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The interleukin-6 regulates the function of monocytes/macrophages (MO/MΦ) via the interleukin-6 receptor β in ayu (*Plecoglossus altivelis*)

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ABSTRACT

Interleukin-6 (IL-6) is one of the most pleiotropic cytokines because of its wide range of effects on cells of the immune and non-immune systems in the body. However, the role of IL-6 in fish monocytes/macrophages (MO/MΦ) is poorly understood. In this study, we cloned the cDNA sequence of the IL-6 gene from ayu (*Plecoglossus altivelis*) and demonstrated using a tissue distribution assay that ayu interleukin-6 (PaIL-6) mRNA is expressed in all tested tissues. Changes in expression were observed in immune tissues as well as in MO/MΦ after a *Vibrio anguillarum* infection; subsequently, PaIL-6 was expressed and purified to prepare anti-PaIL-6 antibodies. Recombinant PaIL-6 protein (rPaIL-6) treatment enhanced pro-inflammatory cytokine expression. Ayu interleukin-6 receptor β (PaIL-6Rβ) knockdown resulted in decreased pro-inflammatory cytokine expression in MO/MΦ treated with rPaIL-6, whereas no significant changes were observed after ayu interleukin-6 receptor α (PaIL-6Rα) knockdown in MO/MΦ. PaIL-6 and PaIL-6Rβ knockdown in MO/MΦ inhibited the phosphorylation of signal transducer and activator of transcription 1. Moreover, PaIL-6Rβ knockdown inhibited the phagocytic and bactericidal ability of ayu MO/MΦ treated with rPaIL-6. These data indicate that PaIL-6 may be able to regulate the function of ayu MO/MΦ.

1. Introduction

Interleukin-6 (IL-6) plays a crucial role in the regulation of inflammatory response in the immune system [1]. The IL-6s comprise four long α-helices, termed A, B, C and D [2]. Further, IL-6 is a pleiotropic cytokine, which plays major roles in inflammation, hematopoiesis, and acute phase reactions [3]. During viral or bacterial pathogenic invasion, IL-6 is up-regulated rapidly and transiently [3]. Therefore, IL-6 functions as the key regulator of the immune system [1,4]. Various types of immune and non-immune cells can produce IL-6; for example, in muscles, IL-6 is secreted into the blood in response to muscle contractions, which stimulates energy mobilization, leading to an increase in the body temperature [5,6]. Further, IL-6 is produced by monocytes/macrophages (MO/MΦ) in response to microbial stimuli [4].

An IL-6 receptor complex consists of an 80-kDa type 1 cytokine α-receptor subunit (IL-6Rα) and a 130-kDa signal-transducing β-receptor subunit (IL-6Rβ); IL-6Rα is only expressed in selected cell populations, whereas IL-6Rβ is ubiquitously expressed in all types of cells [1]. Signaling via IL-6Rβ is vital for hematopoiesis, cell development, cell survival, and cell growth. In addition, IL-6Rβ-dependent signaling mediates downstream activation of the JAK-STAT pathway, which skews the MO/MΦ function into different phenotypes [7,8].

IL-6 acts as an inflammatory biomarker [4]. In the presence of IFN-γ, IL-6 promotes the production of TNF-α and IL-1β, suggesting that IL-6 can enhance the phenotype that MO/MΦ has committed to [9]. In the absence of SOCS3 in MO/MΦ, IL-6 suppresses the LPS-induced expression of TNF and IL-12 [10]. However, the function of IL-6 in an anti-inflammation reaction is conditional; IL-6 is a pro-inflammatory cytokine, and thus, it does not exert anti-inflammatory effects in any case. In the past few years, several IL-6 genes have been identified in teleosts. In rainbow trout (*Oncorhynchus mykiss*), a recombinant IL-6 protein was found to enhance MO/MΦ growth and induce antimicrobial peptide gene expression [11]. In Nile tilapia (*Oreochromis niloticus*), a recombinant IL-6 protein enhanced the secretion of sIgM and production of mIgM in P60 and P70 B cell subsets *in vitro* [12]. However, there is a lack of information on the function of IL-6 in MO/

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Table 1
Primers used in this study.

Primer	Gene	Accession number	Nucleotide sequence (5′→3′)	Sequence information
Pa IL-6F	*IL-6*	MG264003	GGAATTCCTTCATTTCTGGGTACATAATC[a]	Full length
Pa IL-6R			CCGCTCGAGTAGCAATGTTCTGCTTATGGC[b]	
Pa IL-6RαF	*IL-6Rα*	MK564062	GGAATTCCTCGCTCTGGGCACAGAGAGA[a]	Full length
Pa IL-6RαR			CCGCTCGAGTTCTCATGTGCTTGTGACTAG[b]	
Pa IL-6RβF	*IL-6Rβ*	MK564063	GGAATTCAAGCCAAGTAGCAGCACTCA[a]	Full length
Pa IL-6RβR			CCGCTCGAGTAGTCCTCCTTTCACCAAAGA[b]	
Pa IL-6pF	*IL-6*	MG264003	GGAATTCAGCCCGGTGCCGACTACAT[a]	Prokaryotic expression
Pa IL-6pR			CCGCTCGAGTCACATCTGTTCCAGATAAGTG[b]	
Pa IL-6F	*IL-6*	MG264003	CAATACATGGCCTTGCTTCA	RT-qPCR
Pa IL-6R			TTGGTCCTCCTTGTTTACCG	
PaIL-6RαF	*IL-6Rα*	MK564062	CCCTGAGGCAGAAGTTCAAG	RT-qPCR
PaIL-6RαR			ACAGGGCTGTCTCAGAAGGA	
PaIL-6RβF	*IL-6Rβ*	MK564063	GGCTGGGAAACCAATGACTA	RT-qPCR
PaIL-6RβR			GGGTGGTAGGAGGGATTGTT	
PaTNF-αF	*TNF-α*	JP740414	ACATGGGAGCTGTGTTCCTC	RT-qPCR
PaTNF-αR			GCAAACACACCGAAAAAGGT	
PaIL-1βF	*IL-1β*	HF543937	TACCGGTTGGTACATCAGCA	RT-qPCR
PaIL-1βR			TGACGGTAAAGTTGGTGCAA	
PaTGF-βF	*TGF-β*	JP742920	CTGGAATGCCGAGAACAAAT	RT-qPCR
Pa TGF-βR			GATCCAGAACCTGAGGGACA	
PaIL-10F	*IL-10*	JP758157	TGCTGGTGGTGCTGTTTATGTGT	RT-qPCR
PaIL-10R			AAGGAGCAGCAGCGGTCAGAA	
Pa18SF	*18S rRNA*	FN646593	GAATGTCTGCCCTATCAACT	RT-qPCR
Pa18SR			GATGTGGTAGCCGTTTCT	
V.anguillarum 16SF	*16S rRNA*	FM866241	AGAGTTTGATCATGGCTCAG	RT-qPCR
*V.anguillarum*16SR			GGTTACCTTGTTACGACTT	

[a] The underlined nucleotides represent the restriction sites for *Eco*R I.
[b] The underlined nucleotides represent the restriction sites for *Xho* I.

