Molecular cloning, pathologically-correlated expression and functional characterization of the colony-stimulating factor 1 receptor (CSF-1R) gene from a teleost, *Plecoglossus altivelis*

Qiang CHEN[1,2], Xin-Jiang LU[1], Ming-Yun LI[1], Jiong CHEN[1,2,*]

[1] *Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Ningbo University, Ningbo 315211, China*

[2] *Collaborative Innovation Center for Zhejiang Marine High-efficiency and Healthy Aquaculture, Ningbo University, Ningbo 315211, China*

ABSTRACT

Colony-stimulating factor 1 receptor (CSF-1R) is an important regulator of monocytes/macrophages (MO/MΦ). Although several CSF-1R genes have been identified in teleosts, the precise role of CSF-1R in ayu (*Plecoglossus altivelis*) remains unclear. In this study, we characterized the CSF-1R homologue from *P. altivelis*, and named it PaCSF-1R. Multiple sequence alignment and phylogenetic tree analysis showed that PaCSF-1R was most closely related to that of Japanese ricefish (*Oryzias latipes*). Tissue distribution and expression analysis showed that the PaCSF-1R transcript was mainly expressed in the head kidney-derived MO/MΦ, spleen, and head kidney, and its expression was significantly altered in various tissues upon *Vibrio anguillarum* infection. After PaCSF-1R neutralization for 48 h, the phagocytic activity of MO/MΦ was significantly decreased, suggesting that PaCSF-1R plays a role in regulating the phagocytic function of ayu MO/MΦ.

Keywords: Colony-stimulating factor 1 receptor; Pathologically-correlated expression; Monocytes/macrophages; Phagocytosis; Sequence analysis

INTRODUCTION

The innate immune response, a fundamental defense mechanism in fish, is the first line of host defense against pathogens (Akira et al., 2006; Magnadottir, 2006). Cells involved in the innate immune system include monocytes/macrophages (MO/MΦ), neutrophils, and natural killer (NK) cells (Buchmann, 2014). MO/MΦ are critical effectors and regulators of inflammation and the innate immune response. Since this subset of immune cells is of primary importance in combating infections in fish (Magnadottir, 2006), the function and development of MO/MΦ have been investigated in diverse teleosts (Chen et al., 2014; Hanington et al., 2009; Lu et al., 2014; Torraca et al., 2014; Wu et al., 2014).

Colony-stimulating factor 1 receptor (CSF-1R), also known as macrophage colony-stimulating factor receptor (M-CSFR) and cluster of differentiation 115 (CD115), is a member of the protein tyrosine kinase class III (PTK III) family. CSF-1R is structurally related to the prototypic platelet-derived growth factor receptor (PDGFR), mast/stem cell growth factor receptor (SCFR), and fms-like tyrosine kinase III receptor (Flt3) (Elegheert et al., 2011). Similar to other PTK III members, CSF-1R comprises five Ig-like extracellular ligand-binding domains joined by a single membrane-spanning hydrophobic helix to a cytoplasmic protein tyrosine kinase (PTK) domain (Lemmon & Schlessinger, 2010). Upon activation, CSF-1R dimerizes and autophosphorylates on a specific tyrosine residue, creating binding sites for several cytoplasmic SH2-containing signaling molecules that relay and modulate CSF-1R signals (Lemmon & Schlessinger, 2010). In mammals, CSF-1R has two ligands, CSF-1 and IL-34 (Ma et al., 2012). CSF-1R is critical for the proliferation, survival, and differentiation of macrophages, as knockdown of this gene results in large depletions of macrophages in most tissues (Dai et al., 2002; Droin & Solary, 2010). In addition, CSF-1R signaling controls the development of the macrophage lineage under steady conditions and during certain inflammatory reactions (Lenzo et al., 2012).

