phosphorylation of protein kinases.

We next investigated the tissue expression of *poCD39* on RNA levels and found that Japanese flounder *CD39* mRNA was ubiquitously present in all tested tissues with predominant expression in muscle and liver. It has been demonstrated that CD39 is the dominant ecto-nucleotidase in mouse hepatic vascular endothelial cells and plays an

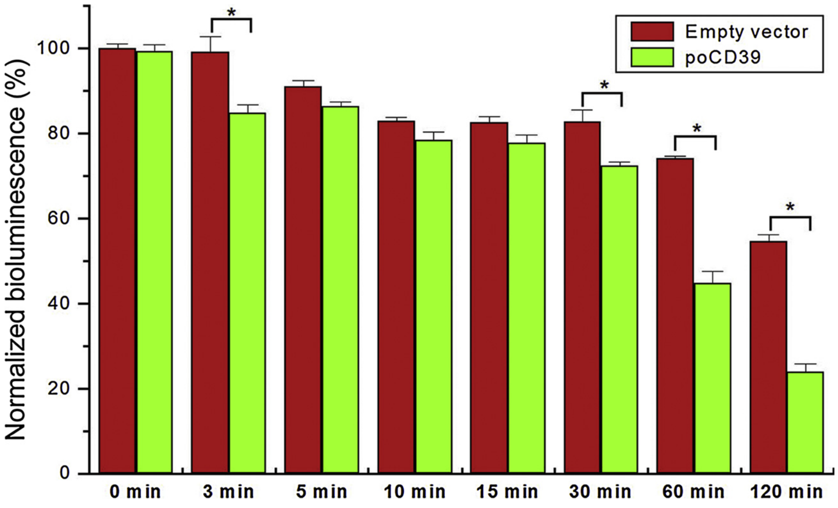

Fig. 6. poCD39 is a functional protein for hydrolysis of eATP. Overnight-cultured HeLa cells (1.2×10^4 cell/well) in a 96-well plate were transiently transfected with Flag-tagged poCD39 plasmid as a source of enzyme or empty vector as a control. After 48 h of transfection, the transfected cells were incubated with 50 μM ATP in culture medium without serum and the changes of ATP concentrations in culture medium were measured with an ATP bioluminescent assay kit CLS II (Roche) in a Tecan Infinite® M200 PRO multimode microplate reader (Switzerland). Progressively decreased ATP content in the culture medium indicated by reduced bioluminescence intensity was observed both in poCD39 plasmid- or empty vector-transfected cells. However, after 30 min incubation poCD39-overexpressed cells show much less ATP content in the culture medium than empty vector-transfected cells, reflecting the enzyme activity of the recombinant poCD39. Data are mean ± standard deviation of triplicate treatments from one representative experiment; similar results were obtained on two other separated occasions.

