Fish and Shellfish Immunology 93 (2019) 200–207

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Functional characterization of purinergic receptor $P2Y_{14}$ in the Japanese flounder (*Paralichthys olivaceus*) head kidney macrophages

Shuo Li[a,1], Nan Wang[a], Yu Feng[a], Jiafang Li[a], Xuyun Geng[b], Jinsheng Sun[a,*]

[a] Tianjin Key Laboratory of Animal and Plant Resistance, College of Life Sciences, Tianjin Normal University, 393 West Binshui Road, Xiqing District, Tianjin, 300387, China

[b] Tianjin Fisheries Research Institute, 442 South Jiefang Road, Hexi District, Tianjin, 300221, China

ARTICLE INFO

Keywords:
P2Y receptor
Inflammation regulation
ERK1/2 signaling
Paralichthys olivaceus

ABSTRACT

Extracellular nucleotides and nucleotide sugars are important danger-associated signaling molecules that play critical roles in regulation of immune responses in mammals through activation of purinergic receptors located on the cell surface. However, the immunological role of extracellular UDP-glucose-activated $P2Y_{14}$ receptor ($P2Y_{14}R$) in fish still remains unknown. In this study, we identified and characterized a $P2Y_{14}R$ paralog in the Japanese flounder (*Paralichthys olivaceus*). The mRNA transcripts of *$P2Y_{14}R$* are detected in all examined Japanese flounder tissues. Compared with the UDP-activated *$P2Y_6$* receptor, however, *$P2Y_{14}R$* gene is highly expressed in Japanese flounder head kidney macrophages (HKMs). In addition, *$P2Y_{14}R$* is significantly upregulated following inflammatory stimulation with LPS and poly (I:C) in the HKMs, suggesting a role of $P2Y_{14}R$ in response to inflammation in fish. Furthermore, activation of $P2Y_{14}$ receptor with its potent and selective agonist MRS 2905 resulted in a decreased expression of LPS-induced pro-inflammatory cytokine *IL-1beta* gene in the HKMs. In contrast, inhibition of $P2Y_{14}$ receptor activity or down-regulation of the endogenous expression of $P2Y_{14}R$ by small interfering RNA significantly upregulates the LPS-induced pro-inflammatory cytokine *IL-1beta* gene expression in the HKMs, demonstrating that $P2Y_{14}R$ is involved in inflammation regulation in fish. Moreover, stimulation of the Japanese flounder HKMs with UDP-glucose evoked a rapid increase of extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in a dose- and time-dependent manner, indicating the involvement of $P2Y_{14}R$ in activation of ERK1/2 signaling in fish immune cells. Taken together, we demonstrated that the inducible $P2Y_{14}R$ plays an important role in regulation of fish innate immunity.

1. Introduction

Extracellular nucleotides such as ATP, ADP, UTP, and UDP and nucleotide sugars such as uridine 5′-diphosphoglucose (UDP-glucose) are important danger signaling molecules that play an important role in the regulation of immune responses through binding to and activating of different members of plasma membrane P2 receptors, which are widely expressed on immune cells [1–3]. P2Y receptors belonging to the G-protein-coupled receptors (GPCRs) superfamily, are a family of the metabotropic purinergic receptors for adenine and uridine nucleotides and nucleotide sugars. Eight P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) with differences in both pharmacology and downstream signaling pathways have been identified in mammals [4–6].

In particular, $P2Y_{14}R$ that is potently and selectively activated by UDP and UDP-sugars [7], is highly expressed in immune and inflammatory cells [7,8]. Activation of $P2Y_{14}R$ regulates a range of immune responses including maturation of dendritic cells [9], inhibition of T lymphocyte proliferation [10], chemotaxis of bone-marrow hematopoietic stem cells [11] and neutrophils [12], and secretion of the potent neutrophil chemoattractant CXCL8/IL-8 in airway epithelial cells [13]. In addition, it has been demonstrated that UDP-glucose and its receptor $P2Y_{14}R$ are key front line players able to trigger innate mucosal immune responses in mouse female reproductive tract [14].

We showed previously that extracellular UDP-activated $P2Y_6$ receptors functions importantly in the Japanese flounder *Paralichthys olivaceus* peripheral blood leukocytes [15]. Because extracellular UDP can also activate $P2Y_{14}$ receptors [8], we presumed the hypothesis that $P2Y_{14}$ receptors could play a role in Japanese flounder innate immunity. For this purpose, we cloned *$P2Y_{14}$* receptor gene and

* Corresponding author. College of Life Sciences, Tianjin Normal University, 393 West Binshui Road, Xiqing District, Tianjin, 300387, China.
E-mail addresses: skyls@tjnu.edu.cn (S. Li), skysjs@tjnu.edu.cn (J. Sun).
[1] S. Li is a senior author.

https://doi.org/10.1016/j.fsi.2019.07.053
Received 22 April 2019; Received in revised form 15 July 2019; Accepted 18 July 2019
Available online 18 July 2019

characterized its expression under normal and inflammatory conditions in Japanese flounder head kidney macrophages (HKMs). By pharmacological activation or inhibition of $P2Y_{14}R$ activities, and down-regulation of the $P2Y_{14}R$ endogenous expression in the HKMs, we explored the potential role of the $P2Y_{14}$ receptor in Japanese flounder innate immunity. In addition, we investigated the consequence of $P2Y_{14}R$ activation on ERK signaling in the HKMs. Taken our findings together, we for the first time demonstrated that the inducible $P2Y_{14}R$ plays an important role in regulation of fish innate immune response and in activation of ERK signaling pathway in fish immune cells.

2. Material and methods

2.1. Experimental animals

Japanese flounder *P. olivaceus* were obtained from a local fish farm in Tianjin, China. Fish were cultured in an aerated recirculating seawater system at 21 °C in the laboratory for two weeks before use in experiments. Fish were handled and clinically examined as described in our previous study [16]. Only animals with healthy appearance and normal activity were used in experiments.

2.2. Chemicals and antibodies

UDP-glucose, LPS and poly (I:C) were obtained from Sigma-Aldrich. The potent and selective $P2Y_{14}$ receptor agonist 2-Thiouridine-5′-O-(α, β-methylene)diphosphate trisodium salt (MRS 2905) and high affinity and selective $P2Y_{14}$ receptor antagonist 4-[4-(4-Piperidinyl)-phenyl]-7-[4-(trifluoromethyl)-phenyl]-2-naphthalenecarboxylic acid (PPTN) were purchased from Tocris Bioscience. Rabbit monoclonal antibodies against phospho-p44/p42 MAPK (p-Erk1/2) and against total p44/p42 MAPK (Erk1/2) were purchased from Cell Signaling Technology. Lipofectamine® RNAiMAX transfection reagent, HRP-labeled goat anti-mouse and goat anti-rabbit secondary antibodies and mouse monoclonal antibody against beta-actin were obtained from ThermoFisher Scientific.

2.3. Preparation and cell culture of Japanese flounder head kidney macrophages

To collect head kidney tissues, Japanese flounder were anesthetized using 0.25 g/L tricaine methanesulfonate (Sigma-Aldrich) and the head kidney was rapidly dissected under sterilized conditions. The dissected head kidney tissues were used to prepare head kidney primary cells with the procedure described previously [17]. The head kidney macrophages (HKMs) were further purified from the obtained primary head kidney cell suspensions by discontinuous Percoll (GE Biosciences) gradient centrifugation with a previously described protocol [18]. The viability of the HKMs was greater than 95% which was examined by trypan blue exclusion assay. The HKMs were grown in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin liquid (Invitrogen) at 21 °C.

