Fish and Shellfish Immunology 95 (2019) 93–104

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Tongue sole (*Cynoglossus semilaevis*) interleukin 10 plays a negative role in the immune response against bacterial infection

Xue-peng Li[a,b,*], Shuai Jiang[a,b], Bin Sun[a,b,c], Jian Zhang[a,b,**]

[a] CAS Key Laboratory of Experimental Marine Biology, CAS Center for Ocean Mega-Science, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China
[b] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China
[c] University of Chinese Academy of Sciences, Beijing, 100039, China

ARTICLE INFO

Keywords:
Interleukin-10
Cynoglossus semilaevis
Peripheral blood leukocytes
Immune defense
Bacterial infection

ABSTRACT

Interleukin-10 (IL-10) is a pleiotropic cytokine and plays a crucial role in immunity. In the current study, we examined the expression patterns and biological functions of tongue sole *Cynoglossus semilaevis* IL-10 (CsIL-10). CsIL-10 is composed of 186 amino acid residues and shares 46.3%–71.7% identities with other teleost IL-10. *Csil-10* expression occurred in multiple tissues and was regulated by bacterial infection. Recombinant CsIL-10 (rCsIL-10) in the form of a dimer bound to a wide range of bacterial species but did not affect bacterial growth. rCsIL-10 could interact with peripheral blood leukocytes (PBL) and significantly reduce the phagocytic activity, ROS production, and apoptosis of PBL. When injected *in vivo*, rCsIL-10 significantly suppressed the expression of proinflammatory cytokines and promoted bacterial dissemination in tongue sole tissues. Consistently, knockdown of *Csil-10* significantly inhibited bacterial infection in tongue sole. Taken together, these results indicate that CsIL-10 plays a negative regulatory role in the immune response against bacterial infection.

1. Introduction

Cytokines are produced by many different immune cells and non-immune cells and play important roles in various immune responses [1]. On the basis of their functions, cytokines are classed into different families such as interleukins (ILs), lymphokines, interferons and chemokines [2]. Interleukins are one of the most crucial families of cytokines and contain a large amount of members. To date, many interleukin family members have been reported, such as IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26 [3]. Several IL-10 viral homologues have also been reported, which mimic the activities of host IL-10 and regulate the immune system of the host to viral infection [4,5].

IL-10 was originally described as a cytokine synthesis inhibitory factor (CSIF) secreted by T-helper (Th) 2 cells [6]. In mammals, the production of IL-10 may be dependent on the stimuli, cell/tissue types and immune processes [7]. IL-10 functions as a homodimer and exerts its activity through the receptor complex IL-10R1/IL-10R2, resulting in the activation of the Jak-Stat signaling pathway [8,9]. Presently, IL-10 is known as a multifunctional cytokine that is indispensable for regulating various immune responses, in particular inhibiting and terminating inflammatory responses, which enhance the host to clear pathogenic microorganism with minimal damage to host tissues [9]. On the other hand, IL-10 is also a co-stimulator of activation of B cells, mast cells, thymocytes and T regulatory cells, and exhibits immunostimulatory properties that give rise to eliminate detrimental particles with limited inflammation [10]. The balance between the immunosuppressive and immunostimulatory effects of IL-10 is greatly affected by the dominant cells function that determine a specific immune phenomenon [11]. The important effect of IL-10 in immune regulation is demonstrated by the study with IL-10 knockout mice, which showed that despite of being raised in a pathogen free environment, IL-$10^{-/-}$ mice exhibited severe enterocolitis in response to their natural enteric flora and displayed greatly polarized Th1 bias with excessive production of pro-inflammatory mediators [12]. Other studies showed that inhibition of IL-10 bioactivity enhanced effective elimination of *Mycobacterium avium* in mice [13]. In general, low and high levels of IL-10 increase resistance and susceptibility, respectively, to intracellular pathogens including bacteria, viruses, and protozoa [9].