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*Corresponding author, E-mail: jchen1975@163.com

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CSF-1R homologues have been identified in a number of teleost species, such as rainbow trout (*Oncorhynchus mykiss*) (Honda et al., 2005), goldfish (*Carassius auratus* L.) (Barreda et al., 2005), gilthead seabream (*Sparus aurata* L.) (Roca et al., 2006), and grass carp (*Ctenopharyngodon idellus*) (Chen et al., 2015). Furthermore, it has been reported that the CSF-1R protein is a specific marker of macrophages in goldfish (Katzenback & Belosevic, 2012), gilthead seabream (Roca et al., 2006), and grass carp (Chen et al., 2015). These studies showed that CSF-1R expression is confined to head kidney-derived MO/MΦ or only detected in purified macrophages. However, the function of CSF-1R in response to infection of teleost MO/MΦ remains unclear.

Ayu is an important commercial teleost widely cultured in Japan, China, and Korea. Recently, the development of ayu aquaculture in China has been severely challenged by *Vibrio anguillarum* infection, which has resulted in both production and animal welfare problems (Li et al., 2009). Considering the key role of MO/MΦ in the innate immune system of fish, it is important to determine their function in disease control. Due to its annual life cycle and accumulation of immunity knowledge, ayu was selected in the present study. We characterized a CSF-1R homologue (PaCSF-1R) from ayu, and analyzed the tissue and cellular distribution pattern before and after *V. anguillarum* infection. In addition, the effects of PaCSF-1R on MO/MΦ phagocytic activity were investigated.

MATERIALS AND METHODS

Fish maintenance

All fish were purchased from a fishery in Ninghai County, Ningbo City, China. Healthy fish, weighing 40-50 g each, were kept in freshwater tanks at 20-22 °C with regular feeding, as described previously (Chen et al., 2014). The fish were acclimatized to laboratory conditions for two weeks before the experiments were conducted. All experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Ethics Committee of Ningbo University.

Molecular characterization of PaCSF-1R cDNA

The cDNA sequence of PaCSF-1R was obtained from transcriptome data of ayu head kidney-derived MO/MΦ deposited in the NCBI SRA database with accession number SRX104781 using BLAST (http: //blast.ncbi.nlm.nih.gov/Blast.cgi). The authenticity of the PaCSF-1R cDNA was confirmed by PCR, cloning, and sequencing. The cleavage sites of the signal peptides were predicted using SignalP 4.1 (http: //www.cbs.dtu.dk/services/SignalP/); ClustalW (http: //clustalw.ddbj.nig.ac.jp/) was used for multiple sequence alignment; ligand-binding domains were predicted using the SMART web server (http: //smart.emblheidelberg.de/); phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura et al., 2011).

Bacterial infection

The *V. anguillarum* challenge was carried out as previously reported (Chen et al., 2014). Briefly, bacteria were grown in nutrient broth on a rotary shaker at 28 °C, and harvested in the logarithmic phase of growth, which was monitored by optical density assay. The *V. anguillarum* cells were washed, resuspended, and diluted to the appropriate concentration in sterile PBS. Head kidney-derived MO/MΦ were purified as described in the Materials and Methods and infected with live *V. anguillarum* at a multiplicity of infection (MOI) of 10. For tissues and peripheral blood leukocytes (PBLs), fish were challenged by intraperitoneal injection with 1.2×10^4 colony forming units (CFUs) of live *V. anguillarum* (in 100 μL PBS) per fish, with PBS alone used as the control. At 0, 4, 8, 12, and 24 h post infection (hpi), the liver, head kidney, spleen, and MO/MΦ were collected and preserved at -80 °C until subsequent use. For the preparation of PBLs, blood was collected through puncture of the caudal vein using a heparinized syringe at 0, 4, 8, 12, and 24 hpi. PBLs were obtained by Ficoll-Hypaque PREMIUM (1.077 g/mL) (GE Healthcare, New Jersey, USA) density gradient centrifugation.

Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed as described previously (Lu et al., 2014). Briefly, total RNA was extracted from fish tissue and MO/MΦ using RNAiso (TaKaRa, Dalian, China). After treatment with DNase I, first strand cDNA was synthesized using AMV reverse transcriptase (TaKaRa). Primers of PaCSF-1R were designed to amplify a 227 bp fragment, PaCSF-1Rtest(+): 5'-TGTACACCGTCCAGAGTGAC-3' and PaCSF-1Rtest(-): 5'-AATTGTTCGGAAAGTGGGCC-3'. The primers: pActin2(+): 5'-TCGTGCGTGACATCAAGGAG-3' and pActin2(-): 5'-CGCACT TCATGATGCTGTTG-3' were used to amplify a 231 bp fragment from a housekeeping β-actin gene, which is a widely used internal control (Huang et al., 2011). RT-qPCR was performed on an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) using SYBR premix Ex Taq II (Perfect Real Time; TaKaRa). The reaction mixture was incubated for 300 s at 95 °C, followed by 40 amplification cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Tissue samples were taken from four fish in each group. MO/MΦ samples were reproduced in three independent experiments.

Prokaryotic expression

The partial sequence encoding a protein fragment at amino acid position 18-207 of PaCSF-1R (PaCSF-1R-Ex) was amplified using the primer pair, PaCSF-1Rp(+): 5'-GGAATTC GCAGAATGGTCCGCCCCAG-3' and PaCSF-1Rp(-): 5'-GCTCGAGTCACTTCTGAATGACGTTGATGGA-3'. After digestion by *Eco*R I and *Xho* I, the amplicon was cloned into the pET-28a expression vector, and the constructed plasmid was subsequently transformed into *Escherichia coli* BL21 (DE3). After induction by IPTG, the recombinant protein (with an N-terminal His_6-tag) was purified using a Ni-NTA column (Qiagen, Shanghai, China) according to the manufacturer's instructions.

Antibody production and Western blot analysis

Antibody production was performed as previously reported (Wu et al., 2015). The purified PaCSF-1R-Ex protein emulsified with

Freund's incomplete adjuvant was used to immunize ICR mice (20-22 g) by intraperitoneal injection once every seven days for a total of four injections. Whole blood was collected and centrifuged to obtain sera. Control mice were injected with complete Freund's adjuvant. Anti-PaCSF-1R-Ex IgG (PaCSF-1R IgG) and control isotype IgG (IsoIgG) were purified by Protein G HP SpinTrap columns (GE Healthcare, USA). The quality of PaCSF-1R IgG was tested by Western blot analysis, and visualization using an enhanced chemiluminescence (ECL) kit (Advansta, Menlo Park, USA).

Primary culture of ayu head kidney-derived MO/MΦ

Ayu head kidney-derived MO/MΦ cells were isolated and cultured as previously described (Zhang et al., 2015). Head kidney was isolated and washed in RPMI 1640 medium (Invitrogen, Shanghai, China) supplemented with 2% fetal bovine serum (FBS) (Invitrogen), penicillin (100 U/mL), streptomycin (100 μg/mL), and heparin (20 U/mL). The cells were separated using Ficoll-Hypaque PREMIUM (1.077 g/mL) (GE Healthcare) in combination with centrifugation according to the manufacturer's instructions. The cells were then seeded in 35 mm dishes at a density of $2×10^7$/mL. Non-adherent cells were washed off, and the attached cells were incubated in complete medium (RPMI 1640, 5% ayu serum, 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) at 24 °C with 5% CO_2. According to Giemsa staining results, over 96% of adherent cells were MO/MΦ.

Phagocytosis assay

Phagocytosis of ayu MO/MΦ was performed as described previously (Zhang et al., 2015). The *E. coli* DH5α in the logarithmic phase of growth were labeled with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, USA) according to the manufacturer's protocols, and cells were thereafter designated FITC-DH5α. MO/MΦ were pre-incubated with PaCSF-1R IgG (250 mg/mL) for 4, 8, 12, 24, and 48 h. IsoIgG (250 mg/mL) was added as a control. FITC-DH5α was added at a MOI of 20, and incubated with cells for 30 min. Cells were washed extensively with sterile PBS to remove extracellular particles. Trypan blue was used to quench the fluorescence that resulted from particles that were outside the cells or stuck to the surface of the cells. Adherent MO/MΦ were loosened by adding 500 μL trypsin-EDTA (0.05% Trypsin, Invitrogen) to each culture well, followed by a 5 min incubation. All cells were collected with no cells remaining in the wells. After centrifuging at 300 g for 5 min, the cells were suspended in FACS buffer (PBS, 5% FCS, 0.1% sodium azide). Cell counting using a hemocytometer showed nearly no loss of cells in this process. The engulfed bacteria were examined by flow cytometry using a Gallios Flow Cytometer (Beckman Coulter, Miami, USA). The results were expressed as the relative mean fluorescence index (MFI) of the control in flow cytometric assay.