2.4. Identification of $P2Y_{14}R$ cDNAs from Japanese flounder head kidney macrophages

To explore the immune functions of $P2Y_{14}R$ in Japanese flounder *P. olivaceus* head kidney macrophages, we initially cloned its cDNA sequences. Based on the available sequence data, a pair of primer (F/R, Table 1) was designed and the ORF region of *$P2Y_{14}R$* (XM_020107896.1) was amplified from the Japanese flounder HKMs by RT-PCR. The amplification products were separated by electrophoresis on a 1.2% agarose gel containing ethidium bromide. The PCR products with the expected sizes were purified with a DiaSpin DNA Gel Extraction Kit (Sangon Biotech Co., Ltd., China) and cloned into a pMD18-T vector (TaKaRa) for DNA sequencing.

Table 1
Sequence of primers used in this study.

Primers	Sequence (5′→3′)
F	ATGGATCCCTTCAACGTCAG
R	TCCTGTTGTCGGTTTTCTCA
q$P2Y_{14}$R-f	GGGTCGCTGTTC TGGGTGA
q$P2Y_{14}$R-r	ATCCAACCGCAGCATCTTC
q$P2Y_{6}$R-f	AGTGCGGAGATGCGGGAC
q$P2Y_{6}$R-r	GGTATCGTGGCTGTGTGAAGTA
q$P2Y_{2}$R-f	TTCCTCCCAGCGAAGACG
q$P2Y_{2}$R-r	CAGACGGACGTAGCAACCAG
q$P2Y_{12}$R-f	ACTGGCCTGCGTGTGTTTG
q$P2Y_{12}$R-r	AGATGGAGCCCGAGAGAAGC
qβ-actin-f	AGGTTCCGTTGTCCCG
qβ-actin-r	TGGTTCCTCCAGATAGCAC
qIL-1β-f	CCTGTCGTTCTGGGCATCAA
qIL-1β-r	CACCCCGCTGTCCTGCTT

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

2.5. Expression analysis of $P2Y_{14}R$ mRNA transcripts in Japanese flounder tissues and head kidney macrophages

qRT-PCR analysis (see section *2.8*) was performed to determine the relative basal gene expression level of *$P2Y_{14}R$* in various Japanese flounder tissues including blood, gill, head kidney, trunk kidney, heart, liver, skin, muscle, intestine and spleen with a protocol described in the previous study [21]. In addition, qRT-PCR analysis was also applied to compare the relative gene expression level of *$P2Y_{2}$*, *$P2Y_{6}$*, *$P2Y_{12}$* and *$P2Y_{14}$* receptors in the Japanese flounder HKMs. To this aim, total RNA from the HKM cells and tissues was purified with a PureLink® RNA Mini Kit and TRIzol reagent (Invitrogen), respectively, according to the manufacturer's recommendations. RNA was quantified by a NanoDrop spectrophotometer and treated with RNase Free DNase I (Invitrogen) to digest possible contaminating genomic DNA following the manufacturer's protocol. The treated RNA was then transcribed into cDNAs using a SuperScript III reverse transcriptase kit (Invitrogen) according to the manufacturer's directions. The relative gene expression levels for the individual *P2Y* receptor genes were determined by qRT-PCR (see section 2.8) with *beta-actin* serving as an internal reference gene.

2.6. In vitro immune challenge experiments in the Japanese flounder HKMs

To study the involvement of $P2Y_{14}R$ in Japanese innate immune responses, Japanese flounder HKMs were challenged with pathogen-associated molecular patterns (PAMPs) LPS and poly (I:C) as described in a previous study [22]. Briefly, overnight-cultured Japanese flounder HKMs (5×10^6 cells/well) were stimulated with 20 μg/ml LPS or poly (I:C) (final concentration, dissolved in cell culture medium) for 4, 8, 12, 24, 36 or 48 h. Cells without PAMP treatment served as controls. After treatment, total RNA was purified and transcribed into cDNAs, and the LPS- and poly (I:C)-induced *$P2Y_{14}R$* gene expression changes were measured by qRT-PCR.

2.7. Pharmacological treatments and knockdown of $P2Y_{14}R$ endogenous expression in the Japanese flounder HKMs

To evaluate the potential role of $P2Y_{14}R$ in inflammatory stimulation-induced innate immune response, $P2Y_{14}R$ activity in the Japanese flounder HKMs were pharmacologically activated or inhibited by pre-incubation with the potent and selective $P2Y_{14}$ receptor agonist MRS 2905 at a final concentration of 20 μM or antagonist PPTN at a final concentration of 10 μM for 30 min, prior to treatment with 20 μg/mL LPS for 2 h in the presence or absence of MRS 2905 or PPTN. The PAMP-induced *IL-1beta* gene expression changes following pharmacological treatment was assessed by qRT-PCR (see below).