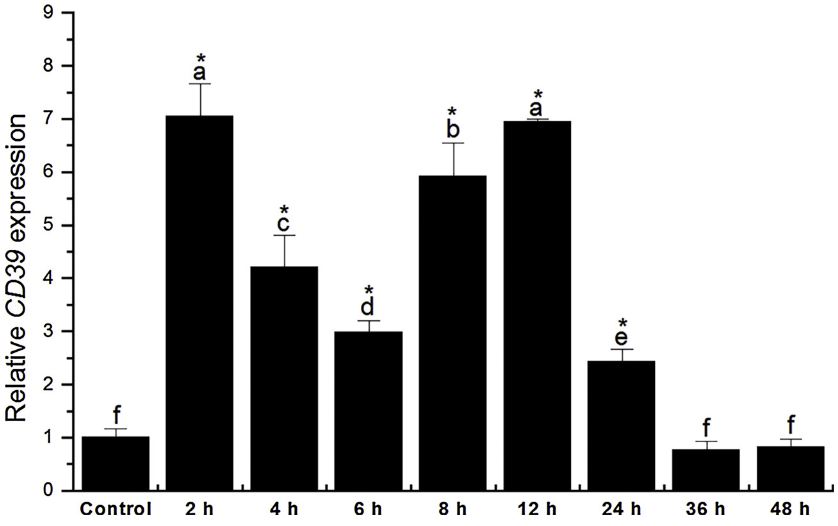

Fig. 7. eATP stimulation-induced *poCD39* gene expression in the HKMs. The induction of *poCD39* gene expression in response to eATP stimulation in the HKMs was evaluated by RT-qPCR with *β-actin* serving as an internal reference gene. Values are presented as means ± standard deviation ($n = 3$). Asterisks (*) mark the significant increase of *poCD39* gene expression compared with the untreated controls ($p < 0.05$). Groups denoted by different lowercase letters represent significant difference at $p < 0.05$. The same letters indicate no significant difference between groups.

essential protective role in hepatic injury and systemic inflammation (Sun et al., 2011). CD39 also has been suggested to play a role in regulating innate immune functions in liver dendritic cells (Yoshida et al., 2013). In addition to liver, dominant expression of CD39 was also found in mouse vascular smooth muscle cells, and deletion of CD39 in mice can result in decreased neointimal formation after vascular injury (Behdad et al., 2009). The relatively high expression of *poCD39* in Japanese flounder liver and muscle tissues thus indicates that poCD39 may play an important role in the regulation of eATP-mediated purinergic signaling in these tissues.

At the cellular level, CD39 was initially described as an activation marker of lymphoid cells (Maliszewski et al., 1994) and was expressed constitutively in a wide range of immune cell types, such as B cells, natural killer (NK) cells, dendritic cells, Langerhans cells, monocytes, macrophages, mesangial cells, neutrophils, leukocytes and regulatory T cells (Dwyer et al., 2007; Koziak et al., 1999). In addition, it has been demonstrated that CD39 is the dominant ecto-nucleotidase responsible for the hydrolysis of ATP and ADP at the surface of mouse primary macrophages (Levesque et al., 2010). Consistent with this observation, we found that *poCD39* was also dominantly expressed in Japanese flounder HKMs. We therefore focused on the immune roles of poCD39 in the HKMs in the present study. Using an immunofluorescence assay, we further showed that poCD39 protein was clearly targeted on the cell membrane, a property required to hydrolyze ATP in the extracellular space.

CD39 is an inducible gene in mammals and remarkable changes in CD39 enzyme activity have been observed in different pathophysiological contexts (Antonioli et al., 2013). It has been reported that LPS, Poly(I:C) and *E. coli* stimulation can result in the upregulation of CD39 in mouse macrophages (Csoka et al., 2015). A strong association has been found between CD39 expression and AIDS disease progression and significant increase of CD39 expression on Tregs was also observed in patients with human immunodeficiency virus (HIV) infection (Nikolova et al., 2011). In agreement with these studies, we found that *poCD39* expression was significantly increased by LPS or zymosan challenges. *E. tarda* infection, however, initially downregulated *poCD39* expression, followed by a significant upregulation. We previously showed that ATP is released from fish cells into the extracellular milieu during inflammatory stimulation and pathogen infection processes (Li et al., 2018d, 2014a, 2016c). We also demonstrated that eATP is a potent signaling molecule in the activation of the Japanese flounder innate immune response (Li et al., 2018a). Downregulation of *poCD39* at the early stage after bacterial infection may favor enhanced inflammation to facilitate bacterial clearance; enhanced expression of *poCD39* in the later stage may favor ATP conversion to adenosine and activation of the P1 receptor to shift from inflammatory to anti-inflammatory status and avoid excessive inflammation.