Statistical analysis

Results are presented as mean±SEM. All data were subjected to one-way or repeated-measures analysis of variance (ANOVA) with SPSS (version 13.0, Chicago, IL, USA). $P<0.05$ were considered statistically significant.

RESULTS

Molecular characterization of PaCSF-1R

The PaCSF-1R sequence was deposited in the GenBank Data Library under accession number KT692936. The cDNA of PaCSF-1R, which was 2 976 nucleotides (nts) long, possessed a large open reading frame that encoded a polypeptide precursor of 992 amino acids (aas). The protein precursor had a calculated molecular weight (MW) of $111.10×10^3$, and its putative isoelectric point (*p*I) was 6.33. Similar to its mammalian counterpart, PaCSF-1R comprised a signal peptide (at *aa* position 1-17), five Ig-like domains (aa 33-500), a short single transmembrane domain (aa 522-543), and a cytoplasmic tyrosine kinase domain (aa 582-915). Multiple alignment with other known PaCSF-1R amino acid sequences revealed that the two Ig-like N-terminal domains, which are important for ligand binding to CSF-1R, were conserved in teleosts and mammals (Figure 1).

Sequence comparisons revealed that PaCSF-1R shared highest amino acid identity with that of Japanese ricefish (68.8%). Phylogenetic tree analysis showed that all fish CSF-1Rs were grouped together to form a distinct cluster that differed from the mammalian cluster (Figure 2), and PaCSF-1R was most closely related to that of Japanese ricefish.

Alteration of PaCSF-1R mRNA expression in response to *V. anguillarum* infection

The mRNA expression of PaCSF-1R was detected in all tested tissues and in the MO/MΦ of healthy ayu, and was found to be extremely high in MO/MΦ, spleen and head kidney (Figure 3). Following infection with *V. anguillarum*, RT-qPCR was performed to analyze the changes in PaCSF-1R mRNA expression in various tissues and in MO/MΦ of ayu. The mRNA expression of PaCSF-1R significantly increased in the spleen, PBLs, and liver at 24 hpi, and decreased in the head kidney at 8 hpi, but not in the MO/MΦ (Figure 3).

Prokaryotic expression of PaCSF-1R N-terminal region and IgG preparation

Previous analysis of human CSF-1R ligand-binding determinants showed that the three N-terminal Ig-like domains in the receptor's extracellular region contain the complete high-affinity CSF-1 binding site (Rieger et al., 2014; Wilhelmsen & Van Der Geer, 2004). Therefore, we selected the sequence comprising the first two N-terminal IgG-like domains of PaCSF-1R (PaCSF-1R-Ex) for prokaryotic expression. The sequence of PaCSF-1R-Ex was amplified from cDNA of MO/MΦ, and was subsequently cloned into a pET-28a vector. The recombinant plasmid pET-28a- PaCSF-1R-Ex was then transformed into *E. coli* BL21 (DE3), expressed by induction with IPTG, purified using the Ni-NTA column (Figure 4A), and used to immunize mice to produce antisera. Using this antibody, we found that the MW of mature PaCSF-1R protein from MO/MΦ was about $1.5×10^5$ by Western blot analysis (Figure 4B); the high MW of