In teleost, studies on IL-10 have been reported in a number of species including fugu [14] rainbow trout [15], zebrafish [16,17],

* Corresponding author. CAS Key Laboratory of Experimental Marine Biology, CAS Center for Ocean Mega-Science, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China.
** Corresponding author. CAS Key Laboratory of Experimental Marine Biology, CAS Center for Ocean Mega-Science, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China.
E-mail addresses: lixuepeng14@mails.ucas.ac.cn (X.-p. Li), zhangjian@qdio.ac.cn (J. Zhang).

https://doi.org/10.1016/j.fsi.2019.10.023
Received 23 July 2019; Received in revised form 9 October 2019; Accepted 9 October 2019
Available online 13 October 2019
1050-4648/

goldfish [18], grass carp [19,20], and Indian Major Carp [21,22]. Fish IL-10 generally are homologous to mammalian IL-10 in structure and some functions, however, they differ from each other in expression patterns [14–16,18,19,21]. In fish, expression of *il-10* is known to be modulated by pathogen infection and LPS stimulation [15,16,18–21,23–28], and some IL-10 exhibit a negative effect on the respiratory burst, phagocytosis, and bactericidal activity in different cell systems [17,18,22,29,30].

Half smooth tongue sole (*Cynoglossus semilaevis*) is a flatfish species farmed widely in China. Tongue sole culture is commonly affected by diseases caused by bacterial pathogens such as *Vibrio* and *Edwardsiella* spp. In the present study, we examined the expression patterns and biological function of tongue sole IL-10 (named CsIL-10). We found that CsIL-10 negatively regulates the immune response of host cells and tissues and plays an important role in bacterial infection.

2. Materials and methods

2.1. Fish

Healthy tongue sole were purchased from a commercial fish farm in Shandong Province, China. These fish were approximately either 5 month old with an average body length of 15.5 cm and an average weight of 15.2 g, or 12 month old with an average body length of 43 cm and an average weight of 505 g. Fish maintenance was carried out as reported previously [31]. Briefly, fish were maintained at 20 °C in aerated seawater fed daily with commercial dry pellets; before the experiment, the fish were verified to be pathogen free as described previously [32]. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) at the dose of 0.1 g/L [33]. The ethics of the animal study was approved by the Ethics Committee of Institute of Oceanology, Chinese Academy of Sciences.

2.2. Sequence analysis

The CsIL-10 sequence (GenBank accession no. XP_008318394.1) was achieved from National Center for Biotechnology Information (NCBI). Domain search, theoretical molecular mass and isoelectric point prediction, multiple sequence alignment, and phylogenetic analysis were performed as described previously [34].

2.3. Quantitative real time reverse transcription PCR (qRT-PCR)

To examine *Csil-10* expression under normal condition, different tissues (intestine, head kidney, blood, liver, spleen, heart, gill, muscle, and brain) were taken aseptically from tongue sole (5 fish) and 30 mg of each tissues were used for total RNA extraction with EZNA Total RNA Kit (Omega Bio-tek, Doraville, USA). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). To examine *Csil-10* expression in response to bacterial infection, *Edwardsiella tarda* and *Vibrio harveyi* [35,36] were cultured in Luria-Bertani broth (LB) medium at 28 °C to an OD_{600} of 0.8; the cells were washed with PBS and resuspended in PBS to 2×10^7 CFU/ml and 5×10^7 CFU/ml for *E. tarda* and *V. harveyi*, respectively. Tongue sole were divided randomly into three groups (20/group) and injected intraperitoneally (i.p.) with 50 μl E. *tarda*, *V. harveyi* or PBS. At 6 h, 12 h, 24 h, and 48 h post-infection (hpi), head kidney, spleen, and blood (5 fish/group) were taken and used to examine *Csil-10* expression. qRT-PCR was performed as reported previously [37]. The expression level of *Csil-10* was analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with β-actin (ACTB) or 60S ribosomal protein L18a (RPL18a) as an internal reference [37]. The primers for qRT-PCR were listed in Table 1.

Table 1
Primers used in this study.