MΦ in teleosts.

Innate immunity is a semi-specific and widely distributed form of immunity; it is the first line of defense against pathogens, representing a vital systemic response to prevent infection and maintain homeostasis [13]. MO/MΦ play a pivotal role in the immune system [14,15], owing to their ability to phagocytose and kill microorganisms [16], and produce cytokines [17,18]. Ayu (*Plecoglossus altivelis*) is the sole member of Osmeriformes family Plecoglossidae living in streams and coastal waters in some regions of Asia. Ayu is an important commercial teleost owing to its delicious taste. IL-6 is produced promptly in response to infections and tissue injuries to contribute to host defense mechanisms in mammals [2]. In teleost, IL-6 has been found to participate in antibody production after infection [12]. Hence, the identification and characterization of IL-6 is very important to illustrate the mechanism of an inflammatory response in ayu. In our study, we identified an IL-6 gene in ayu and investigated the mRNA expression level of the ayu IL-6 (PaIL-6) in various tissues of healthy and infected ayu. In addition, the effects of recombinant ayu interleukin-6 (rPaIL-6) on the expression of inflammatory cytokines were investigated. The effects of ayu interleukin-6 receptor beta (PaIL-6Rβ) knockdown on phagocytosis, intracellular bacterial killing, and phosphorylation level of signal transducer and activator of transcription 1 (PaSTAT1) protein of MO/MΦ were also determined.

2. Materials and methods

2.1. Fish rearing

All experimental ayu (Zhemin No. 1, length 17–20 cm, weighing 40–50 g each) were obtained from Ninghai County, Ningbo City. Fish were kept in recirculating fresh water at an ambient temperature of approximately 20–22 °C for two weeks for acclimation, prior to the start of experiments. The fish used in this study were healthy and did not show any pathological signs. All experiments were approved by the Animal Ethics Committee of Ningbo University and performed according to the Experimental Animal Management Law of China.

2.2. Molecular characterization of IL-6 and its receptor cDNAs

The cDNA sequence of the ayu IL-6 (PaIL-6), IL-6 receptor alpha (PaIL-6Rα) and IL-6 receptor beta (PaIL-6Rβ) genes were obtained from the transcriptome analysis of ayu head kidney-derived MO/MΦ; transcriptomic data were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE40221. The full-length cDNA sequence of PaIL-6, PaIL-6Rα, and PaIL-6Rβ were subsequently confirmed by PCR, each construct was confirmed by sequencing at BGI, Shenzhen, China. The primers for the full-length cDNA sequences of PaIL-6, PaIL-6Rα, and PaIL-6Rβ are shown in Supplementary Table 1. The PCR program used was 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s; followed by 72 °C for 10 min, with a final hold at 4 °C. The accession numbers of PaIL-6, PaIL-6Rα, and PaIL-6Rβ sequences are shown in Supplementary Table 1. The cleavage sites of the signal peptides were predicted using the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignmentswere generated by the ClustalW program (http://clustalw.ddbj.nig.ac.jp/). The molecular weight and isoelectric point were predicted using the ExPASy Compute pI/MW tool (http://web.expasy.org/compute_pi/) and the ligand-binding domains were predicted using the SMART program (http://smart.embl-heidelberg.de/). Phylogenetic and molecular evolutionary analyses were conducted using the molecular evolutionary genetics analysis (MEGA) tool (version 5) [19]. The sequences used in this study are listed in Supplementary Table 1.

2.3. Prokaryotic expression, antibody preparation, and purification of recombinant protein

The primers PaIL-6F and PaIL-6R were designed to amplify the sequence encoding PaIL-6 (aa23 - 220) (Table 1). After digestion with *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 isopropyl-β-d-thiogalactopyranoside (IPTG), the recombinant protein (with a N-terminal 6 × histidine [His] tag) was purified using the Ni-NTA column

(QIAGEN, Shanghai, China) according to the manufacturer's instructions. The pure inclusion bodies containing rPaIL-6 were recovered by centrifugation and resuspended in 3 mL solubilization buffer (pH < 8.0). Denatured rPaIL-6 was purified and refolded by urea gradient size-exclusion chromatography. The possibility of contamination of rPaIL-6 preparations with endotoxins was tested using the *Limulus amebocyte* lysate test according to the manufacturer's instructions. In brief, endotoxin standard solutions covering the desired concentration range were prepared to generate a standard curve (OD 545 nm). A 0.1 mL sample was added to the working fluid to test the endotoxins. Less than 0.1 EU/mg of endotoxin was detected in the recombinant proteins after toxin removal with an endotoxin-removal column (Pierce, Rockford, IL). The purified rPaIL-6 was used as an immune antigen to produce antisera in ICR mice. In brief, the groups of animals (n = 5) were intraperitoneally and subcutaneously immunized with 0.25 mL recombinant PaIL-6 protein (0.1 mg/mL) in a 50% emulsion of Freud's adjuvant (FCA, Sigma, USA), respectively. Thereafter, the mice were injected intraperitoneally at two-week intervals with the same doses. Two days after the last immunization, the mice were placed under anesthesia (pelltobarbitalum natricum) and sacrificed for blood collection from the heart using a syringe; sera were collected by centrifuging the blood samples at 14,000 g for 15 min at 4 °C and stored at −80 °C until use. Protein A HP SpinTrap columns (GE healthcare, New Jersey, USA) were used to purify the anti-PaIL-6 IgG. The IgG from saline-injected ICR mice (isotype IgG) was used as an isotype control. The specificity of the antibody was tested by western blotting and the protein band was visualized using an enhanced chemiluminescence (ECL) kit (Advansta, Menlo Park, USA), as described in Section 2.7.

2.4. Bacterial challenge test

V. anguillarum were grown at 28 °C in nutrient broth with shaking and collected in the logarithmic growth stage. Further, they were washed, resuspended, and diluted to the appropriate concentration in sterile phosphate-buffered saline (PBS). For the in vivo challenge test, ayu were infected by intraperitoneal (i.p.) injection of 1.2×10^4 colony-forming units (CFU) of *V. anguillarum* (in 100 μL PBS) per fish for the infected group, while the same volume of PBS was used for the control group. No clinical changes were observed in the first 24 h; however, on the next day, a few fish succumbed without external signs. External signs appeared from the third day, starting with small red spots developing into ulcers, erythema of the fin bases, and hemorrhage from the anus. We collected the blood of injected ayu, using the spread plate method on TSB medium to obtain a single bacterial colony. The single bacterial colony was confirmed by sequencing, showing that ayu were infected with *V. anguillarum*. The head kidney, body kidney, spleen, liver, gills, intestine, muscle, and skin were obtained 4, 8, 12, 24, and 48 h after bacterial challenge, after which they were snap-frozen in liquid nitrogen immediately and processed for subsequent real-time quantitative PCR analysis. At each time point, we challenged 5 fish (n = 5). For *in vitro* MO/MΦ treatment, head kidney-derived MO/MΦ were isolated and cultured at a concentration of 2×10^6/mL, as described in Section 2.5. We used 2 mL cell suspension per well, and *V. anguillarum* was added at a multiplicity of infection (MOI) of 2. We obtained cell samples at 4, 8, 12, and 24 h after bacterial challenge, and processed them for subsequent real-time quantitative PCR analysis.