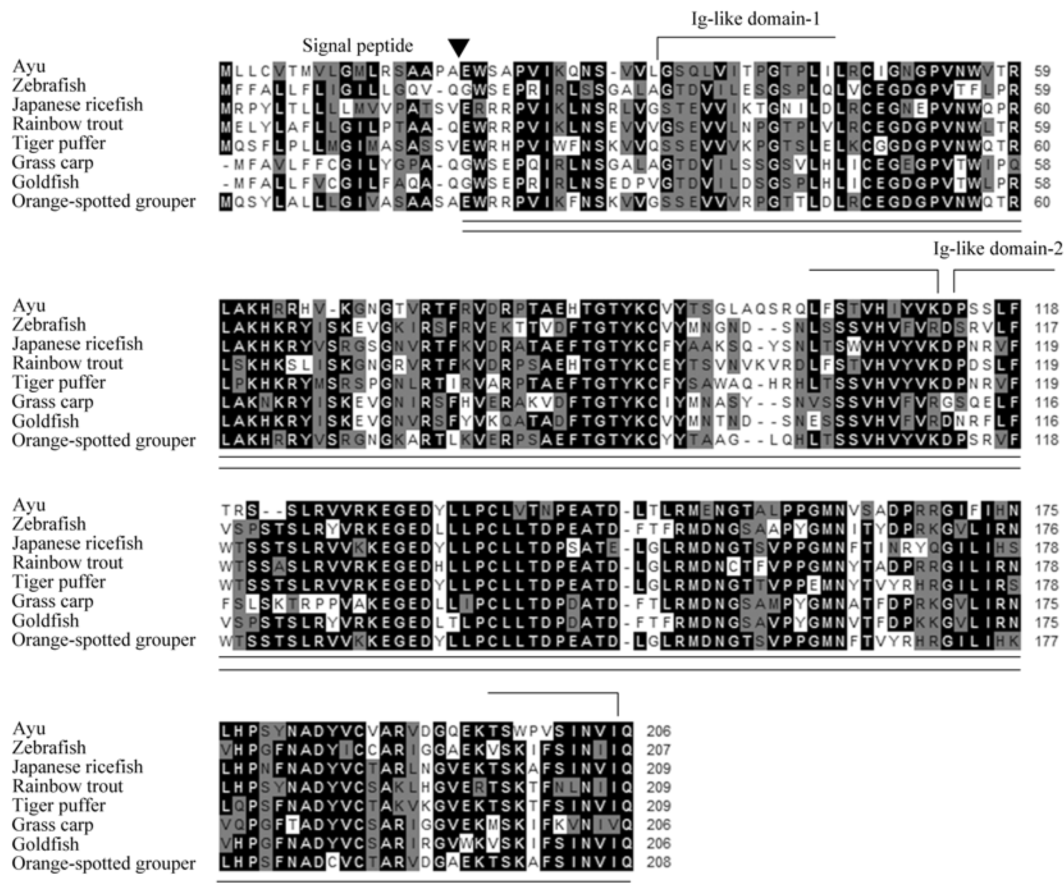

Figure 1 Multiple alignment of the amino acid sequences of two Ig-like N-terminal CSF-1R domains in several animals

Threshold for shading was 60%; similar residues are highlighted in gray; identical residues are highlighted in black; alignment gaps are denoted "-". Inverted triangle indicates cleavage site of the CSF-1R signal peptide. The sequence for prokaryotic expression is double underlined. Ig-like domain-1 and Ig-like domain-2 are indicated. The sequences used in the analyses are ayu (*Plecoglossus altivelis*), KT692936; zebrafish (*Danio rerio*), NM_131672; Japanese ricefish (*Oryzias latipes*), XM_004073307; rainbow trout (*Oncorhynchus mykiss*), NM_001124738; tiger puffer (*Takifugu rubripes*), XM_004073307; grass carp (*Ctenopharyngodon idellus*), KP244336; goldfish (*Carassius auratus*); and, orange-spotted grouper (*Epinephelus coioides*), HQ594531.

native PaCSF-1R may be caused by post-translational modification, as previously reported (Wilhelmsen & Van Der Geer, 2004). Anti-PaCSF-1R IgG was subsequently purified from antisera using the Protein G HP SpinTrap (GE Healthcare), and was stored at -80ºC for subsequent use.