Primer	Sequences (5′→ 3′)[a]
CsIL-10-F	CTATTCAAGGCCATGGGCGA
CsIL-10-R	CAGAGGTCACTCGCGTTCTT
β-actin F	GCACGGTATTGTGACCAACTGG
β-actin R	CAGGGGAGCCTCTGTGAGC
RPL18a-F:	GAACCCTACCCCTCCTCTGT
RPL18a-R:	TACGAGAGTCGTAACGCAGC
CsIL-10-pF	GATATCATGGGACCCATGTGCACCAACCAG (EcoRV)
CsIL-10-pR	GATATCGGCAGAGGTCACTCGCGTTCTT (EcoRV)
NF-κB-F	ACCGTCGCTGAGCTAACAAA
NF-κB-R	GGGTCAAGCCCCATCCATAC
TNF-α-F	TGACCAACTCGGTGGAATGG
TNF-α-R	GGAACGACACCTGGCTGTAA
IL-1β-F	CCTTCCCACTTCCTGGCTAC
IL-1β-R	TGGCGTGTGCTGGCTTTTAT
IL8-F	ATTCCTGCCAGCTCTCACTG
IL8-R	CTTCACCCAAGGAGCCTCAG
IL6-F	TCCTGCGTGATGGCATAGTG
IL6-R	CTCATCAGGACGTCAGAGCC

[a] Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.

2.4. Recombinant protein preparation and antibody preparation

To prepare recombinant CsIL-10 (rCsIL-10), the expression plasmid pETCsIL-10 was constructed as follows. CsIL-10 was amplified by PCR with primers CsIL-10-pF and CsIL-10-pR (Table 1); the PCR products were ligated with the T-A cloning vector T-Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the CsIL-10-containing fragment, which was inserted into pET259 [38] at the SwaI site, resulting in plasmid pETCsIL-10, which expresses His-tagged rCsIL-10. For purification of rCsIL-10, *Escherichia coli* BL21 (DE3) (TransGen Biotech Beijing, China) was transformed with pETCsIL-10. For purification of His-tagged rTrx, BL21 (DE3) was transformed with the plasmid pET32a (Novagen, San Diego, USA). The transformants were cultured in LB medium at 37 °C to mid-logarithmic phase. Isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM. After growing at 16 °C for an additional 12 h, the cells were harvested by centrifugation, and His-tagged rCsIL-10 and rTrx proteins were purified using Ni-NTA agarose (QIAGEN, Valencia, USA) as recommended by the manufacturer. The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. Native electrophoresis was carried out as described previously [33], using NativeMARK™ Unstained Protein Standard (Invitrogen, Carlsbad, CA, USA). Mouse antibodies against rCsIL-10 and rTrx were prepared as reported previously [39].

2.5. Extracellular production of CsIL-10 by peripheral blood leukocytes (PBL)

Tongue sole PBL were prepared as reported previously [39]. To examine extracellular production of CsIL-10, PBL (1×10^7 cells) were treated with LPS (10 μg/ml) for 12 h and then harvested by centrifugation 600*g* for 10 min. The extracellular proteins were prepared by TCA-acetone precipitation method [40]. The whole-cell proteins were prepared by lysing the cells in RIPA lysis buffer (Beyotime, Shanghai, China), followed by centrifugation at 15000*g* for 30 min at 4 °C. Western bolt analysis was performed as described previously [41] with anti-rCsIL-10 antibody (prepared above) (1:1000 dilution) or mouse anti-β-actin antibody (ABclonal Biotech, Woburn, USA) (1:2000 dilution) as primary antibodies and horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibody (Tiangen, Beijing, China; 1:2000 dilution) as secondary antibody. The immunoblotted protein bands were visualized by using an enhanced chemiluminescence kit