To test whether poCD39 is a functional protein, we overexpressed poCD39 protein in HeLa cells and examined the resultant effects on eATP levels. We found that the ATP levels in the cell culture medium were significantly reduced in poCD39-overexpressed cells compared with the empty vector-transfected cells. These data suggest that poCD39

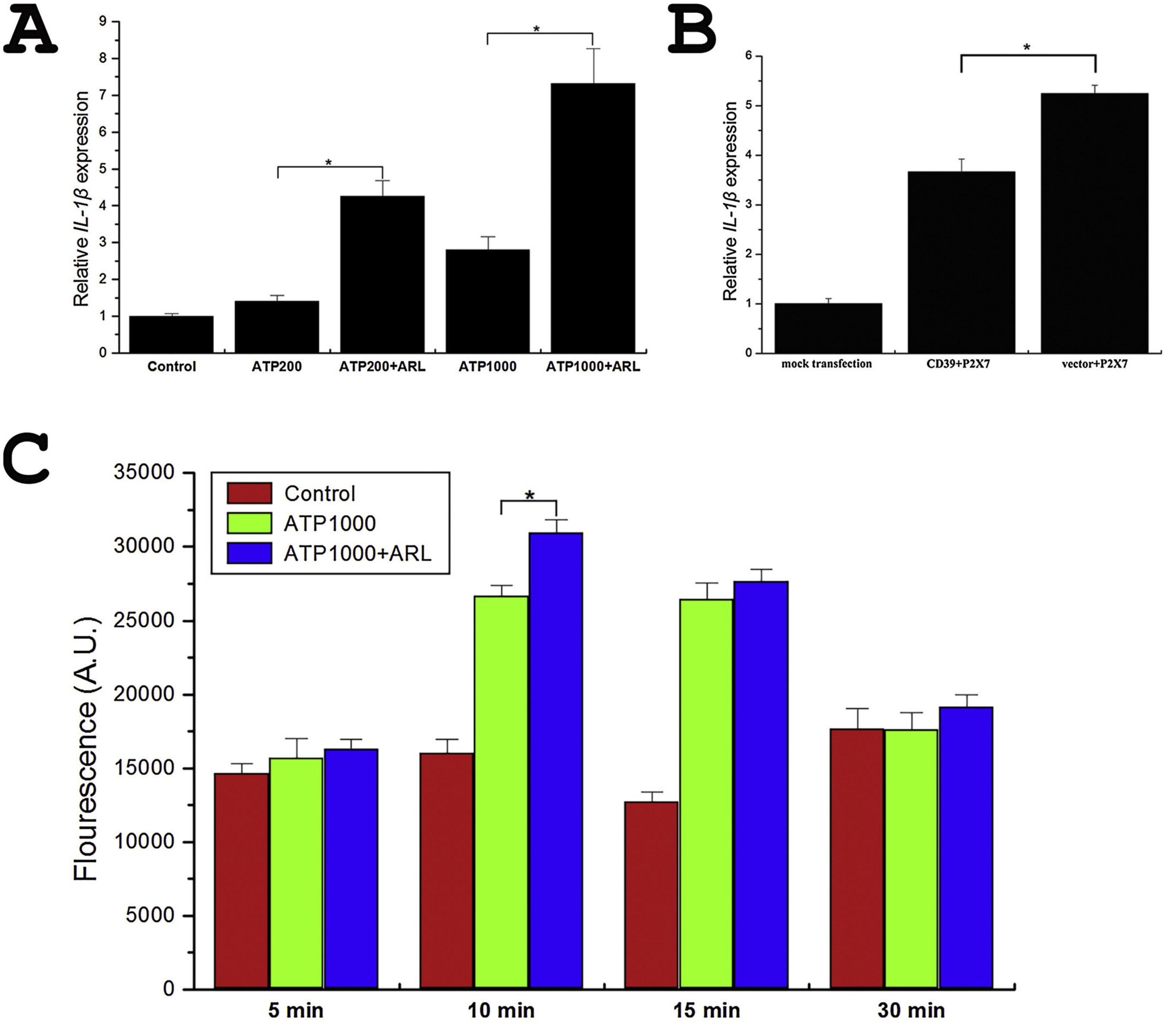

Fig. 8. poCD39 regulates *IL-beta* gene expression and ROS production in Japanese flounder HKMs. (A) Inhibition of poCD39 enzyme activity with ecto-NTPDase inhibitor ARL 67156 increases eATP-induced *IL-1beta* gene expression in the HKMs. Overnight-cultured HKMs (5×10^6 cells/well) were pretreated with or without 100 μM ecto-NTPDase pharmacological inhibitor ARL 67156 for 2 h to inhibit the endogenous enzymatic activity of poCD39. The cells were then stimulation with 200 or 1000 μM ATP for 8 h in the presence or absence of ARL 67156. *IL-1beta* gene expression changes by ARL 67156 treatment was measured by RT-qPCR. (B) Overexpression of poCD39 in Japanese flounder FG-9307 cells resulted in decreased *IL-1beta* gene expression induced by eATP. Japanese flounder FG-9307 cells were mock transfected or co-transfected with Flag-tagged poCD39/P2X7R or P2 X7R/empty vector for 48 h and then stimulated with 1000 μM ATP for 2 h. eATP -induced *IL-1beta* gene expression was measured by RT-qPCR with *β-actin* serving as an internal reference gene. (C) Inhibition of poCD39 enzyme activity with ecto-NTPDase inhibitor ARL 67156 increases eATP-induced ROS production in Japanese flounder HKMs. Japanese flounder HKMs cultured overnight were pre-incubated with or without 100 μM ecto-NTPDase inhibitor ARL 67156 for 2 h and then stimulated with 1000 μM ATP in the presence or absence of ARL 67156 for the indicated time points. After treatment, culture medium was collected and centrifuged. ROS production in the culture supernatant was determined by a ROS assay kit and was presented as fluorescence changes in arbitrary units. Values are presented as the means ± standard deviation (n = 3). Asterisks (*) mark the significant differences of *IL-1beta* gene expression (A and B) or ROS production (C) at $p < 0.05$.

is a functional enzyme for phosphohydrolysis of eATP, and may play a potential role in regulating the duration and extent of eATP-induced innate immune responses by controlling the available ATP levels in the extracellular environment.