In addition, the endogenous expression of *$P2Y_{14}R$* in the Japanese

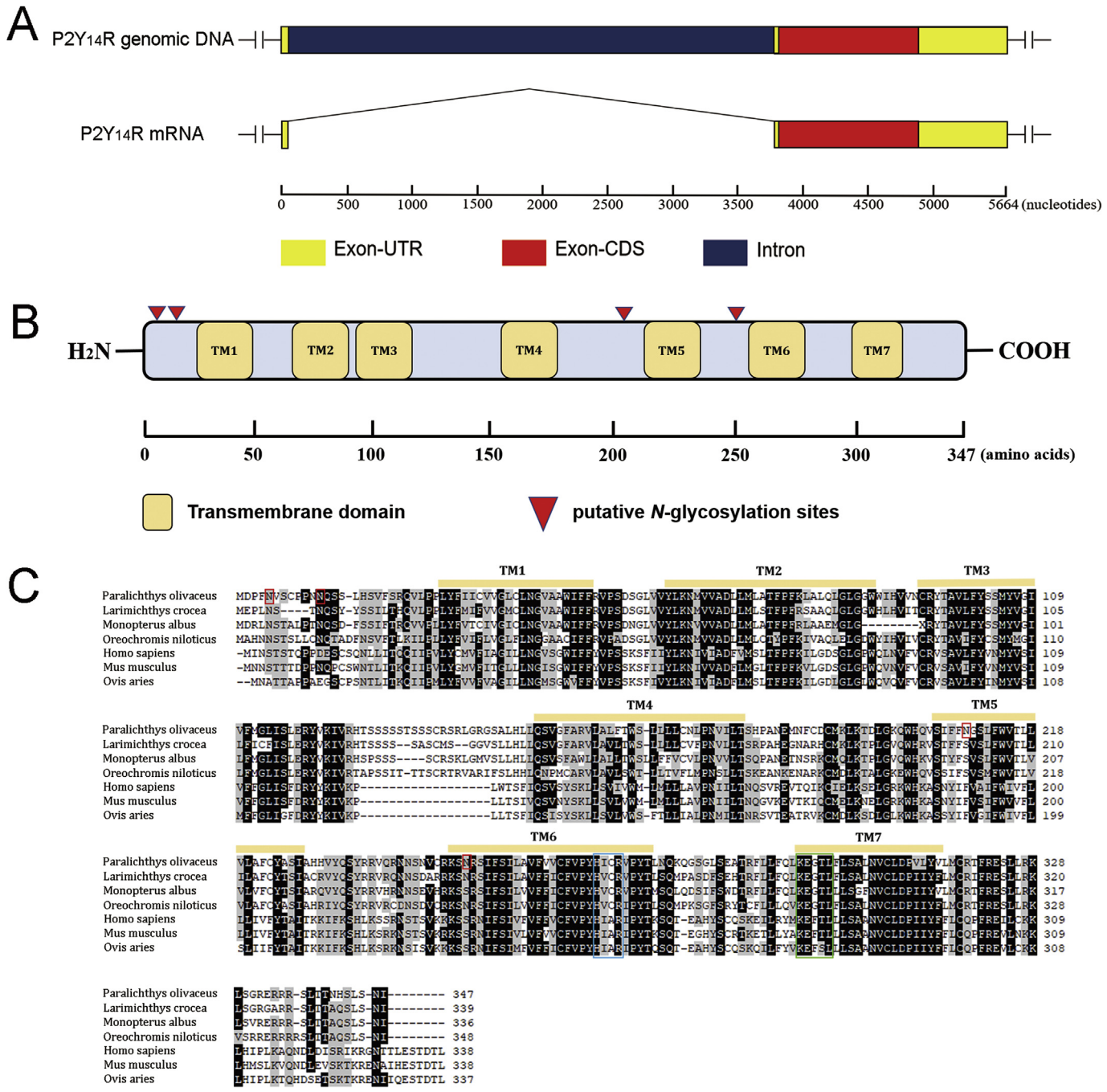

Fig. 1. Scheme showing the genomic and domain structures of Japanese flounder P2Y$_{14}$R and sequence alignment of selected mammalian and teleost P2Y$_{14}$R paralogs. (A) Genomic DNA structure of Japanese flounder *P2Y$_{14}$R* gene. Top panel shows the extron and intron structure of Japanese flounder *P2Y$_{14}$R* gene. Bottom panel shows the *P2Y$_{14}$R* mRNA structure derived from the genomic DNA sequence of Japanese flounder *P2Y$_{14}$R*. (B) Schematic domain structures of Japanese flounder P2Y$_{14}$R protein. The predicted *N*-glycosylation sites are indicated by red invert arrows on top of the rectangle. The transmembrane domains are shown as yellow boxes. (C) Multiple alignment of the amino acid sequences of Japanese flounder P2Y$_{14}$R and its counterpart proteins from representative vertebrate species using the ClustalW program. The GenBank accession numbers of P2Y$_{14}$ receptor proteins from selected vertebrate species are Japanese flounder *Paralichthys olivaceus* P2Y$_{14}$R (XP_019963455.1), yellow croaker *Larimichthys crocea* P2Y$_{14}$R (XP_019133030.1), rice swampeel *Monopterus albus* P2Y$_{14}$R (XP_020478366.1), tilapia *Oreochromis niloticus* P2Y$_{14}$R (XP_005474964.1), human *Homo sapiens* P2Y$_{14}$R (NP_001074924.1), mouse *Mus musculus* P2Y$_{14}$R (AAH58558.1) and sheep *Ovis aries* P2Y$_{14}$R (XP_011956798.1). Identity is denoted by shaded white letters and similarity is shown by shaded black letters. The seven transmembrane domains are indicated on the top of the sequences. The predicted *N*-glycosylation sites in Japanese flounder P2Y$_{14}$R protein were boxed in red. The H-X-X-R/K motif that is crucial for agonist activity in the TM6 and the featured K-E-X-X-L motif for extracellular nucleotides binding in TM7 of all the P2Y$_{14}$ receptors are boxed in blue 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.)

flounder HKMs was down-regulated by small interfering RNA. For this purpose, negative control siRNA (sequence was not disclosed by the provider) and a set of synthetic small interfering RNAs (siRNAs) for *P2Y$_{14}$R* were ordered from RiboBio Inc. (Guangzhou, China). Because of the lack of reliable *anti*-P2Y$_{14}$ Abs, knockdown efficiency of the three *P2Y$_{14}$R* siRNA candidates was evaluated 48 h post-transfection by qRT-PCR as described in section *2.8*. The knockdown efficiency of the optimized siRNA with the target sequence of 5′-GGTGGATCCATGTGGT

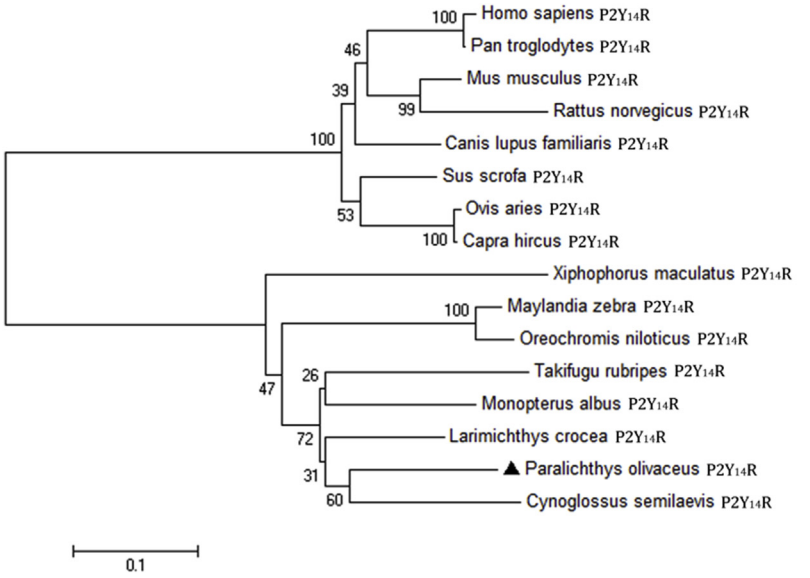

Fig. 2. Phylogenetic relationship of $P2Y_{14}$ receptor paralogs from Japanese flounder and other vertebrate species. The maximum-likelihood phylogenetic tree was constructed using MEGA 5.0 program. The GenBank accession numbers for the P2Y receptor paralogs from different species are *Cynoglossus semilaevis* $P2Y_{14}R$ (XP_016887698.1), *Xiphophorus maculatus* $P2Y_{14}R$ (XP_005808340.1), *Maylandia zebra* $P2Y_{14}R$ (XP_004565057.2), *Takifugu rubripes* $P2Y_{14}R$ (XP_003979142.1), *Capra hircus* $P2Y_{14}R$ (XP_005675495.2), *Rattus norvegicus* $P2Y_{14}R$ (NP_598261.1), *Canis lupus familiaris* $P2Y_{14}R$ (XP_005634627.1), *Pan troglodytes* $P2Y_{14}R$ (XP_001145005.1), *Sus scrofa* $P2Y_{14}R$ (XP_020925281.1) and the list in the legend of Fig. 1.