2.5. Primary culture of ayu head kidney-derived MO/MΦ

Ayu MO/MΦ, a mixed cell population enriched with macrophages, were isolated and cultured as described previously [20]. Briefly, ayu (length: 17–20 cm; weight: 40–50 g) were sacrificed by an overdose of anesthetic (0.03% [v/v] ethyleneglycol monophenyl ether). Head kidneys were removed immediately to isolate the leukocytes. Tissue was homogenized through a 100 μm nylon cell strainer (Falcon). A Ficoll density gradient (Invitrogen, Shanghai, China) was used to obtain head kidney leukocyte-enriched fractions. After washing twice with RPMI1640, ayu MO/MΦ were cultured in 6-well plates at a concentration of 2×10^7 cells/mL. Cells (2 mL) were then incubated for at least 12 h at 24 °C with 5% CO_2. Non-adherent cells were removed by aspiration and adherent cells were incubated with a complete medium (RPMI1640, 5% ayu serum, 5% fetal bovine serum (FBS), 1% penicillin/streptomycin). The purity of ayu MO/MΦ was 96% according to the data of flow cytometry analysis (Fig. 5A).

2.6. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from fish tissues and MO/MΦ using RNA iso (Takara, Dalian, China). After treatment with DNase I, first-strand cDNA was synthesized using AMV reverse transcriptase (Takara). RT-qPCR was performed on an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq II (Takara). The primer sequences for the target genes are listed in Table 1. Amplifications were carried out in a 25 μL reaction volume containing the sample, primers, and SYBR Premix Ex TaqII. 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.The mRNA expression of the target genes was normalized against that of 18S rRNA using the $2^{-\Delta\Delta Ct}$ method.

2.7. Western blotting

To detect PaIL-6 protein expression in the supernatant of ayu MO/MΦ after infection, live *V. anguillarum* was diluted to appropriate concentrations in PBS to infect MO/MΦ at an MOI of 2. The supernatant of ayu MO/MΦ was concentrated in Amicon-Ultra 4 centrifugal filter units with a 3-kDa cutoff (Millipore). MO/MΦ were pelleted and lysed in a buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 100 mM NaCl, 0.2 mM DTT, 0.5 mM sodium orthovanadate, and 0.4 mM PMSF [pH 7.4]) containing phosphatase inhibitor (Phosphatase Inhibitor Cocktail; Sigma, St. Louis, MO), following which, 10 μL samples were boiled in an SDS loading buffer containing β-mercaptoethanol for 3 min. The proteins were resolved using 12% SDS-PAGE, transferred to PVDF membrane (Pall, NY, USA), and incubated with the anti-PaIL-6 antibody (1:200). Thereafter, membranes were washed and incubated for 1 h with a secondary antibody, horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (1:5000), and the protein bands visualized using an ECL Western blotting detection system, as previously described [21]. STAT1-specific and Tyr^{694}-phosphorylated PaSTAT1-specific antibodies used for this study were prepared previously [20].

2.8. RNA interference

PaIL-6 siRNA (5′-CAUUACUAACCUGGUCACAUCUGUU-3′), PaIL-6Rα siRNA (5′-CAGCCUGUUUCUGUGCUCGUUAUAU-3′), PaIL-6Rβ siRNA (5′-GCCGUUUCUAUCUUUGGUATT-3′), and scrambled siRNA (5′-CAUAAUCGUCCACUGCUACUAUGUU-3′) were designed and purchased from Invitrogen. The transfection reagent, Lipofectamine 2000 (Invitrogen), was used for the siRNA transfection process according to the manufacturer's protocol. In brief, 5 mL of Lipofectamine 2000 in 250 mL of Opti-MEM (Invitrogen) was mixed with 100 pmoL PaIL-6 siRNA, PaIL-6Rα siRNA, PaIL-6Rβ siRNA, or scrambled siRNA in 250 μL of Opti-MEM. The mixture was then incubated for 20 min at room temperature, before being added to MO/MΦ, with a final siRNA concentration of 40 nM. The cell culture medium was replaced with a complete medium after incubation for 5.5 h, following which, cells were cultured for another 48, 72, or 96 h for later use. The knockdown efficiency was confirmed by RT-qPCR.

2.9. Phagocytosis assay

Escherichia coli DH5α in the logarithmic growth phase were prepared and labeled with FITC (Sigma) according to the manufacturer's protocol; thereafter, these cells were called FITC-DH5α. Ayu MO/MΦ were cultured in 6-well platesata concentration of 2×10^6 cells/mL. We used 2 mL cell suspension per well (4×10^6 cells per well). The cells were treated with 100 ng/mL rPaIL-6 and PaIL-6Rβ siRNA. BSA, a globulin in bovine serum containing 583 amino acid residues, is widely used in protein control because it has no detectable effect on cell activities. FITC-DH5α was added at an MOI of 10, followed by incubation for another 30 min. Sterile PBS was used to extensively wash the cells to remove extracellular particles. The fluorescence emitted by particles outside the cells or sticking to the cell surface was quenched with 10 μL Trypan blue (0.4%) for 1 min. A Gallios flow cytometer (Beckman Coulter, Miami, USA) and FlowJo software were used to analyze the bacteria engulfed by the cells. Relative mean fluorescence intensity (MFI) of the rPaIL-6- and BSA-treated groups was expressed as the fold change relative to the value of the group not treated with bacteria, and the value of the BSA-treated group was assigned a unit of 100.

2.10. Bacterial killing assay

The bacterial killing assay was performed by analyzing CFU, as previously described, with modifications [22]. A standard curve was generated with the RT-qPCR results to assess the bacterial number. RNA obtained from the *V. anguillarum* culture at a concentration of 10^9 CFU/mL was serially diluted 10-fold. Each RNA dilution was used to construct a standard curve. After pre-incubation with 100 ng/mL rPaIL-6 and PaIL-6Rβ siRNA as previously described, ayu MO/MΦ were infected with live *V. anguillarum* at an MOI of 10 for 30 min. Thereafter, the remaining bacteria were killed using 80 μL gentamicin (50 μg/mL) for 15 min to avoid the effect of gentamicin on intracellular bacteria and the MO/MΦ were washed extensively with sterile PBS. Cells of the uptake groups were collected, while those of the kill groups were further incubated for 1.5 h to allow for bacterial killing before cell lysis, following which, these cells were collected for RNA template preparation. As a molecular indicator, 16S rRNA has gradually become a powerful tool for microbial detection and classification [23]. The bacterial killing efficiency was confirmed by RT-qPCR for *V. anguillarum* 16S rRNA, using primers 16SF and 16SR (Table 1) [24]. We collected the cells and counted use 4×10^6 cells in each group. The Ct values based on the standard curve that was previously generated were used to assay the total CFU/mL in all samples.