We therefore examined the potential role of poCD39 in eATP-driven innate immune responses in Japanese flounder HKMs. We found that inhibition of poCD39 enzyme activity with the ecto-NTPDase inhibitor ARL 67156 significantly augmented eATP-induced pro-inflammatory cytokine *IL-1beta* gene expression, suggesting that poCD39 is involved in the regulation of eATP-mediated pro-inflammatory cytokine gene expression in fish. Similarly, recent studies have shown that CD39 plays an essential role in regulating P2X7R-dependent IL-1beta production in murine macrophages (Levesque et al., 2010). Furthermore, ROS production in the HKMs was also significantly increased by pre-incubation with ARL 67156, suggesting a role for poCD39 in the attenuation of eATP-activated innate immune responses. We attempted to down-regulate *poCD39* endogenous expression by siRNA in Japanese flounder HKMs. Unfortunately, a set of siRNAs that target either the non-coding region or coding region of *poCD39* failed to downregulate *poCD39* expression. Since ARL 67156 can also block the activities of other ecto-NTPDases (Lévesque et al., 2007), we could not exclude the possible contributions from other ecto-NTPDase members in these processes.

Further studies are therefore needed to clarify this possibility. However, given the high expression level of *poCD39* in Japanese flounder HKMs and the reduced *IL-1beta* expression in poCD39 overexpressing FG-9307cells, we propose that poCD39 may serve as a “brake” on the pro-inflammatory actions of eATP in fish by controlling the available eATP levels.

Considering the important role of poCD39 in modulating eATP-induced innate immune responses, we next asked whether eATP could exert a feedback role in regulating *poCD39* expression. We found that 200 μM ATP stimulations can significantly upregulate *poCD39* expression (approximately 7-fold than untreated control) in Japanese flounder HKMs, suggesting that the dominantly-expressed P2X2R in the HKMs (Li et al., 2016b) that is activated by lower ATP concentrations (usually at the micromolar level) is likely implicated in this process. The upregulated *poCD39* expression may lead to enhanced poCD39 enzymatic activity, allowing the HKMs to adjust the outcome of eATP-mediated innate immune responses by reducing the available eATP concentrations and attenuating eATP-activated immune responses, and thereby preventing excessive inflammation.