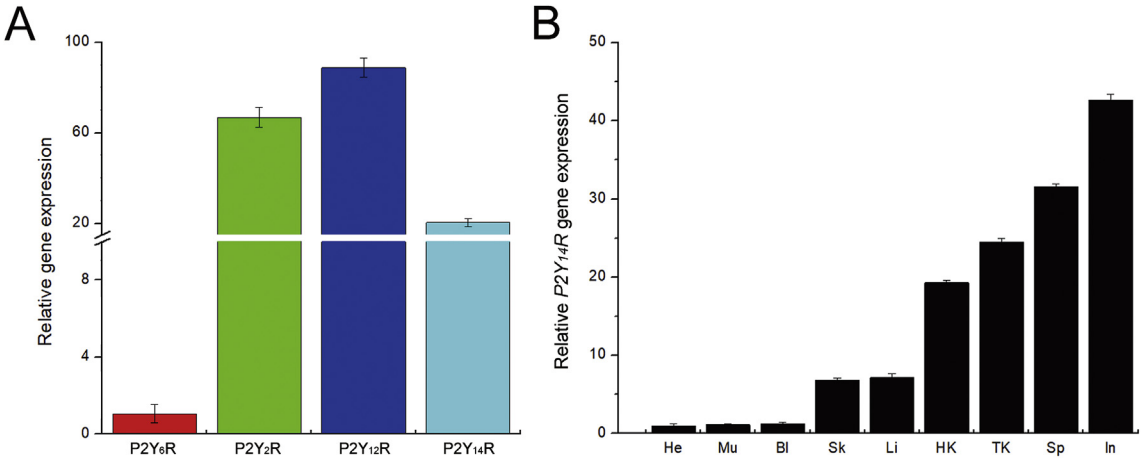

Fig. 3. qRT-PCR analysis of the relative gene expression of *P2YR* paralogs in the Japanese flounder head kidney macrophages and tissues. (A) Comparative analysis of the relative gene expression levels of $P2Y_2$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{14}$ receptors in Japanese flounder head kidney macrophages. (B) qRT-PCR analysis of the gene expression pattern of $P2Y_{14}R$ in healthy *P. olivaceus* tissues. Bl: blood; Mu: muscle; HK: head kidney; TK: trunk kidney; He: heart; Li: liver; Sk: skin; Sp: spleen; In: intestine. Error bars indicate standard deviation (n = 3).

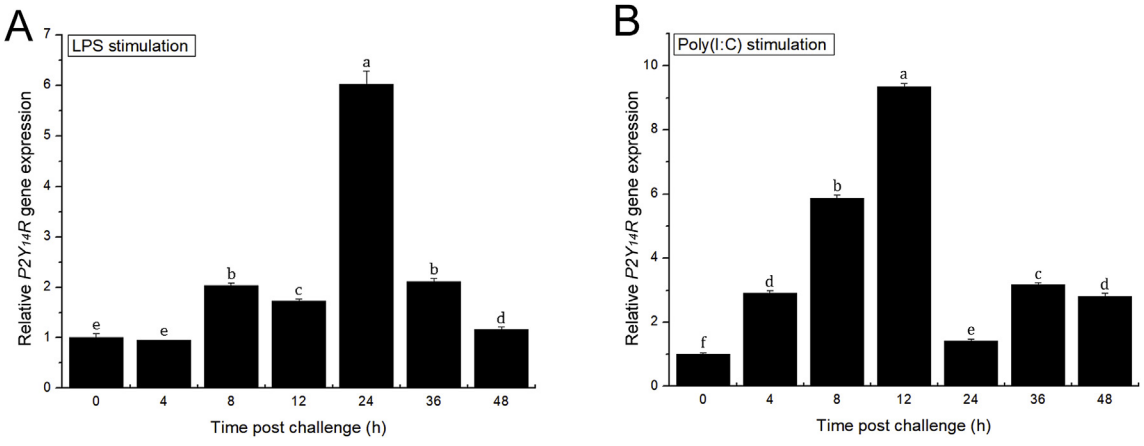

Fig. 4. Inflammatory stimulation-induced $P2Y_{14}R$ gene expression in *P. olivaceus* head kidney macrophages. Japanese flounder HKMs were stimulated with 20 μg/ml (final concentration) LPS (A) or poly (I:C) (B) for the indicated time points. After 0, 4, 8, 12, 24, 36 and 48 h post stimulation, total RNA was extracted and the $P2Y_{14}R$ gene expression changes relative to the untreated controls were determined by qRT-PCR. *Beta-actin* was served as an internal reference gene. Values labeled with different *lowercase* letters indicated significant difference at $p < 0.05$ among treatments. Data are the mean ± standard deviation of triplicate treatments from one representative experiment; similar results were obtained on two other separate occasions.