2.11. Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). The biological repeats are indicated by "n." All data were subjected to one-way ANOVA with SPSS (version 13.0, Chicago, IL, USA). P values < 0.05 were considered statistically significant.

3. Results

3.1. Molecular characterization and sequence analysis of PaIL-6

The PaIL-6 sequence was deposited in the GenBank Data Library under accession number MG264003. The cDNA of PaIL-6 ORF consisted of 663 bp of nucleotide sequence and encoded a polypeptide of 221 amino acids. The molecular mass was 25.1 kDa and the predicted theoretical isoelectric point (pI) was 7.66. Signal peptide was detected at the N-terminal between residues 1 and 23, suggesting that PaIL-6 is a secretory protein. The mature PaIL-6 comprised of 197 amino acids, with a molecular weight of 22.5 kDa and a pI of 6.88. Multiple sequence alignment revealed four conserved α-helixes of the IL-6 protein family (Fig. 1). Sequence comparisons showed that PaIL-6 shared the highest identity with Atlantic salmon (*Salmo salar*) IL-6 (28.5%) and rainbow trout (*Oncorhynchus mykiss*) IL-6 (28%). The phylogenetic tree analysis showed that the IL-6s were grouped together to form a fish cluster, which is distinct from a mammalian cluster (Fig. 2). The PaIL-6 amino acid sequence clustered within the fish IL-6 group.

3.2. Tissue PaIL-6 mRNA expression in response to V. anguillarum infection

In healthy fish, RT-qPCR was performed to analyze the transcripts of PaIL-6 in different tissues including skin, muscle, intestine, body kidney, head kidney, liver, gill, and spleen (Fig. 3A). After *V. anguillarum* infection, the mRNA expression of PaIL-6 significantly increased in the head kidney and liver, at 4, 8, and 12 hpi (Fig. 3B and C). In the gill and spleen, the PaIL-6 transcript level increased at all time points after infection (Fig. 3D and E).

3.3. Preparation of recombinant PaIL-6 (rPaIL-6) and corresponding antibody

We selected the mature peptide that encoded a protein fragment from amino acid position 24–221 of PaIL-6, for prokaryotic expression. This sequence of PaIL-6 was cloned into pET-28a vector and transformed into BL21 (DE3), which was induced by IPTG. The soluble recombinant protein was purified using the Ni-NTA column and analyzed by SDS-PAGE. The SDS-PAGE analysis showed that the MW of purified rPaIL-6 was approximately 31 kDa (with Trx-Tag, 6 × His-tag, Fig. 4A). Thereafter, antiserum was generated by immunizing mice with purified rPaIL-6. Using this antiserum, Western blot analyses could detect PaIL-6 protein in the supernatant of *V. anguillarum*-infected ayu MO/MΦ (Fig. 4B). The MW of the PaIL-6 secretory protein without signal peptide was approximately 22.5 kDa, which corresponded with our results. In our previous work, PaIL-8 (PaCXCL-8) was identified [25]. We further found that the anti-PaIL-6 antibody only detects PaIL-6, but not PaIL-8 (Fig. 4B).

3.4. Altered PaIL-6 expression after V. anguillarum infection in MO/MΦ

To ascertain whether PaIL-6 expression was altered in MO/MΦ after infection, RT-qPCR and western blotting were performed to analyze the expression levels of PaIL-6 in normal and infected ayu MO/MΦ. The mRNA expression of PaIL-6 was significantly up-regulated at all time points after *V. anguillarum* infection (Fig. 5B). Protein levels detected by Western blot analysis revealed that PaIL-6 expression was up-regulated at all time points (Fig. 5C).

3.5. Effect of PaIL-6 on cytokine mRNA expression of MO/MΦ

We investigated whether rPaIL-6 affects the mRNA expression of cytokines in ayu MO/MΦ. The mRNA expression of TNF-α and IL-1β was significantly up-regulated in ayu MO/MΦ treated with rPaIL-6. Moreover, the mRNA levels of TGF-β and IL-10 showed no significant change in ayu MO/MΦ treated with rPaIL-6 (Fig. 6).

3.6. Effect of PaIL-6Rα and PaIL-6Rβ on cytokine mRNA expression of MO/MΦ

We first obtained the PaIL-6Rα and PaIL-6Rβ gene sequences from the transcriptome data; these gene sequences were identified in ayu. Phylogenetic tree analysis grouped teleost PaIL-6Rα and PaIL-6Rβ together in a cluster, respectively, distinct from the mammal PaIL-6Rα and PaIL-6Rβ cluster (Supplementary Fig. 1 and Supplementary Fig. 2). Then, we used PaIL-6Rα siRNA or PaIL-6Rβ siRNA to explore whether they affect the mRNA expression of cytokines in MO/MΦ upon incubation with rPaIL-6. We assessed the transfection efficiency for PaIL-6Rα siRNA and PaIL-6Rβ siRNA. The PaIL-6Rα mRNA level in MO/MΦ treated with PaIL-6Rα siRNA was 23.04% of that of the control (treated