In summary, our data demonstrated for the first time that 1) fish CD39 is a functional glycosylated membrane protein involved in regulating eATP-mediated innate immune responses in fish and 2) inflammatory stimuli, pathogen infection and eATP are important regulators of fish *CD39* transcription. Our findings suggest that CD39 is a potential therapeutic target for limiting the inflammatory responses in fish.

Declarations of interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.04.024.

References

Antonioli, L., Pacher, P., Vizi, E.S., Haskó, G., 2013. CD39 and CD73 in immunity and inflammation. Trends Mol. Med. 19, 355–367.

Behdad, A., Sun, X., Khalpey, Z., Enjyoji, K., Wink, M., Wu, Y., Usheva, A., Robson, S.C., 2009. Vascular smooth muscle cell expression of ectonucleotidase CD39 (ENTPD1) is required for neointimal formation in mice. Purinergic Signal. 5, 335–342.

Boeynaems, J.-M., Communi, D., 2006. Modulation of inflammation by extracellular nucleotides. J. Invest. Dermatol. 126, 943–944.

Brisevac, D., Adzic, M., Laketa, D., Parabucki, A., Milosevic, M., Lavrnja, I., Bjelobaba, I., Sevigny, J., Kipp, M., Nedeljkovic, N., 2015. Extracellular ATP selectively upregulates ecto-nucleoside triphosphate diphosphohydrolase 2 and ecto-5’-nucleotidase by rat cortical astrocytes in vitro. J. Mol. Neurosci.

Colgan, S.P., Eltzschig, H.K., Eckle, T., Thompson, L.F., 2006. Physiological roles for ecto-5’-nucleotidase (CD73). Purinergic Signal. 2, 351–360.

Csoka, B., Nemeth, Z.H., Toro, G., Koscso, B., Kokai, E., Robson, S.C., Enjyoji, K., Rolandelli, R.H., Erdelyi, K., Pacher, P., Hasko, G., 2015. CD39 improves survival in microbial sepsis by attenuating systemic inflammation. FASEB J. 29, 25–36.

Dwyer, K.M., Deaglio, S., Gao, W., Friedman, D., Strom, T.B., Robson, S.C., 2007. CD39 and control of cellular immune responses. Purinergic Signal. 3, 171–180.

Heine, P., Braun, N., Sevigny, J., Robson, S.C., Servos, J., Zimmermann, H., 2001. The C-terminal cysteine-rich region dictates specific catalytic properties in chimeras of the ectonucleotidases NTPDase1 and NTPDase2. Eur. J. Biochem. 268, 364–373.

Hunter, T., 1995. Protein kinases and phosphatases: the Yin and Yang of protein phosphorylation and signaling. Cell 80, 225–236.

Imai, M., Kaczmarek, E., Koziak, K., Sévigny, J., Goepfert, C., Guckelberger, O., Csizmadia, E., Schulte am Esch, J., Robson, S.C., 1999. Suppression of ATP Diphosphohydrolase/CD39 in human vascular endothelial cells†. Biochemistry 38, 13473–13479.

Koziak, K., Sevigny, J., Robson, S.C., Siegel, J.B., Kaczmarek, E., 1999. Analysis of CD39/ATP diphosphohydrolase (ATPDase) expression in endothelial cells, platelets and leukocytes. Thromb. Haemost. 82, 1538–1544.

Kuhny, M., Hochdörfer, T., Ayata, C.K., Idzko, M., Huber, M., 2014. CD39 is a negative regulator of P2X7-mediated inflammatory cell death in mast cells. Cell Commun. Signal.: CCS 12 40-40.

Kukulski, F., Bahrami, F., Ben Yebdri, F., Lecka, J., Martin-Satue, M., Levesque, S.A., Sevigny, J., 2011. NTPDase1 controls IL-8 production by human neutrophils. J. Immunol. 187, 644–653.

Lévesque, S.A., Lavoie, É.G., Lecka, J., Bigonnesse, F., Sévigny, J., 2007. Specificity of the ecto-ATPase inhibitor ARL 67156 on human and mouse ectonucleotidases. Br. J. Pharmacol. 152, 141–150.