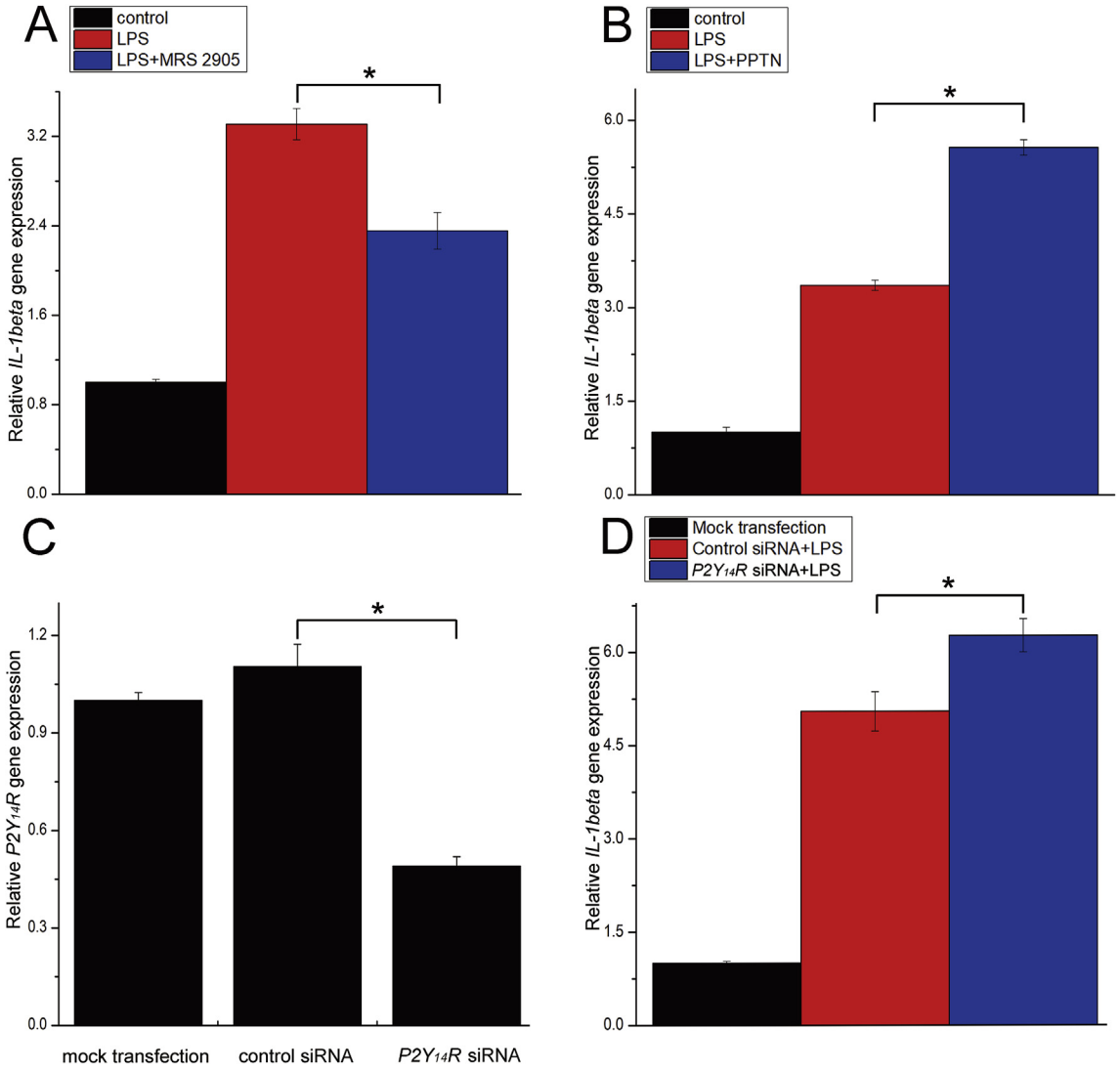

Fig. 5. The role of $P2Y_{14}R$ in inflammatory stimulation-induced pro-inflammatory cytokine *IL-1beta* gene expression in the Japanese flounder HKMs. (A and B) Pharmacological activation or inhibition of $P2Y_{14}$ receptors activity affects LPS-induced *IL-1beta* gene expression in the Japanese flounder HKMs. Japanese flounder HKMs were pre-incubated with or without 20 μM potent and selective $P2Y_{14}R$ agonist MRS 2905 (A) or 10 μM antagonist PPTN (B) for 30 min followed by stimulation with 20 μg/mL LPS for 2 h in the presence or absence of MRS 2905 or PPTN. The relative pro-inflammatory cytokine *IL-1beta* gene expression levels were examined by qRT-PCR and were normalized to untreated control cells (set to 1). Significant difference between PAMP treated groups and PAMP plus MRS2905 or between PAMP treated groups and PAMP plus PPTN treated groups was determined by the Student's t-test and is indicated by brackets and asterisks at $p < 0.05$. Data are presented as means ± standard deviations of triplicate determinants from one representative experiment. Similar results were obtained in other two separate experiments. (C) Evaluation of the silencing efficiency of optimized $P2Y_{14}R$ siRNA. Japanese flounder HKMs were mock transfected or transfected with negative control siRNA or $P2Y_{14}R$ siRNA for 48 h at a final concentration of 150 nM. The relative $P2Y_{14}R$ gene expression levels were determined by qRT-PCR and normalized to the expression level in mock transfected cells (set to 1). Asterisks (*) mark the significant decrease of $P2Y_{14}R$ gene expression in $P2Y_{14}R$ siRNA transfected cells compared with the negative control siRNA transfected control cells ($p < 0.05$). (D) Down-regulation of $P2Y_{14}R$ expression upregulates the LPS-induced pro-inflammatory cytokine *IL-1beta* gene expression in the Japanese flounder HKMs. Japanese flounder HKMs were mock transfected or transfected with negative control siRNA or optimized $P2Y_{14}R$ siRNA for 48 h at a final concentration of 150 nM. After transfection, the HKMs were stimulated with 20 μg/mL LPS for 2 h. The LPS-induced gene expression of *IL-1beta* in the $P2Y_{14}R$ down-regulated HKMs were evaluated by qRT-PCR with *beta-actin* as an internal reference gene. Asterisks (*) indicate the significant increase of *IL-1beta* gene expression in $P2Y_{14}R$ siRNA transfected cells compared with the negative control siRNA transfected control cells ($p < 0.05$). Values are presented as means ± standard deviation (n = 3).

CAA-3′on the Japanese flounder $P2Y_{14}R$ was about 50% as determined by qRT-PCR (Fig. 5C). After evaluation, overnight-cultured Japanese flounder HKM cells at a cell density of 5×10^6 cells/well in a 24-well plate were transfected with 150 nM optimized $P2Y_{14}R$ siRNA or equal amount of negative control siRNA using Lipofectamine® RNAiMAX reagent (Invitrogen) followed by the manufacturer's protocol. After 48 h of silencing, the cells were stimulated with 20 μg/ml LPS for 2 h and the gene expression changes of *IL-1beta* were determined by quantitative real-time PCR (qRT-PCR) (see below).

2.8. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed on an Applied Biosystems® 7500 Fast Real-Time PCR System (ThermoFisher Scientific) using a FastStart Universal SYBR Green Master (Rox) kit (Roche) according to the manufacturer's instructions. The primers for the detection of Japanese flounder $P2Y_2$, $P2Y_6$, $P2Y_{12}$, $P2Y_{14}$, *IL-1beta* and *beta-actin* genes are listed in Table 1. Gene expression levels were evaluated using the comparative $2^{-\triangle\triangle Ct}$ quantification method with *beta-actin* as an internal reference gene. Melting curve analysis and gel

electrophoresis were performed at the end of amplification. The PCR products were further sequenced to ensure the specificity of amplification.

2.9. Western blots analysis of UDP-glucose-induced ERK1/2 phosphorylation in the HKMs

Overnight-cultured Japanese flounder HKMs were incubated with UDP-glucose at different concentrations for the indicated time points. The effect of UDP-glucose treatment on ERK signaling in the HKMs was evaluated by Western blot analysis. The procedure for Western blot analysis was described previously [16]. Briefly, after treatment, cells were washed twice with cold TBS and lysed at 4 ˚C in a lysis buffer containing 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). The cell lysates were then centrifuged at 13000 rpm for 15 min. The extracted supernatants were separated by electrophoresis through an SDS-polyacrylamide gel and subsequently transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were incubated with rabbit monoclonal antibodies specific for phospho-p44/p42 MAPK (1:600) and total p44/p42 MAPK (1:1000) followed by incubation with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:3000, Pierce). Beta-actin (1:5000) was served as a loading control to show that equal amount of protein was applied. Target proteins were visualized using a SuperSignal West Pico detection kit (Pierce) on a Molecular Imager ChemiDoc™ XRS + Imaging System (Bio-Rad) according to the manufacturer's directions.

2.10. Statistical analysis

Data are presented as the mean ± standard deviation from triplicate experiments. Statistical analysis was performed with Student's t-test for the comparison between two groups. Multiple group comparison was conducted by one-way ANOVA followed by Duncan's analysis using SPSS software version 11.0. Statically differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Sequence analysis of Japanese flounder $P2Y_{14}R$ proteins

The GenBank database was researched and two putative Japanese flounder *$P2Y_{14}R$* cDNA sequences with accession numbers XM_020107896.1 and XM_020107895.1, respectively, were retrieved. Blast against the Japanese flounder genomic DNA sequence available in the NCBI database revealed that these two genes are located in different regions of the assembled Japanese flounder genomic scaffold, indicating that they are duplicated genes in *P. olivaceus* [19,20]. However, no significant sequence similarity was found between the two putative Japanese flounder *$P2Y_{14}R$* genes, and the latter expresses much lower than the former in the Japanese flounder HKMs (data not shown). Further sequence analysis revealed that the putative Japanese flounder $P2Y_{14}R$ with accession number XM_020107895.1 does not harbor the conserved signature motif K-E-X-X-L for extracellular nucleotides binding in TM7 of all identified $P2Y_{14}$ receptors. In addition, phylogenetic analysis revealed that it can not be clustered into either the clade of mammalian $P2Y_{14}$Rs or the clade of fish $P2Y_{14}$Rs. These data indicate that XM_020107895.1 is a non-functional receptor. Thus, XM_020107896.1 was served as a target in the current study and was amplified from the Japanese flounder HKMs.