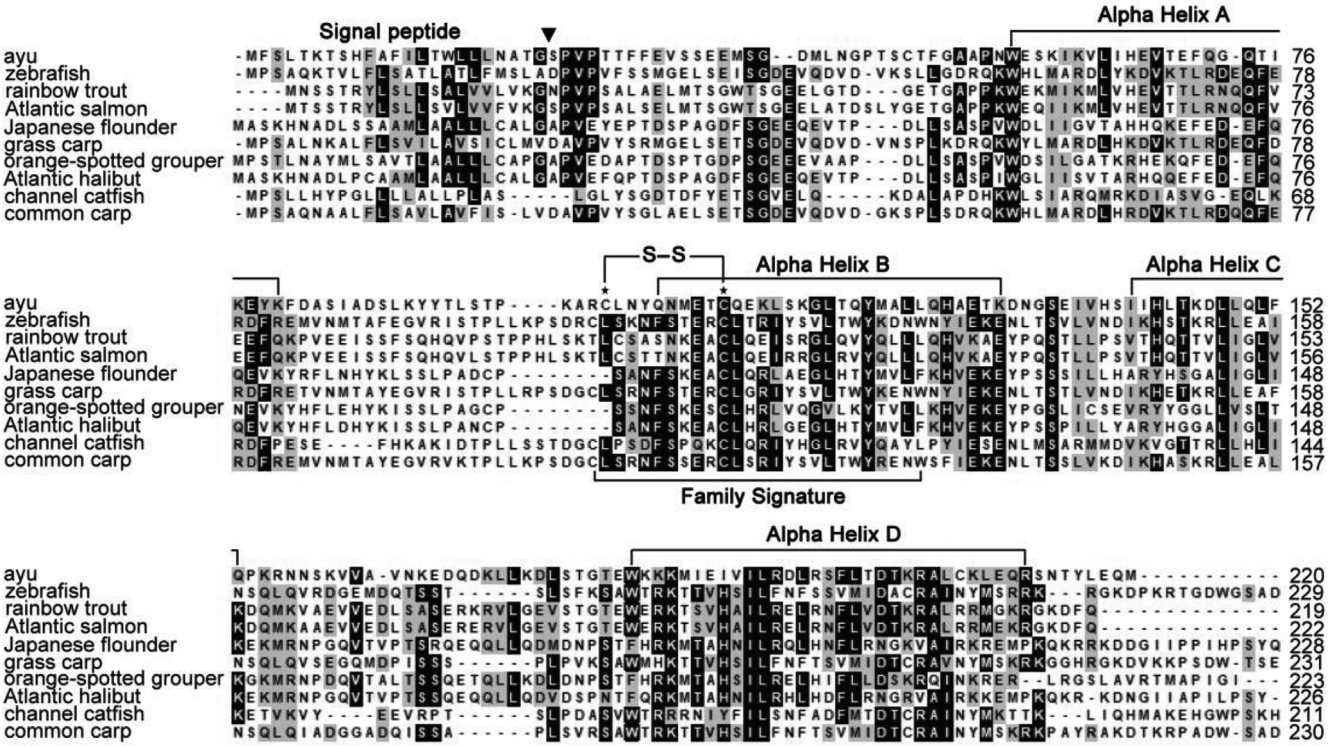

Fig. 1. Multiple alignment of the amino acid sequences of PaIL-6 with other vertebrate IL-6 proteins. Identical and similar residues identified using CLUSTALW are highlighted in black and grey, respectively; alignment gaps are denoted as"-." The threshold for shading is > 60%. The inverted triangle shows the cleavage site of the PaIL-6 signal peptide. The location of the four predicted alpha-helices (A–D) are indicated above the sequence. The family signature is underlined; the positions of the conserved putative disulfide bridges are also shown with S–S. GenBank accession numbers of amino acid sequences are listed in Supplementary Table 1.

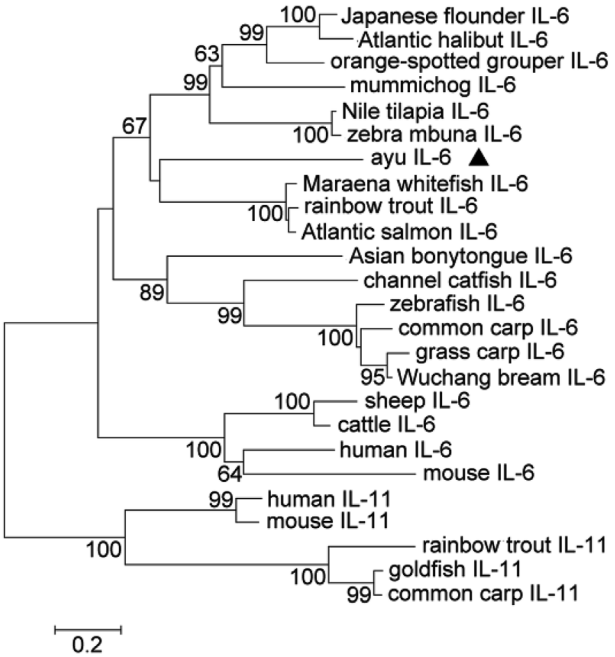

Fig. 2. Phylogenetic tree analysis of IL-6 amino acid sequences. The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping (1000 replicates; shown only when > 60%). The scale bar represents the number of substitutions per base position. The GenBank accession numbers of the sequences used are listed in Supplementary Table 1.

with scrambled siRNA) at 48 h. The PaIL-6Rα mRNA level in MO/MΦ treated with PaIL-6Rα siRNA was 15.85% of that of the control at 96 h (Fig. 7A). The PaIL-6Rβ mRNA level in MO/MΦ treated with PaIL-6Rβ siRNA was 19.04% of that of the control at 48 h. The PaIL-6Rβ mRNA level in MO/MΦ treated with PaIL-6Rβ siRNA was 16.08% of that of the control at 96 h (Fig. 7B). Next, we measured the production of pro-inflammatory cytokines in MO/MΦ. RT-qPCR analysis showed that the mRNA expression of TNF-α and IL-1β was significantly down-regulated in the PaIL-6Rβ siRNA-treated group. However, the mRNA expression of TNF-α and IL-1β were not changed in the PaIL-6Rα siRNA-treated group. These results indicate that IL-6Rβ mediates the effect of IL-6 on ayu MO/MΦ.

3.7. Effect of PaIL-6 and PaIL-6Rβ on phosphorylation levels of PaSTAT1 in MO/MΦ

STAT1 is essential for the function of IL-6. First, we assessed the transfection efficiency for PaIL-6 siRNA. The PaIL-6 mRNA level in MO/MΦ treated with PaIL-6 siRNA was 15.82% of that of the control (treated with scrambled siRNA) at 48 h and 16.17% of that of the control at 96 h (Fig. 8A). Then, we used PaIL-6 siRNA to explore whether PaIL-6 affects the phosphorylation levels of PaSTAT1 proteins in MO/MΦ, upon LPS stimulation. Western blot analysis showed that LPS significantly the phosphorylation level of PaSTAT1 in MO/MΦ. However, the phosphorylation level of PaSTAT1 was significantly down-regulated after treatment with PaIL-6 siRNA (Fig. 8B). Moreover, we explored whether PaIL-6Rβ affected the phosphorylation level of PaSTAT1 in MO/MΦ upon incubation with rPaIL-6. Western blot analysis showed that rPaIL-6 significantly upregulated the phosphorylation level of PaSTAT1 in MO/MΦ in the control group. However, the phosphorylation level of PaSTAT1 was significantly down-regulatedin the PaIL-6Rβ siRNA-treated group (Fig. 8C).