PaCSF-1R mediation of ayu MO/MΦ phagocytosis

We analyzed the phagocytic activity of ayu MO/MΦ after PaCSF-1R IgG neutralization. After antibody neutralization for 4, 8, 12, and 24 h, the phagocytic activity of MO/MΦ was similar to that of the IsoIgG-treated control (Figure 5A-D). However, the phagocytosis of MO/MΦ was significantly downregulated after antibody neutralization for 48 h (Figure 5E).

DISCUSSION

In mammals, CSF-1R is a specific marker of the MO/MΦ lineage, and is critical for macrophage proliferation and development (Chitu & Stanley, 2006; Dai et al., 2002). However, the function of CSF-1R in teleost MO/MΦ remains unclear. In the present work, we characterized a CSF-1R homologue from ayu (PaCSF-1R). Multiple alignments revealed that PaCSF-1R contained the N-terminal ligand-binding domain, which was highly conserved in fish and mammals, suggesting that the function of CSF-1R may be conserved from teleosts to mammals.

A previous orange-spotted grouper study showed that CSF-1R mRNA expression was highest in the spleen, followed by the gill and kidney (Dan et al., 2013). RT-qPCR analysis in rainbow trout showed that M-CSFR mRNA was mainly expressed in the spleen, head kidney and kidney (Honda et al., 2005). In the present study, PaCSF-1R mRNA was expressed in all tested tissues, with the highest expression in MO/MΦ, followed by the spleen and head kidney, similar to that reported

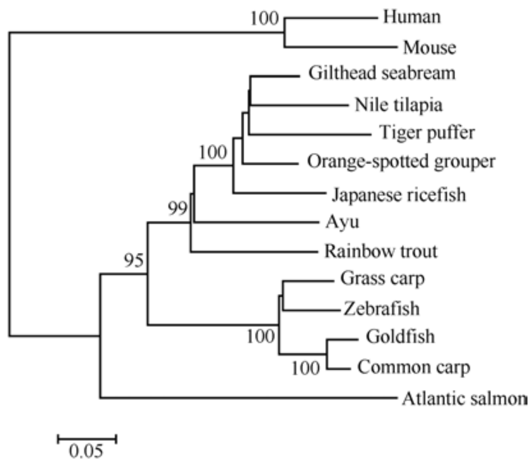

Figure 2 Phylogenetic (neighbor-joining) analysis of the complete amino acid sequence of PaCSF-1R with other known CSF-1Rs using MEGA 5.0 software

Values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1 000 replicates; shown only when >60%). Scale bar shows number of substitutions per base. In addition to those listed in Figure 1, accession numbers of the other sequences used are common carp (*Cyprinus carpio*), AB526448; zebrafish (*Danio rerio*), NM_131672; orange-spotted grouper (*Epinephelus coioides*), HQ594531; gilthead seabream (*Sparus aurata*), CAJ18352; Nile tilapia (*Oreochromis niloticus*), XM_003455186; and, Atlantic salmon (*Salmo salar*), NM_001171807.

in other teleosts (Chen et al., 2015). Further bacterial infection results showed that PaCSF-1R mRNA expression in the spleen, PBLs, and liver were upregulated after *V. anguillarum* treatment, which is in consistent with the situation in orange-spotted grouper and gilthead seabream (Dan et al., 2013, Reyes-Becerril et al., 2011). Furthermore, the mRNA expression of PaCSF-1R in the head kidney was downregulated after *V. anguillarum* treatment. It has been reported that low doses of lipopolysaccharide (LPS) injected intravenously recruit monocytes from the bone marrow to the bloodstream 4 h after injection in mice (Ludin et al., 2012). Since the head kidney is the main hematopoietic and lymphoid tissue in teleosts, like mammalian bone marrow, the downregulation of mRNA expression of PaCSF-1R in the head kidney may be due to the decrease of MO/MΦ in the head kidney. These results suggested that PaCSF-1R may play an essential role in the anti-bacterial process. Considering its highest expression in MO/MΦ, the role of PaCSF-1R in MO/MΦ-mediated phagocytosis is at the top priority of function analysis.