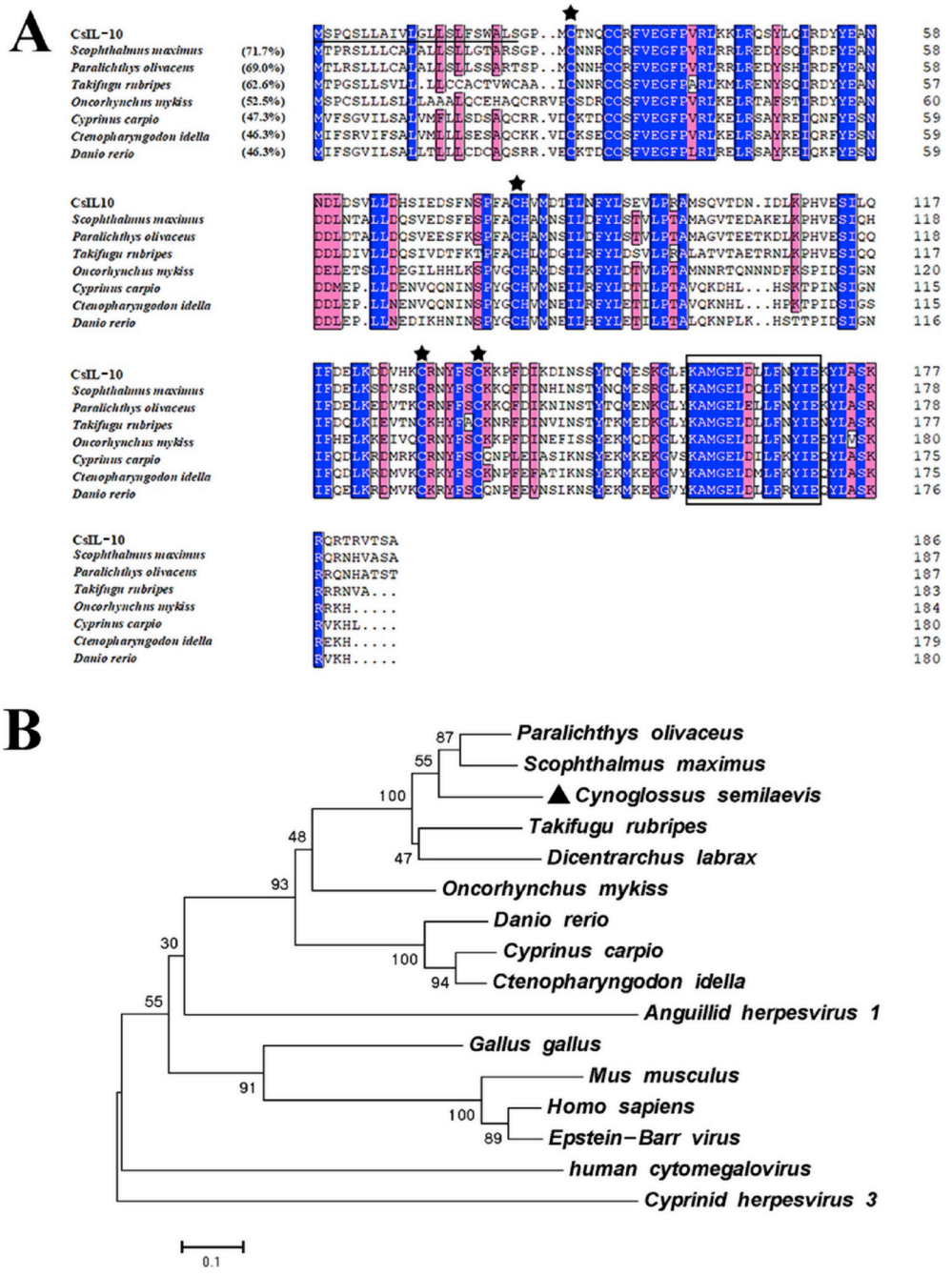

Fig. 1. Alignment of the sequences of IL-10 homologues (A) and phylogenetic analysis of CsIL-10 (B). (A) Dots denote gaps introduced for maximum matching. Numbers in brackets indicate overall sequence identities between CsIL-10 and the compared sequences. The consensus residues are in blue, the residues that are ≥75% identical among the aligned sequences are in pink, the IL-10 family signature motif is boxed, and the signal peptide is underlined. The cysteine residues involved in formation of disulphide bridge are labeled with star. (B) The phylogenetic tree was constructed based on the amino acid sequences of vertebrate and viral IL-10 homologues with MEGA 5.0 using the neighbor-joining method. Numbers beside the internal branches indicate bootstrap values based on 1000 replications. The GenBank accession numbers of the IL-10 sequences are as follows: *Paralichthys olivaceus*, AHX22595; *Scophthalmus maximus*, AWP03065; *Danio rerio*, AAW78362; *Takifugu rubripes*, CAD62446; *Oncorhynchus mykiss*, BAD20648; *Cyprinus carpio*, BAC76885; *Ctenopharyngodon idella*, AFH58708; *Homo sapiens*, NP_000563; *Mus musculus*, NP_034678; *Gallus gallus*, NP_001004414; *Cyprinid herpesvirus* 3, ABG42961; *Anguillid herpesvirus* 1, ADA57788; *human cytomegalovirus*, P17150; *Epstein-Barr virus*, P03180. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Beyotime, Shanghai, China).

2.6. Binding activity of rCsIL-10 to bacteria and PBL