Levesque, S.A., Kukulski, F., Enjyoji, K., Robson, S.C., Sevigny, J., 2010. NTPDase1 governs P2X7-dependent functions in murine macrophages. Eur. J. Immunol. 40, 1473–1485.

Li, S., Li, X., Chen, X., Geng, X., Sun, J., 2014a. ATP release channel Pannexin1 is a novel immune response gene in Japanese flounder Paralichthys olivaceus. Fish Shellfish Immunol. 40, 164–173.

Li, S., Li, X., Coddou, C., Geng, X., Wei, J., Sun, J., 2014b. Molecular Characterization and Expression Analysis of ATP-Gated P2X7 Receptor Involved in Japanese Flounder (*Paralichthys olivaceus*) Innate Immune Response. PLoS One 9, e96625.

Li, S., Chen, X., Geng, X., Zhan, W., Sun, J., 2015a. Identification and expression analysis of nascent polypeptide-associated complex alpha gene in response to immune challenges in Japanese flounder Paralichthys olivaceus. Fish Shellfish Immunol. 46, 261–267.

Li, S., Chen, X., Li, X., Geng, X., Lin, R., Li, M., Sun, J., 2015b. Molecular characterization of purinergic receptor P2X4 involved in Japanese flounder (Paralichthys olivaceus) innate immune response and its interaction with ATP release channel Pannexin1. Fish Shellfish Immunol. 47, 100–109.

Li, S., Chen, X., Hao, G., Geng, X., Zhan, W., Sun, J., 2016a. Identification and characterization of a novel NOD-like receptor family CARD domain containing 3 gene in response to extracellular ATP stimulation and its role in regulating LPS-induced innate immune response in Japanese flounder (Paralichthys olivaceus) head kidney macrophages. Fish Shellfish Immunol. 50, 79–90.

Li, S., Chen, X., Hao, G., Geng, X., Zhan, W., Sun, J., 2016b. Identification and characterization of ATP-gated P2X2 receptor gene dominantly expressed in the Japanese flounder (Paralichthys olivaceus) head kidney macrophages. Fish Shellfish Immunol. 54, 312–321.

Li, S., Peng, W., Chen, X., Geng, X., Zhan, W., Sun, J., 2016c. Expression and role of gap junction protein connexin43 in immune challenge-induced extracellular ATP release in Japanese flounder (Paralichthys olivaceus). Fish Shellfish Immunol. 55, 348–357.

Li, S., Peng, W., Li, J., Hao, G., Geng, X., Sun, J., 2017. Characterization of Japanese flounder (Paralichthys olivaceus) Caspase1 involved in extracellular ATP-mediated immune signaling in fish. Fish Shellfish Immunol. 67, 536–545.

Li, S., Chen, X., Li, J., Li, X., Zhang, T., Hao, G., Sun, J., 2018a. Extracellular ATP is a potent signaling molecule in the activation of the Japanese flounder (Paralichthys olivaceus) innate immune responses. Innate Immun In press.

Li, S., Hao, G., Xu, Y., Wang, N., Li, J., Geng, X., Sun, J., 2018b. Functional characterization of purinergic P2Y2 and P2Y12 receptors involved in Japanese flounder (Paralichthys olivaceus) innate immune responses. Fish Shellfish Immunol. 75, 208–215.

Li, S., Li, J., Wang, N., Hao, G., Sun, J., 2018c. Characterization of UDP-Activated purinergic receptor P2Y6 involved in Japanese flounder Paralichthys olivaceus innate immunity. Int. J. Mol. Sci. 19, 2095.

Li, S., Li, J., Wang, N., Zhang, T., Xu, Y., Sun, J., 2018d. Expression analysis of Pannexin1 channel gene in response to immune challenges and its role in infection-induced ATP release in tilapia (Oreochromis niloticus). Fish Shellfish Immunol. 81, 470–475.

Li, S., Li, J., Peng, W., Hao, G., Sun, J., 2019. Characterization of the responses of the caspase 2, 3, 6 and 8 genes to immune challenges and extracellular ATP stimulation in the Japanese flounder (Paralichthys olivaceus). BMC Vet. Res. 15, 20.