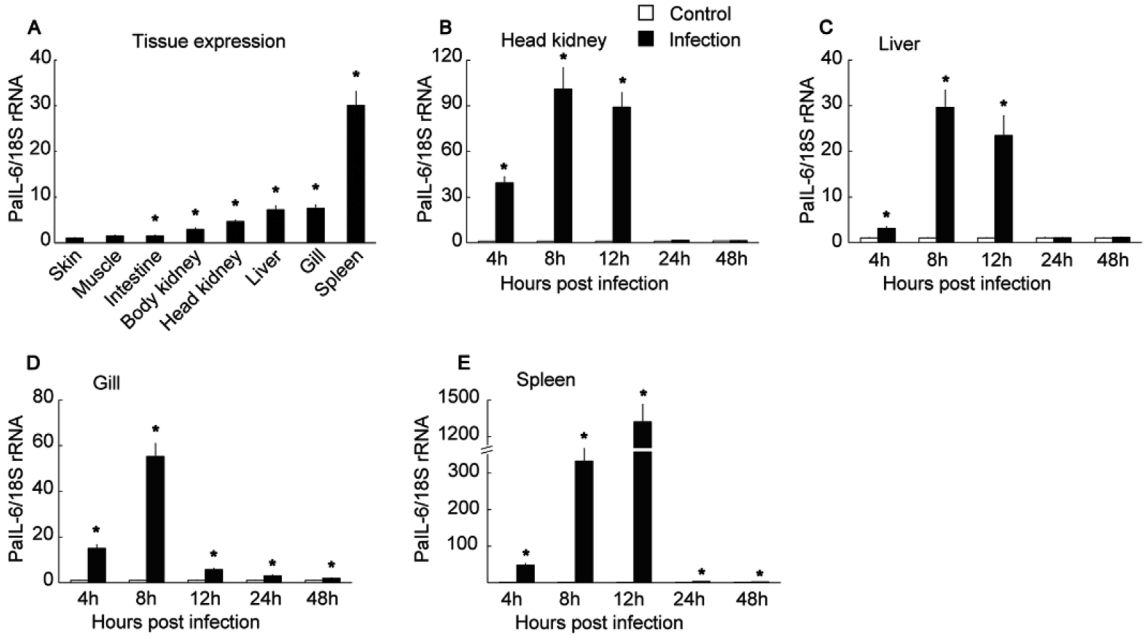

Fig. 3. RT-qPCR analysis of PaIL-6 mRNA expression in different tissues. (A) PaIL-6 mRNA expression in healthy ayu tissues. The PaIL-6 expression level of skin was set to 1. (B–E) PaIL-6 mRNA expression at different time points following *V. anguillarum* infection. The PaIL-6 expression level of control at 4 hpi was set to 1 in every graph. Fish were injected intraperitoneally with *V. anguillarum* for 4, 8, 12, 24 and 48 h. The mRNA transcript levels were normalized to the 18S rRNA level. Data are expressed as the mean ± SEM. n = 5. $^*P < 0.05$.

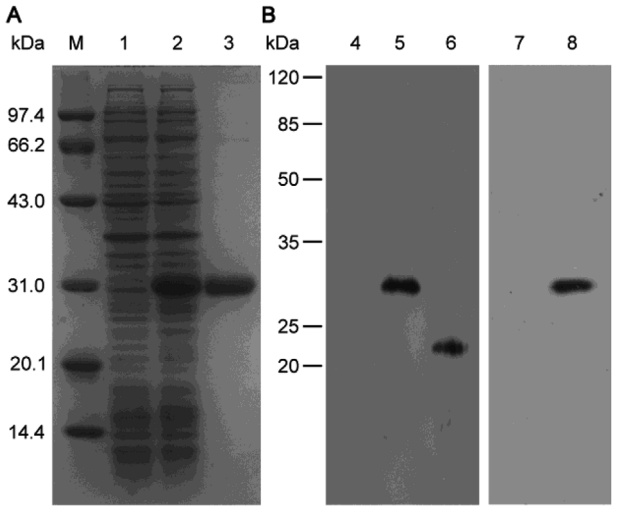

Fig. 4. Prokaryotic expression and Western blot analysis of PaIL-6. (A) SDS-PAGE analysis of the prokaryotic expression of PaIL-6. Lane M: protein marker; 1 and 2: protein from BL21 (DE3) transformed with the pET-28a-PaIL-6 plasmid before and after IPTG induction, respectively; 3: purified recombinant PaIL-6. (B) Western blot analysis of the specificity of the antibody for PaIL-6. 4: negative control; 5: purified recombinant PaIL-6; 6: The supernatant of *V. anguillarum*-infected ayu MO/MΦ; 7: purified recombinant PaIL-8 (PaCXCL-8); and 8: purified recombinant PaIL-6.

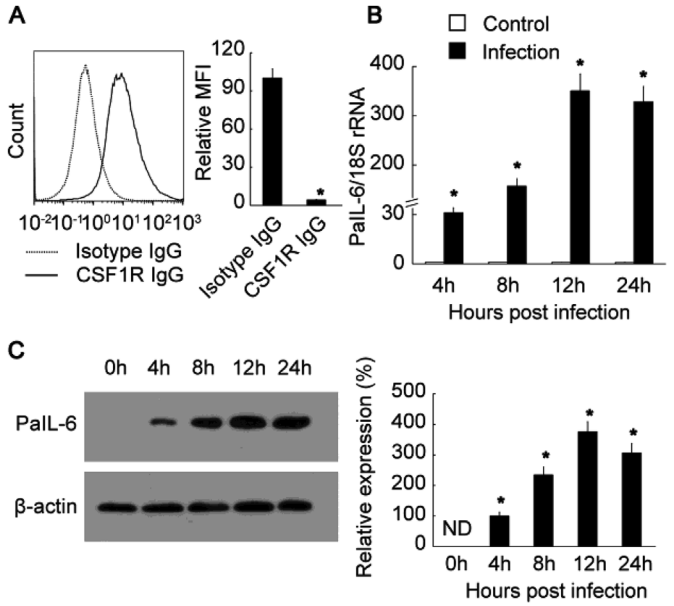

Fig. 5. Alteration of PaIL-6 expression in *V. anguillarum*-infected MO/MΦ. (A) Flow cytometric analysis of the PaCSF1R$^+$ cells using anti-PaCSF1R-Ex IgG or Isotype IgG; a total of 10000 events were analyzed. (B) PaIL-6 mRNA expression in ayu MO/MΦ. RT-qPCR was performed to analyze changes in PaIL-6 mRNA in MO/MΦ. PaIL-6 transcript levels were normalized to that of 18S rRNA. The PaIL-6 expression level of control at 4 hpi was set to 1. Data are expressed as mean ± SEM; n = 5, $^*P < 0.05$. (C) PaIL-6 protein expression in the supernatant of ayu MO/MΦ. Western blot analysis was performed to analyze the variation in PaIL-6 protein expression in the supernatant of MO/MΦ. Histogram displaying changes in the relative band intensity of PaIL-6 in samples collected at 0, 4, 8, 12, and 24 hpi. PaIL-6 protein expression was normalized to that of β-actin. The representative blots of three independent experiments are shown. Data are expressed as mean ± SEM; $^*P < 0.05$.

3.8. Effect of PaIL-6 and PaIL-6Rβ on the phagocytosis and bacterial killing of MO/MΦ

We further explored the effect of rPaIL-6 or PaIL-6Rβ siRNA on phagocytosis and bacterial killing activity of MO/MΦ. The phagocytosis of FITC-DH5α by rPaIL-6-pre-treated MO/MΦ was significantly higher than that in the control group (Fig. 9A and B), which was consistent with the cytokine expression results; however, the phagocytosis activity of the PaIL-6Rβ siRNA-treated MO/MΦ was lower than that in the control group (Fig. 9A and B). In addition, direct measurement of

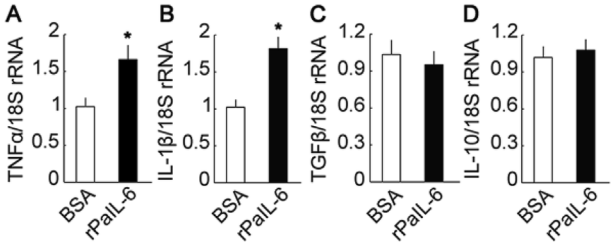

Fig. 6. Effect of rPaIL-6 on the mRNA expression of TNF-α, IL-1β, TGF-β, and IL-10 in MO/MΦ. The BSA-treated group was used as control. MO/MΦ was treated with rPaIL-6 for 24 h. The levels of TNF-α (A), IL-1β (B), TGF-β (C), and IL-10 (D) transcripts were determined. The mRNA transcript levels were normalized to Pa18S rRNA level. Data are expressed as mean ± SEM; n = 5, $^*P < 0.05$.