The Gram-negative bacteria *E. tarda*, *Pseudomonas fluorescens*, and *V. harveyi* have been reported previously [35,36,42]. The Gram-positive bacteria *Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus aureus* were purchased from China General Microbiological Culture Collection Center (CGMCC); *Streptococcus iniae* has been reported previously [43]. Bacteria were cultured in LB medium to OD_{600} 0.8. Bacteria-protein interaction was determined by enzyme-linked immunosorbent assay (ELISA), Western blot, and immunofluorescence microscopy as reported previously [41]. For ELISA, bacteria were resuspended in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) to 10^8 CFU/ml; the bacterial suspension (100 μl/well) was added to ELISA plates (Sangon, Shanghai, China), and the plates were incubated at 4 °C overnight. After incubation, the plates were blocked with 5% skim milk powder and washed with PBST. rCsIL-10, rTrx, or PBS at various concentrations were added to the plates, followed by incubation at 22 °C for 2 h. The plates were washed and treated with anti-His antibody (Abcam, Cambridge, UK; 1:1000 dilution) and goat anti-mouse IgG-HRP antibody (Tiangen, Beijing, China; 1:2000 dilution). Color development was performed using TMB Kit (Tiangen, Beijing, China). The plate was read at 450 nm with a precision microplate reader (Molecular Devices, Toronto, Canada). Protein binding index is defined as follows: A_{450} of protein/A_{450} of PBS. To determine the effect of proteins on bacterial growth, bacteria were suspended in PBS to 1×10^6 CFU/ml and treated with rCsIL-10 (16 μg/ml), rTrx (16 μg/ml), or PBS; the mixtures were placed into in 96-well ELISA plates (100 μl/well). The plates were incubated at 28 °C, and bacterial growth was determined by measuring OD_{600} at every hour.