Compared with the *$P2Y_{14}R$* reference sequence (accession No. XM_020107896.1) retrieved from the GenBank database, the cloned Japanese flounder *$P2Y_{14}R$* cDNA has 4 nucleotide changes. These differences resulted in 4 amino acid substitutions. Genomic structure analysis showed that the Japanese flounder *$P2Y_{14}R$* gene has two extrons and one intron, and the 5′-UTR is comprised of the entire first extron and the partial second extron (Fig. 1A). Sequence analysis of the Japanese flounder *$P2Y_{14}R$* revealed that it encoded a full-length open reading frame of 347 amino acids with a predicted molecular mass of 39 kDa and an isoelectric point of 9.71. Domain analysis revealed that the Japanese flounder $P2Y_{14}R$ protein processes seven transmembrane domains (TMs) and an extracellular amino-termini and a cytoplasmic carboxyl-termini. In addition, the Japanese flounder $P2Y_{14}R$ possess four potential *N*-linked glycosylation sites including ^{5}Asn, ^{12}Asn, ^{209}Asn and ^{250}Asn, respectively (Fig. 1B). The Japanese flounder $P2Y_{14}R$ shares about 42% sequence identity and 65% sequence similarity with human being $P2Y_{14}$ receptor. In addition, Japanese flounder $P2Y_{14}R$ exhibits 24%, 22%, and 38% sequence identity with the known Japanese flounder $P2Y_2$, $P2Y_6$ and $P2Y_{12}$ receptors [15,23], respectively, indicating their different structures for ligand binding. Multiple sequence alignment of Japanese flounder $P2Y_{14}R$ with its counterparts from the selected vertebrate species revealed that the TM6 and TM7 are highly conserved among the $P2Y_{14}R$ members from different species (Fig. 1C). Specially, the H-X-X-R/K motif that is crucial for agonist activity in the TM6 shared by all cloned P2Y receptors, and the featured K-E-X-X-L motif for extracellular nucleotides binding in TM7 of mammalian $P2Y_{14}$ receptors [24] are found in the fish $P2Y_{14}$ receptors, suggesting that a conserved mode of agonist binding remains in $P2Y_{14}$ receptors during evolution. Phylogenetic analysis further showed that the $P2Y_{14}$ receptors are well separated into the mammalian and fish clusters and the Japanese flounder $P2Y_{14}R$ shows a closer relationship with *Cynoglossus semilaevis* $P2Y_{14}R$ (Fig. 2).

3.2. Differential expression of P2Y receptors in the Japanese flounder HKMs

In human beings, the prominent expression of $P2Y_{14}R$ was found in immune cells including neutrophils, lymphocytes, and megakaryocytic cells [25]. Using $P2Y_{14}R$ knockout mice, it was demonstrated that $P2Y_{14}R$ plays a key role in recruitment of macrophages [26]. GPCRs are usually co-expressed in mammalian macrophages [27]. Our previous studies showed that multiple P2Y receptor subtypes are expressed in the Japanese flounder HKMs [15,23]. We therefor compared the gene expression level of *$P2Y_{14}R$* with three known P2Y receptor subtypes including *$P2Y_2$*, *$P2Y_6$* and *$P2Y_{12}$* in the Japanese flounder HKMs. As shown in Fig. 3A, *$P2Y_{14}$* together with *$P2Y_2$*, *$P2Y_6$* and *$P2Y_{12}$* receptors are co-expressed in the HKMs. Considering that several P2X receptors also express in the Japanese flounder HKMs [28], the presence of multiple P2 receptors indicates that diverse and complicated purinergic immune signaling pathways may exist in the Japanese flounder HKMs. However, the expression level of ATP/UTP-activated *$P2Y_2R$* and ADP-activated *$P2Y_{12}R$* is much higher than that of other P2Y receptors in the HKMs. Interestingly, compared with *$P2Y_{14}Rs$*, the UDP-activated *$P2Y_6R$* expresses much lower in the HKMs. As $P2Y_{14}R$ also could be activated by UDP, the relative high expression level of *$P2Y_{14}R$* suggests that $P2Y_{14}R$ may play a primary role in UDP-activated immune signaling in the Japanese flounder HKMs.

3.3. Constitutive expression of $P2Y_{14}R$ in Japanese flounder tissues

The relative gene expression level of *$P2Y_{14}R$* in Japanese flounder tissues was examined by qRT-PCR. As shown in Fig. 3B, *$P2Y_{14}R$* is present in all examined Japanese flounder tissues including blood, heart, head kidney, intestine, liver, muscle, spleen, skin and trunk kidney. This is in general agreement with the constitutive tissue expression properties of other P2 receptors in the Japanese flounder [15,23,28–30]. Similarly, *$P2Y_{14}R$* is also broadly expressed in several human tissues, including the placenta, adipose tissue, stomach, intestine, spleen, lung and brain [5,31], and mouse tissues such as brain, heart, kidney, spleen and thymus [32]. Compared with the UDP-activated Japanese flounder $P2Y_6$ receptor that is dominantly expressed in liver [15], however, the most abundant expression of *$P2Y_{14}R$* was

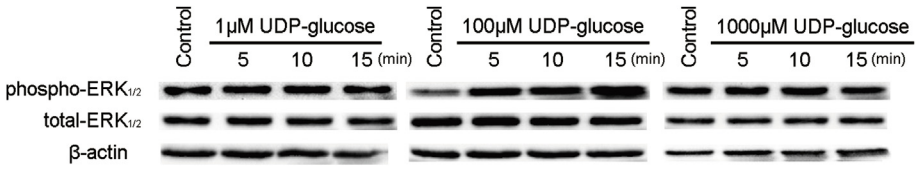

Fig. 6. UDP-glucose promotes ERK1/2 activation in the Japanese flounder HKMs. The Japanese flounder HKMs were incubated with 1, 100 or 1000 μM UDP-glucose for the indicated durations. The untreated cells served as controls. Western blot analysis using *anti*-phospho-ERK1/2 revealed that ERK1/2 protein phosphorylation increased only after 100 μM UDP-glucose treatment while total ERK1/2 phosphorylation did not change. Beta-actin served as a loading control to show equal amount of proteins was loaded. Results shown are representative of three individual experiments.

observed in Japanese flounder intestine. In addition, the lowest expression of Japanese flounder *P2Y$_6$R* is in spleen while the least expression of *P2Y$_{14}$R* is in Japanese flounder heart. These observations revealed that the Japanese flounder P2Y$_6$ and P2Y$_{14}$ receptors have different tissue distribution preference even though both of them can be activated by UDP. The preferred tissue expression of P2Y$_6$ and P2Y$_{14}$ receptors also indicate that they may contribute differently in the UDP-mediated purinergic signaling in a given Japanese flounder tissue.

3.4. Inflammatory stimulation-regulated *P2Y$_{14}$R* expression in the Japanese flounder HKMs

To examine the response of *P2Y$_{14}$R* to inflammatory stimulation, the Japanese flounder HKMs were stimulated with canonic inflammatory stimuli LPS and poly (I:C) to mimic different pathogen infections. As shown in Fig. 4, *P2Y$_{14}$R* expression was significantly induced (greater than 6-fold than untreated controls) upon both LPS and poly (I:C) challenges in the HKMs. The highly inducible expression of *P2Y$_{14}$R* in the HKMs suggests that P2Y$_{14}$R may play an important role in responses to inflammatory challenges in the Japanese flounder. Similar to our observation, P2Y$_{14}$R was also markedly upregulated after immunological challenge with LPS in rat [25]. In addition, it has been demonstrated that P2Y$_{14}$R plays an important role in protecting mice from vesicular stomatitis virus infection [1]. Notably, the Japanese flounder *P2Y$_{14}$R* was upregulated as early as 4 h after poly (I:C) stimulation and was maximally induced (approximate 10-fold than control) 12 h after treatment, indicating that P2Y$_{14}$R may perform an essential role in response to viral infection in fish.