Maliszewski, C.R., Delespesse, G.J., Schoenborn, M.A., Armitage, R.J., Fanslow, W.C., Nakajima, T., Baker, E., Sutherland, G.R., Poindexter, K., Birks, C., 1994. The CD39 lymphoid cell activation antigen. Molecular cloning and structural characterization. J. Immunol. 153, 3574–3583.

Mizumoto, N., Kumamoto, T., Robson, S.C., Sevigny, J., Matsue, H., Enjyoji, K., Takashima, A., 2002. CD39 is the dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness. Nat. Med. 8, 358–365.

Nikolova, M., Carriere, M., Jenabian, M.-A., Limou, S., Younas, M., Kök, A., Huë, S., Seddiki, N., Hulin, A., Delaneau, O., Schuitemaker, H., Herbeck, J.T., Mullins, J.I., Muhtarova, M., Bensussan, A., Zagury, J.-F., Lelievre, J.-D., Lévy, Y., 2011. CD39/Adenosine pathway is involved in AIDS progression. PLoS Pathog. 7, e1002110.

Papanikolaou, A., Papafotika, A., Christoforidis, S., 2011. CD39 reveals novel insights into the role of transmembrane domains in protein processing, apical targeting and activity. Traffic 12, 1148–1165.

Schulze zur Wiesch, J., Thomssen, A., Hartjen, P., Tóth, I., Lehmann, C., Meyer-Olson, D., Colberg, K., Frerk, S., Babikir, D., Schmiedel, S., Degen, O., Mauss, S., Rockstroh, J., Staszewski, S., Khaykin, P., Strasak, A., Lohse, A.W., Fätkenheuer, G., Hauber, J., van Lunzen, J., 2011. Comprehensive analysis of frequency and phenotype of t regulatory cells in HIV infection: CD39 expression of FoxP3+ t regulatory cells correlates with progressive disease. J. Virol. 85, 1287–1297.

Smith, T.M., Kirley, T.L., 1998. Cloning, sequencing, and expression of a human brain ecto-apyrase related to both the ecto-ATPases and CD39 ecto-apyrases. Biochim. Biophys. Acta (BBA) - Protein Structure and Molecular Enzymology 1386, 65–78.

Sun, X., Imai, M., Nowak-Machen, M., Guckelberger, O., Enjyoji, K., Wu, Y., Khalpey, Z., Berberat, P., Munasinghe, J., Robson, S., 2011. Liver damage and systemic inflammatory responses are exacerbated by the genetic deletion of CD39 in total hepatic ischemia. Purinergic Signal. 7, 427–434.

Wink, M., Lenz, G., Rodnight, R., Sarkis, J.F., Battastini, A.O., 2000. Identification of brain ecto-apyrase as a phosphoprotein. Mol. Cell. Biochem. 213, 11–16.

Wu, J.J., Choi, L.E., Guidotti, G., 2005. N-linked Oligosaccharides Affect the Enzymatic Activity of CD39: Diverse Interactions between Seven N-linked Glycosylation Sites. Mol. Biol. Cell 16, 1661–1672.

Yoshida, O., Kimura, S., Jackson, E.K., Robson, S.C., Geller, D.A., Murase, N., Thomson, A.W., 2013. CD39 expression by hepatic myeloid dendritic cells attenuates inflammation in liver transplant ischemia-reperfusion injury in mice. Hepatology 58, 2163–2175.

Zhang, H.-J., Tian, J., Qi, X.-K., Xiang, T., He, G.-P., Zhang, H., Yu, X., Zhang, X., Zhao, B., Feng, Q.-S., Chen, M.-Y., Zeng, M.-S., Zeng, Y.-X., Feng, L., 2018. Epstein-Barr virus activates F-box protein FBXO2 to limit viral infectivity by targeting glycoprotein B for degradation. PLoS Pathog. 14, e1007208.

Zimmermann, H., 2001. Ectonucleotidases: some recent developments and a note on nomenclature. Drug Dev. Res. 52, 44–56.