For Western blot, rCsIL-10 or rTrx was incubated with bacterial cells

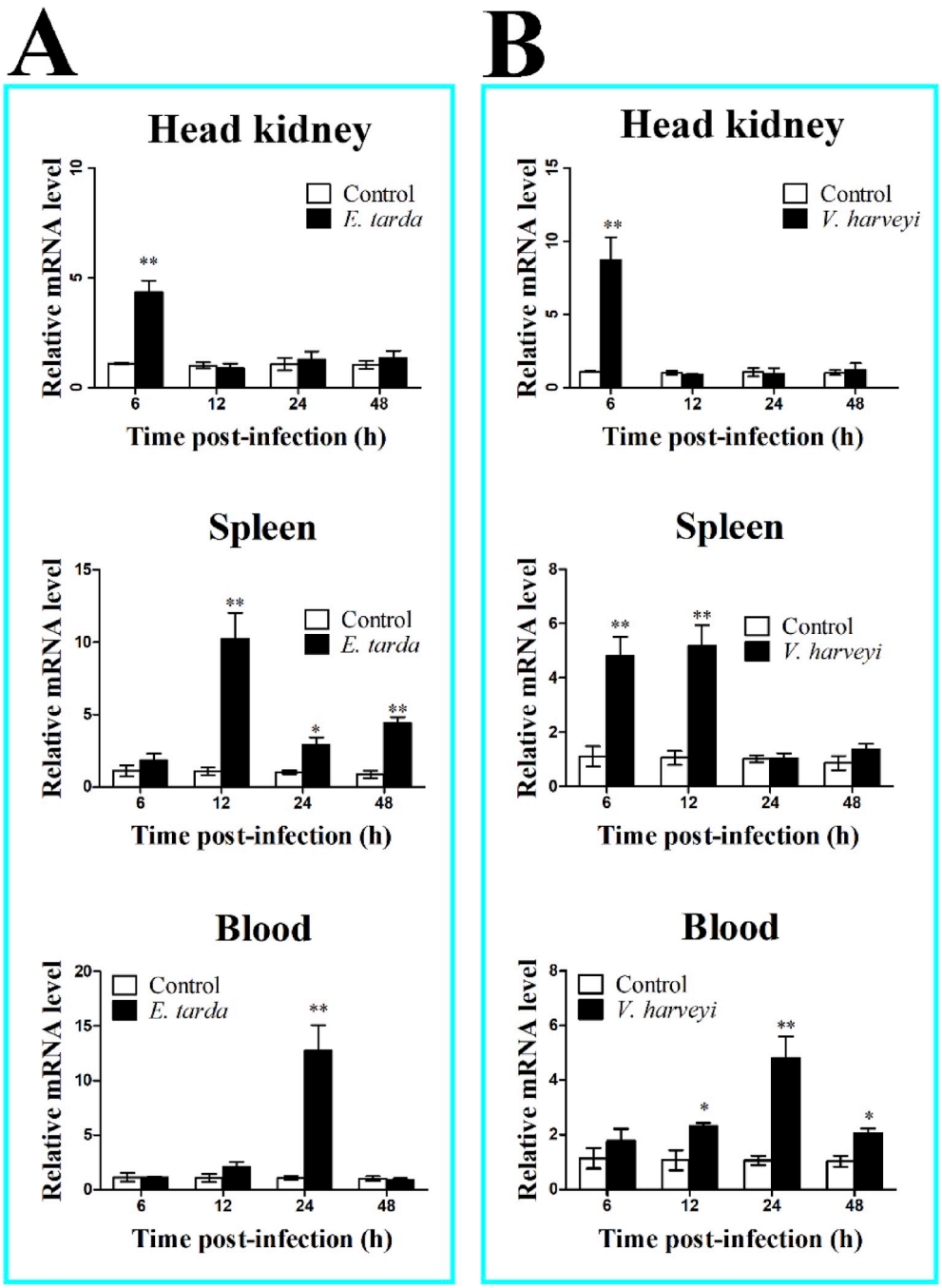

Fig. 2. *Csil-10* expression in fish tissues during bacterial infection. Tongue sole were infected with or without (control) *Edwardsiella tarda* (A) or *Vibrio harveyi* (B), and *Csil-10* expression in head kidney, spleen, and blood was determined by quantitative real time RT-PCR at various time points. In each case, the expression level of the control fish was set as 1. 60 fish were used in each experiment. Data are the means of three independent assays and presented as means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

(1×10^9 CFU/ml) at a final concentration of 100 μg/ml at room temperature for 1 h. The bacteria were harvested by centrifugation, washed four times with PBS, and resuspended in SDS-PAGE loading buffer. After boiling at 100 °C for 5 min, the samples were separated by SDS-PAGE. Western blot was performed as described above with mouse antibody against His-tag (1:1000 dilution) or mouse anti-rTrx antibody (1:1000 dilution).

For microscopic examination of rCsIL-10 binding to bacteria, *V. harveyi* was resuspended in PBS to 10^8 CFU/ml; rCsIL-10 or rTrx (16 μg/ml) was added to the bacterial cells, and the mixture was incubated at 22 °C for 1 h. The cells were treated with mouse anti-His monoclonal antibody (Abcam, Cambridge, UK) (1:1000 dilution) and FITC-labeled goat anti-mouse IgG (Abcam, Cambridge, UK) (1:2000 dilution) as reported previously [44]. After washing with PBS, the cells were stained with 4, 6-diamino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, USA) and examined with a fluorescence microscope (E800; Nikon Corporation, Tokyo, Japan). For microscopic examination of rCsIL-10 binding to PBL, PBL were resuspended in PBS to 10^7 cells/ml, rCsIL-10 or rTrx (16 μg/ml) was added to the PBL, and the mixture was incubated at 22 °C for 1 h. The cells were treated first with mouse anti-His monoclonal antibody and then with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG as above. After washing with PBS, the cells were stained with DAPI and CM-DiI (Invitrogen, Carlsbad, USA), and then examined with a fluorescence microscope as above.