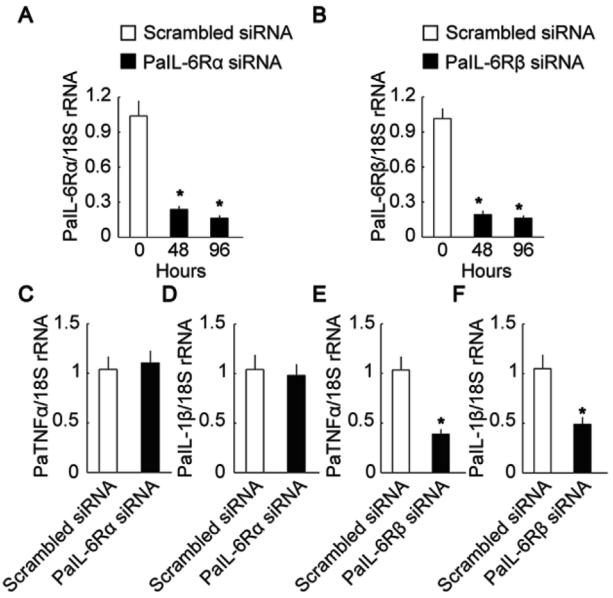

Fig. 7. Effect of PaIL-6Rα or PaIL-6Rβ on pro-inflammatory cytokine expression in ayu MO/MΦ. PaIL-6Rα or PaIL-6Rβ siRNA was transfected into MO/MΦ. The scrambled siRNA-transfected MO/MΦ group was used as control. MO/MΦ were treated with PaIL-6Rα or PaIL-6Rβ siRNA or scrambled siRNA to knockdown the expression of PaIL-6Rα or PaIL-6Rβ, after which cells were incubated with rPaIL-6 for 24 h. (A) The mRNA expression of PaIL-6Rα in MO/MΦ treated with PaIL-6Rα siRNA. (B) The mRNA expression of PaIL-6Rβ in MO/MΦ treated with PaIL-6Rβ siRNA. (C and D) The mRNA expression of TNF-α and IL-1β in MO/MΦ treated with PaIL-6Rα siRNA and further incubated with rPaIL-6. (E and F) mRNA expression of TNF-α and IL-1β in MO/MΦ treated with PaIL-6Rβ siRNA incubated with rPaIL-6. The mRNA transcript levels were normalized to Pa18S rRNA level. Data are expressed as mean ± SEM. n = 5. $^*P < 0.05$.

intracellular *V. anguillarum* CFUs in ayu MO/MΦ showed that the bacterial survival rate in the rPaIL-6-treated group was lower than that in the control group (Fig. 9C and D). However, the bacterial survival rate in the PaIL-6Rβ siRNA-treated group was higher than that in the rPaIL-6 treated group (Fig. 9C and D).

4. Discussion

IL-6 plays an important role in the vertebrate immune system. In the present study, we cloned the cDNA sequence of IL-6 from ayu and detected the mRNA and protein of IL-6 from ayu MO/MΦ. The sequence comparisons showed that, whereas PaIL-6 was grouped with the IL-6 of other fish, the identity and similarity of PaIL-6 was relatively low compared to its counterparts. However, the protein structure revealed that PaIL-6 shares high structural homology with other reported IL-6

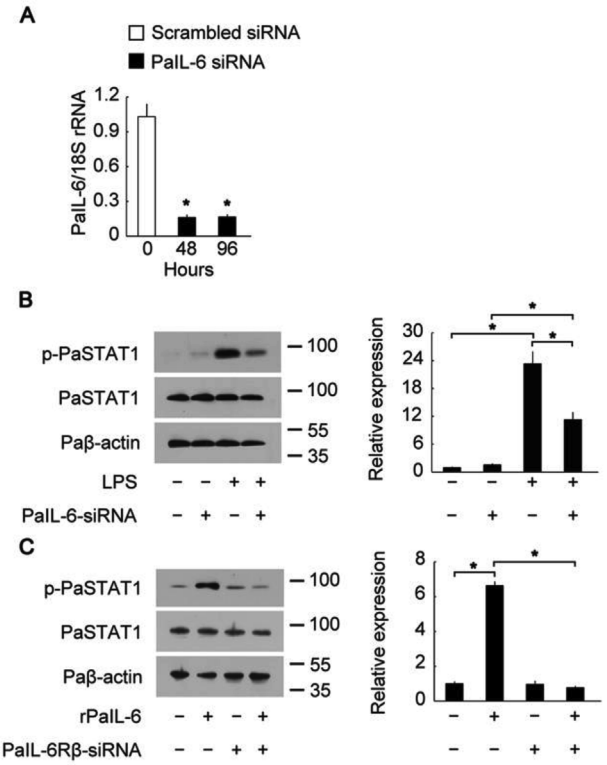

Fig. 8. Effect of PaIL-6 on phosphorylation level of PaSTAT1. (A) The mRNA expression of PaIL-6 in MO/MΦ treated with PaIL-6 siRNA. (B) MO/MΦ were transfected with PaIL-6 siRNA or scrambled siRNA before stimulation with LPS. (C) MO/MΦ were transfected with PaIL-6Rβ siRNA or scrambled siRNA before stimulation with rPaIL-6. Cell lysates were then subjected to Western blot analysis to compare the phosphorylation levels of PaSTAT1. Data are expressed as mean ± SEM. n = 3. $^*P < 0.05$.

[26,27]. PaIL-6 has a signal peptide and four long α-helices (A, B, C, and D), which are also observed in other vertebrates. Therefore, in this study, we characterized a novel IL-6 gene in teleosts.

Tissue distribution analysis showed that PaIL-6 expression was high in the head kidney, liver, gill, and, especially in the spleen, which was in accordance with the findings in the blunt snout bream (*Megalobrama amblycephala*) [28], and Japanese pufferfish (*Fugu rubripes*) [29]. In bacterial challenge test, we found that PaIL-6 mRNA was up-regulated in the head kidney, liver, gill, and spleen upon *V. anguillarum* infection, which was in accordance with the findings in the orange-spotted grouper (*Epinephelus coioides*) [30] and blunt snout bream (*Megalobrama amblycephala*) [31]. Furthermore, both mRNA and protein levels of PaIL-6 were significantly up-regulated in MO/MΦ after infection with live *V. anguillarum*. Collectively, our results provide evidence that PaIL-6 is tightly associated with bacterial challenges and has great importance in host defense in teleosts.