In mammals, overexpression of CSF-1R on microglia accelerates the phagocytosis of aggregated amyloid beta (Aβ) through macrophage scavenger receptors and expression of Fcγ receptors (Mitrasinovic et al., 2003). In the present study, after PaCSF-1R neutralization for 48 h, the phagocytic activity of MO/MΦ was significantly downregulated compared with that of the isotype control. Therefore, we suggest that PaCSF-1R may be involved in phagocytosis of ayu MO/MΦ through macrophage scavenger receptors and Fcγ receptors. The function of CSF-1R in MO/MΦ may result from binding with its ligands, and neutralization of PaCSF-1R may block PaCSF-1R-mediated phagocytosis of MO/MΦ. In mammals, CSF-1 and IL-34 are two ligands of CSF-1R. CSF-1 competes with IL-34 for binding to CSF-1R (Wei et al., 2010), and may compensate for the absence of IL-34 in the brainstem and cerebellum (Wang et al., 2012; Wei et al., 2010). IL-34 does not control the recruitment of blood monocytic cells and their subsequent differentiation into Langerhans cells in inflammation, but is crucial for their maintenance in situ (Nakamichi et al., 2013). Macrophages are important participants in the phagocytosis of foreign material in most tissues (Pixley et al., 2004). Therefore, we speculate that PaCSF-1R may play a role in the regulation of MO/MΦ function in response to bacterial infection. Since the characterization of CSF-1R ligands in teleosts is still unclear, further investigation is needed to determine the detailed mechanism underlying the phagocytic function of PaCSF-1R.

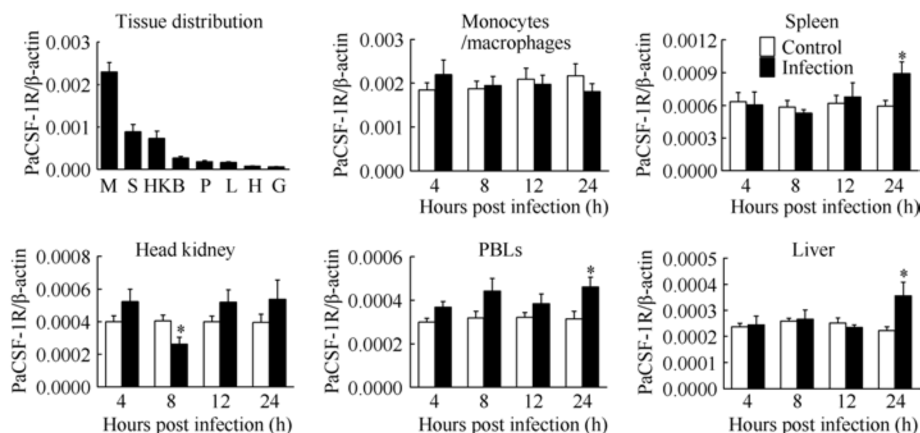

Figure 3 RT-qPCR analysis of PaCSF-1R mRNA expression in various tissues and MO/MΦ of ayu

Fish were sacrificed 4, 8, 12, and 24 h after intraperitoneal injection of *V. anguillarum a*; MO/MΦ were collected at 4, 8, 12, and 24 h post-infection. M, MO/MΦ, S, spleen, HK, head kidney; B, brain; P, PBLs; L, liver; H, heart; G, gill. PaCSF-1R transcript levels were normalized to β-actin. Data are expressed as the mean±*SEM* of the results from four fish. *P<0.05.