3.5. *P2Y$_{14}$R* modulates PAMP-induced inflammatory response in the HKMs

It has been demonstrated that P2Y$_{14}$R is a key cell surface signaling protein in mammalian innate immune responses [33]. To explore the potential role of P2Y$_{14}$R in Japanese flounder innate immunity, we treated the HKMs with canonical inflammatory molecule LPS to induce inflammatory responses and examined the consequence of inflammatory stimulation-induced multifunctional pro-inflammatory cytokine *IL-1beta* gene expression when P2Y$_{14}$ receptor activity was activated by the potent and selective agonist MRS 2905 [34] or inhibited by its selective and potent antagonist PPTN [33]. As expected, LPS stimulation significantly promotes *IL-1beta* expression in the HKMs. However, the LPS-induced *IL-1beta* expression was attenuated when P2Y$_{14}$ receptor was activated by its potent agonist MRS 2905 (Fig. 5 A). In contrast, the LPS-induced *IL-1beta* expression was significantly augmented when P2Y$_{14}$R was inhibited with the high affinity and selective P2Y$_{14}$ receptor antagonist PPTN (Fig. 5 B). These observations suggest that P2Y$_{14}$R may negatively regulate the PAMP-induced immune responses in fish.

To support this hypothesis, we down-regulated the endogenous expression of P2Y$_{14}$R by small interfere RNA in the HKMs. For this purpose, a set of siRNA that targets on the different regions of *P2Y$_{14}$R* was designed and one optimized siRNA that could significantly down-regulate the endogenous expression level of *P2Y$_{14}$R* in the HKMs was obtained (Fig. 5C). In consistent with the results obtained by pharmacologic experiments, the enhanced gene expression of inflammatory stimuli-induced *IL-1beta* was also observed in the *P2Y$_{14}$R* down-regulated HKMs that were silenced by the optimized *P2Y$_{14}$R* siRNA (Fig. 5D). The above complementary experiments demonstrated that in addition to the UDP-activated P2Y$_6$ receptor, the UDP/UDP-glucose-activated P2Y$_{14}$ receptor could represent an additional molecule for "fine-tuning" PAMP-induced inflammatory responses in fish.

3.6. Activation of ERK signaling by UDP-glucose in the Japanese flounder HKMs

Mitogen-activated protein (MAP) kinase signaling pathways are often stimulated by activation of Gi-coupled receptors [7]. P2Y$_{14}$R is known to couple to heterotrimeric G proteins of the G$_{\alpha i/o}$ family [7]. Although P2Y$_{14}$R could be activated by UDP, it is the only subtype known to be uniquely and potently activated by UDP-glucose [35], an endogenous damage-associated molecular pattern molecule (DAMP) released by injured cells [36,37]. We therefore examined the phosphorylation status of ERK1/2 protein following P2Y$_{14}$R activation in the HKMs. As shown in Fig. 6, ERK1/2 protein was quickly phosphorylated as early as 5 min following 100 μM UDP-glucose treatment in the HKMs; associated with the extended stimulation process, ERK1/2 protein phosphorylation was increased. In addition, the UDP-glucose-activated ERK1/2 phosphorylation shows a concentration-dependent manner as ERK1/2 phosphorylation did not changed at lower (1 μM) or higher (1 mM) concentration of UDP-glucose stimulation. These findings indicate that the Japanese flounder P2Y$_{14}$R is functional and that activation of this receptor by UDP-glucose results in the activation of ERK signaling in the fish macrophages.

In conclusion, we characterized an inducible UDP-glucose–activated P2Y$_{14}$R that is involved in regulation of fish innate immunity.

Acknowledgements

This research was funded by the Natural Science Foundation of Tianjin (NO. 18JCZDJC33600), the National Key R&D Program of China (No. 2018YFD0901301), the University Yong Talent Cultivation Program provided by Tianjin Municipal Education Commission, the Program for Innovative Research Team in Universities of Tianjin (TD13-5076), the Tianjin Development Program for Innovation and Entrepreneurship, and the Innovation Team of Tianjin Fisheries Research System (NO. ITTFRS2017007).

References

[1] R. Li, B. Tan, Y. Yan, X. Ma, N. Zhang, Z. Zhang, M. Liu, M. Qian, B. Du, Extracellular UDP and P2Y$_6$ function as a danger signal to protect mice from vesicular stomatitis virus infection through an increase in IFN-β production, J. Immunol. 193 (9) (2014) 4515–4526.
[2] E.R. Lazarowski, T.K. Harden, UDP-sugars as extracellular signaling molecules: cellular and physiologic consequences of P2Y$_{14}$ receptor activation, Mol. Pharmacol. 88 (1) (2015) 151–160.
[3] L. Vitiello, S. Gorini, G. Rosano, A. la Sala, Immunoregulation through extracellular nucleotides, Blood 120 (3) (2012) 511–518.
[4] M.P. Abbracchio, G. Burnstock, J.-M. Boeynaems, E.A. Barnard, J.L. Boyer, C. Kennedy, G.E. Knight, M. Fumagalli, C. Gachet, K.A. Jacobson, G.A. Weisman, International union of pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy, Pharmacol. Rev. 58 (3) (2006) 281–341.
[5] I. von Kügelgen, Pharmacological profiles of cloned mammalian P2Y-receptor subtypes, Pharmacol. Ther. 110 (3) (2006) 415–432.
[6] S. Li, I. Bjelobaba, S.S. Stojilkovic, Interactions of Pannexin1 channels with