Inflammatory response is a critical factor in the defense mechanism of teleosts [32,33]. Cytokines such as TNF-α, IL-1β, IL-10, and TGF-β are a broad and loose category of small proteins that play an important role in inflammatory response [4,34,35]. Through the cascades of intracellular signaling, cytokines can alter gene expression and that of related transcription factors to influence the production of other cytokines [4]. In mammals and some teleosts, the ability of MO/MΦ to produce TNF-α and IL-1β was decreased after treatment with IL-6 in response to lipopolysaccharide [11,36–38]. These findings indicate that IL-6 possibly exerts an anti-inflammatory effect, with or without infection, in MO/MΦ. In our study, rPaIL-6 treatment increased the mRNA expression of pro-inflammatory cytokines; however, it did not significantly change the levels of anti-inflammatory cytokines in ayu MO/MΦ. Similarly, a study in large yellow croaker (*Larimichthys crocea*)

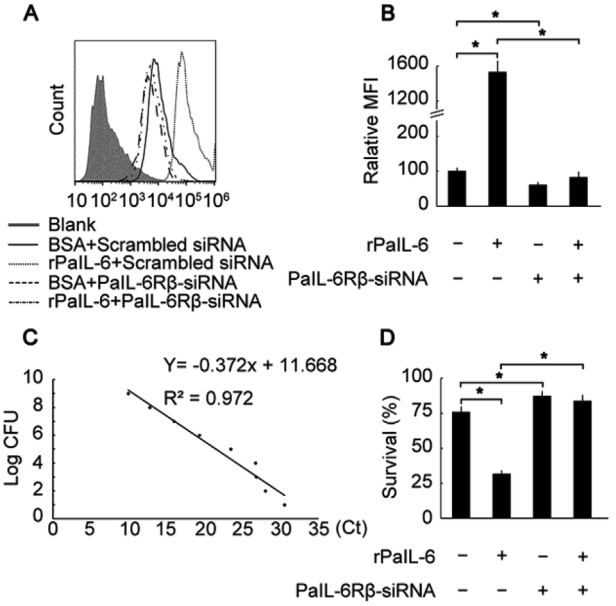

Fig. 9. Effect of PaIL-6 on phagocytosis and bacterial killing in ayu MO/MΦ. (A and B) Ayu MO/MΦ were pre-treated with BSA, rPaIL-6, or PaIL-6Rβ siRNA. Thereafter, FITC-DH5α was added at an MOI of 10 and incubated for an additional 30 min. The phagocytosis of FITC-DH5α was determined by flow cytometric analysis. MFI is presented asa fold change over the value for the BSA-treated group, which was assigned a unit of 100. (C and D) Effect of rPaIL-6 or PaIL-6Rβ on the bacterial killing of MO/MΦ. MO/MΦ were infected with live *V. anguillarum* after treatment with BSA, rPaIL-6, or PaIL-6Rβ siRNA. The viability of *V. anguillarum* (MOI = 10) was determined by RT-qPCR. Data are expressed as mean ± SEM; n = 5, $^*P < 0.05$.

showed that incubation with recombinant IL-6 fusion protein significantly increased the expression of TNF-α in the head-kidney cells; however, it did not affect IL-1β expression significantly [27]. In flounder (*Paralichthys olivaceus*), vaccination with rOmpV plus rIL-6 or pcIL-6 after challenge with *E. tarda* resulted in up-regulationof TNF-α and IL-1β expression [39]. In grass carp (*Ctenopharyngodon idella*), treatment with recombinant IL-6 significantly increased the expression of TNF-α and IL-1β in head-kidney leucocytes [40]. Our results revealed that PaIL-6 may play a pro-inflammatory role in resting MO/MΦ in ayu. It may be envisaged that the different roles of IL-6 in MO/MΦ may be determined by the differences in species.

We further analyzed the underlying mechanism behind the pro-inflammatory role of ayu IL-6 in MO/MΦ. In mammalian resting MO/MΦ, the mRNA expression of IL-6Rβ is only 40% higher than that of IL-6Rα [41]. However, in ayu, the mRNA expression of IL-6Rβ was approximately four times higher than that of IL-6Rα (data not shown). The pro-inflammatory role played by IL-6 in ayu MO/MΦ may be attributed to the differential expression of receptors. In our study, RNAi knockdown data showed that the expression of pro-inflammatory cytokines in ayu MO/MΦ was related to the expression of PaIL-6Rβ, but not PaIL-6Rα. Therefore, it may be envisaged that PaIL-6Rβ is required for regulating the pro-inflammatory response of IL-6. However, it is necessary to further confirmthe pro-inflammatory association of IL-6Rβ and IL-6 in other fish.

Owing to the wide expression of IL-6Rβ on cells, IL-6 is thought to signal ubiquitously via IL-6Rβ complex formation with membrane-bound IL-6Rα [1]. IL-6Rβ signaling primarily induces p-STAT1, which is an important transcription factor that mediates the expression of a variety of genes in response to cell stimuli and plays a key role in MO/MΦ polarization [20]. We found that both the autocrine and exogenous PaIL-6 could activate the phosphorylation of PaSTAT1 in ayu MO/MΦ. In Japanese eel (*Anguilla japonica*) and Malabar grouper (*Epinephelus malabaricus*), STAT1 proteins could be activated by LPS, which indicates that the function of STAT1 is conservative in fish [42,43]. It is possible that PaIL-6 plays a pro-inflammatory function via PaIL-6Rβ by inducingthe phosphorylation of PaSTAT1.

Phagocytosis and killing of bacteriaare two important components of MO/MΦ for defending against pathogenic bacterial infection [44,45]. The teleost fish MO/MΦ have been extensively studied; the results indicate that they rapidly kill pathogens through phagocytosis [46]. Related research shows that pro-inflammatory cytokines can enhance the phagocytic activity of MO/MΦ [47]. In mice, IL-6 is essential for MO/MΦ to phagocytose virus-infected cells, and mice deficient in IL-6 have decreased survival and more severe lung injury [48]. In addition, the pro-inflammatory properties of IL-6 have been shown to enhance bacterial clearance during infection [49]. However, the effect of IL-6 on phagocytosis and killing of bacteria in fish has not been reported. The results of our study support the notion that both PaIL-6 and PaIL-6Rβ havea positive effect on phagocytosis and bacterial digestion by ayu MO/MΦ, which coincides with the reports in mammals.

In conclusion, we characterized an IL-6 gene in ayu. The transcripts of PaIL-6 were dramatically up-regulated in various ayu immune tissues upon bacterial infection. We showed that the function of PaIL-6 in ayu MO/MΦ is to improve the production of pro-inflammatory cytokines, phagocytosis, and bactericidal activity. Additionally, we proved that the functions of PaIL-6 were mediated by PaIL-6Rβ. This work broadens our knowledge of the functions and mechanisms of IL-6 in fish, and will help in the control of various fish diseases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.049.

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