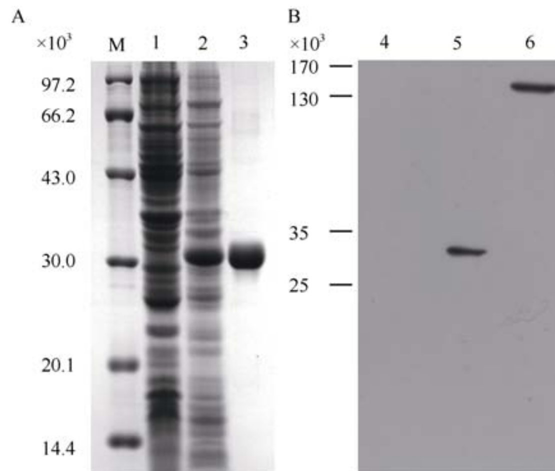

Figure 4 Prokaryotic expression of PaCSF-1R-Ex and Western blot analysis for PaCSF-1R

A: SDS-PAGE analysis of prokaryotic expressed PaCSF-1R. Lane M: protein marker; 1 and 2: protein from BL21 (DE3) transformed with pET-28a-PaCSF-1R-Ex plasmid before and after IPTG induction; 3: purified recombinant PaCSF-1R-Ex. B: Western blot analysis of recombinant PaCSF-1R-Ex and native PaCSF-1R. Lane 4: negative control; 5: purified recombinant PaCSF-1R-Ex; 6: total proteins of MO/MΦ.

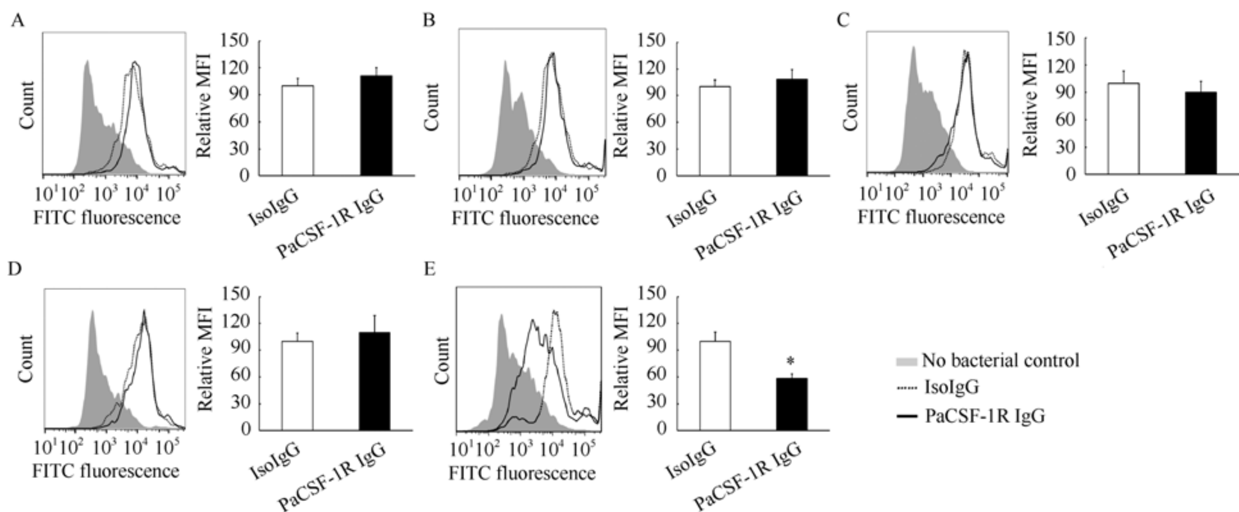

Figure 5 Effect of PaCSF-1R neutralization on phagocytosis of FITC-DH5α by ayu MO/MΦ

MO/MΦ were incubated with PaCSF-1R IgG for 4 (A), 8 (B), 12 (C), 24 (D), and 48 h (E). IsoIgG was added as the control. Then, FITC-DH5α was added at an MOI of 20, followed by incubation for an additional 30 min. The engulfed bacteria were also examined by flow cytometry. A total of 10 000 events were analyzed by flow cytometry. Relative mean fluorescence intensity (MFI) was presented as fold change over the control, which was assigned a unit of 100. Data are expressed as the mean±*SEM* of three independent experiments. *: $P<0.05$.

In summary, we identified a CSF-1R gene from ayu. PaCSF-1R expression was pathologically correlated with *V. anguillarum* infection, and may play a role in the regulation of MO/MΦ function in response to bacterial infection. Further investigation is needed to determine the detailed mechanism underlying CSF-1R function in teleosts.

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