purinergic and NMDA receptor channels, Biochim. Biophys. Acta 1860 (1) (2018) 166–173.
[7] T. Harden, J. Sesma, I. Fricks, E. Lazarowski, Signalling and pharmacological properties of the P2Y receptor, Acta Physiol. 199 (2) (2010) 149–160.
[8] K. Jacobson, S. Paoletta, V. Katritch, B. Wu, Z. Gao, Q. Zhao, R. Stevens, E. Kiselev, Nucleotides acting at P2Y receptors: connecting structure and function, Mol. Pharmacol. 88 (2) (2015) 220–230.
[9] L. Skelton, M. Cooper, M. Murphy, A. Platt, Human immature monocyte-derived dendritic cells express the G protein-coupled receptor GPR105 (KIAA0001, P2Y14) and increase intracellular calcium in response to its agonist, uridine diphosphoglucose, J. Immunol. 171 (4) (2003) 1941–1949.
[10] M. Scrivens, J.M. Dickenson, Functional expression of the P2Y14 receptor in murine T-lymphocytes, Br. J. Pharmacol. 146 (3) (2005) 435–444.
[11] B.C. Lee, T. Cheng, G.B. Adams, E.C. Attar, N. Miura, S.B. Lee, Y. Saito, I. Olszak, D. Dombkowski, D.P. Olson, J. Hancock, P.S. Choi, D.A. Haber, A.D. Luster, D.T. Scadden, P2Y-like receptor, GPR105 (P2Y14), identifies and mediates chemotaxis of bone-marrow hematopoietic stem cells, Genes Dev. 17 (13) (2003) 1592–1604.
[12] J.I. Sesma, S.M. Kreda, N. Steinckwich-Besancon, H. Dang, R. García-Mata, T.K. Harden, E.R. Lazarowski, The UDP-sugar-sensing P2Y(14) receptor promotes Rho-mediated signaling and chemotaxis in human neutrophils, Am. J. Physiol. Cell Physiol. 303 (5) (2012) C490–C498.
[13] T. Müller, H. Bayer, D. Myrtek, D. Ferrari, S. Sorichter, M.W. Ziegenhagen, G. Zissel, J.J. Christian Virchow, W. Luttmann, J. Norgauer, F.D. Virgilio, M. Idzko, The P2Y14 receptor of airway epithelial cells, Am. J. Respir. Cell Mol. Biol. 33 (6) (2005) 601–609.
[14] T. Arase, H. Uchida, T. Kajitani, M. Ono, K. Tamaki, H. Oda, S. Nishikawa, M. Kagami, T. Nagashima, H. Masuda, H. Asada, Y. Yoshimura, T. Maruyama, The UDP-glucose receptor P2RY14 triggers innate mucosal immunity in the female reproductive tract by inducing IL-8, J. Immunol. 182 (11) (2009) 7074–7084.
[15] S. Li, J. Li, N. Wang, G. Hao, J. Sun, Characterization of UDP-activated purinergic receptor P2Y6 involved in Japanese flounder Paralichthys olivaceus innate immunity, Int. J. Mol. Sci. 19 (7) (2018) 2095.
[16] S. Li, X. Li, X. Chen, X. Geng, J. Sun, ATP release channel Pannexin1 is a novel immune response gene in Japanese flounder Paralichthys olivaceus, Fish Shellfish Immunol. 40 (1) (2014) 164–173.
[17] S. Li, X. Li, X. Gen, Y. Chen, J. Wei, J. Sun, Identification and characterization of lipopolysaccharide-induced TNF-alpha factor gene from Japanese flounder Paralichthys olivaceus, Vet. Immunol. Immunopathol. 157 (3–4) (2014) 182–189.
[18] S. Li, X. Chen, N. Wang, J. Li, Y. Feng, J. Sun, Identification and characterization of ecto-nucleoside triphosphate diphosphohydrolase 1 (CD39) involved in regulating extracellular ATP-mediated innate immune responses in Japanese flounder (Paralichthys olivaceus), Mol. Immunol. 112 (2019) 10–21.
[19] W. Wang, J. Wang, F. You, L. Ma, X. Yang, J. Gao, Y. He, J. Qi, H. Yu, Z. Wang, X. Wang, Z. Wu, Q. Zhang, Detection of alternative splice and gene duplication by RNA sequencing in Japanese flounder, Paralichthys olivaceus, G3: Genes|Genomes|Genetics 4 (12) (2014) 2419.
[20] J. Zahradník, L. Kolářová, H. Pařízková, P. Kolenko, B. Schneider, Interferons type II and their receptors R1 and R2 in fish species: evolution, structure, and function, Fish Shellfish Immunol. 79 (2018) 140–152.
[21] S. Li, J. Li, W. Peng, G. Hao, J. Sun, 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 (1) (2019) 20.
[22] S. Li, X. Chen, X. Geng, W. Zhan, J. Sun, 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 (2) (2015) 261–267.
[23] S. Li, G. Hao, Y. Xu, N. Wang, J. Li, X. Geng, J. Sun, Functional characterization of purinergic P2Y2 and P2Y12 receptors involved in Japanese flounder (Paralichthys olivaceus) innate immune responses, Fish Shellfish Immunol. 75 (2018) 208–215.
[24] M.P. Abbracchio, J.-M. Boeynaems, E.A. Barnard, J.L. Boyer, C. Kennedy, M.T. Miras-Portugal, B.F. King, C. Gachet, K.A. Jacobson, G.A. Weisman, G. Burnstock, Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family, Trends Pharmacol. Sci. 24 (2) (2003) 52–55.
[25] D.J. Moore, P.R. Murdock, J.M. Watson, R.L.M. Faull, H.J. Waldvogel, P.G. Szekeres, S. Wilson, K.B. Freeman, P.C. Emson, GPR105, a novel Gi/o-coupled UDP-glucose receptor expressed on brain glia and peripheral immune cells, is regulated by immunologic challenge: possible role in neuroimmune function, Mol. Brain Res. 118 (1) (2003) 10–23.
[26] J. Xu, H. Morinaga, D. Oh, P. Li, A. Chen, S. Talukdar, Y. Mamane, J.A. Mancini, A.R. Nawrocki, E. Lazarowski, J.M. Olefsky, J.J. Kim, GPR105 ablation prevents inflammation and improves insulin sensitivity in mice with diet-induced obesity, J. Immunol. 189 (4) (2012) 1992–1999.
[27] J.E. Lattin, K. Schroder, A.I. Su, J.R. Walker, J. Zhang, T. Wiltshire, K. Saijo, C.K. Glass, D.A. Hume, S. Kellie, M.J. Sweet, Expression analysis of G Protein-Coupled Receptors in mouse macrophages, Immunome Res. 4 (2008) 5-5.
[28] S. Li, X. Chen, G. Hao, X. Geng, W. Zhan, J. Sun, Identification and characterization of ATP-gated P2X2 receptor gene dominantly expressed in the Japanese flounder (Paralichthys olivaceus) head kidney macrophages, Fish Shellfish Immunol. 54 (2016) 312–321.
[29] S. Li, X. Chen, X. Li, X. Geng, R. Lin, M. Li, J. Sun, 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 (1) (2015) 100–109.
[30] S. Li, X. Li, C. Coddou, X. Geng, J. Wei, J. Sun, Molecular characterization and expression analysis of ATP-gated P2X7 receptor involved in Japanese flounder (< italic > Paralichthys olivaceus < /italic >) innate immune response, PLoS One 9 (5) (2014) e96625.
[31] J.K. Chambers, L.E. Macdonald, H.M. Sarau, R.S. Ames, K. Freeman, J.J. Foley, Y. Zhu, M.M. McLaughlin, P. Murdock, L. McMillan, J. Trill, A. Swift, N. Aiyar, P. Taylor, L. Vawter, S. Naheed, P. Szekeres, G. Hervieu, C. Scott, J.M. Watson, A.J. Murphy, E. Duzic, C. Klein, D.J. Bergsma, S. Wilson, G.P. Livi, AG Protein-coupled receptor for UDP-glucose, J. Biol. Chem. 275 (15) (2000) 10767–10771.
[32] K. Freeman, P. Tsui, D. Moore, P.C. Emson, L. Vawter, S. Naheed, P. Lane, H. Bawagan, N. Herrity, K. Murphy, H.M. Sarau, R.S. Ames, S. Wilson, G.P. Livi, J.K. Chambers, Cloning, pharmacology, and tissue distribution of G-protein-coupled receptor GPR105 (KIAA0001) rodent orthologs, Genomics 78 (3) (2001) 124–128.
[33] M.O. Barrett, J.I. Sesma, C.B. Ball, P.S. Jayasekara, K.A. Jacobson, E.R. Lazarowski, T.K. Harden, A selective high-affinity antagonist of the P2Y14 receptor inhibits UDP-glucose-stimulated chemotaxis of human neutrophils, Mol. Pharmacol. 84 (1) (2013) 41–49.
[34] A. Das, H. Ko, L.E. Burianek, M.O. Barrett, T.K. Harden, K.A. Jacobson, Human P2Y (14) receptor agonists: truncation of the hexose moiety of uridine-5'-diphosphoglucose and its replacement with alkyl and aryl groups, J. Med. Chem. 53 (1) (2010) 471–480.
[35] T.K. Harden, J.I. Sesma, I.P. Fricks, E.R. Lazarowski, Signalling and pharmacological properties of the P2Y receptor, Acta Physiol. 199 (2) (2010) 149–160.
[36] A. Azroyan, V. Cortez-Retamozo, R. Bouley, R. Liberman, Y.C. Ruan, E. Kiselev, K.A. Jacobson, M.J. Pittet, D. Brown, S. Breton, Renal intercalated cells sense and mediate inflammation via the P2Y14 receptor, PLoS One 10 (3) (2015) e0121419.
[37] I.R. Beacham, J.P. Headrick, Bacterial UDP-glucose hydrolases and P2 receptor-mediated responses to infection: a commentary, Adv. Infect. Dis. 3 (2) (2013) 100–104.