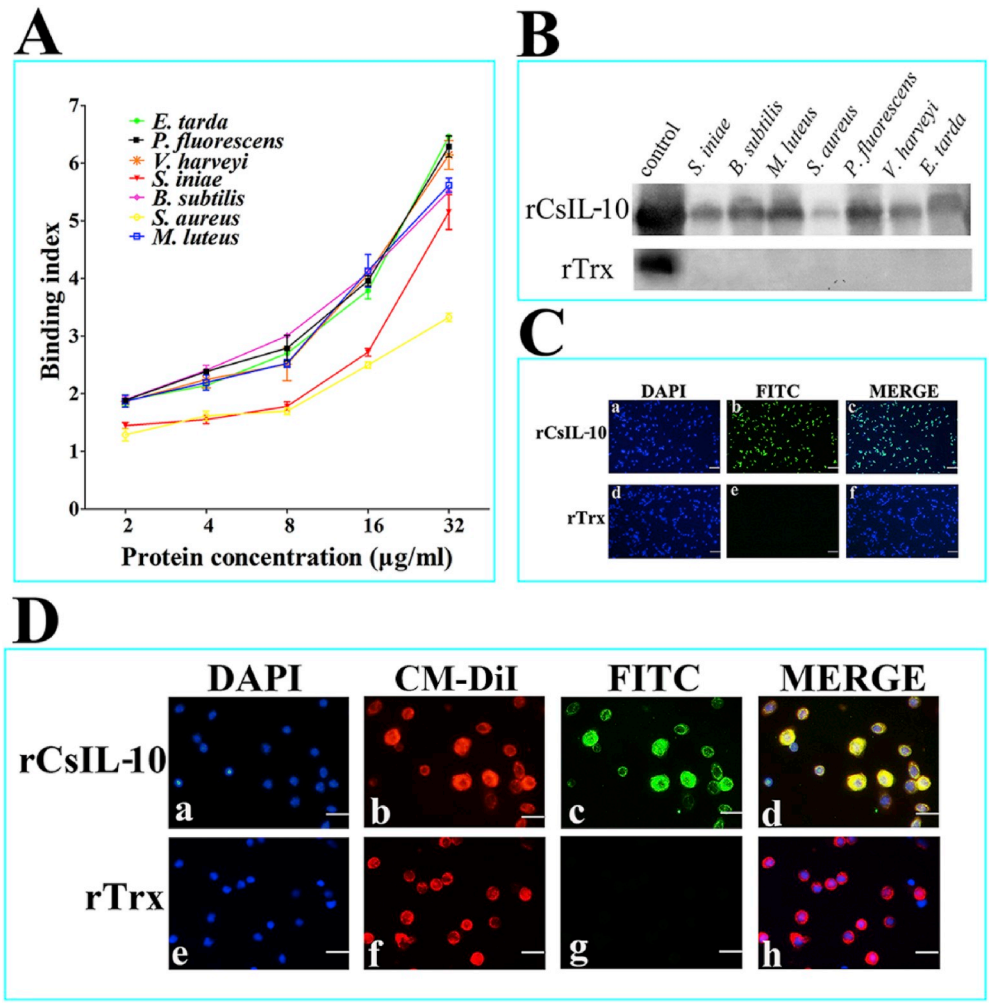

Fig. 3. Binding of rCsIL-10 to bacteria and peripheral blood leukocytes (PBL). (A and B) *Edwardsiella tarda*, *Pseudomonas fluorescens*, *Vibrio harveyi*, *Streptococcus iniae*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Micrococcus luteus* were incubated with or without (control) different concentrations of rCsIL-10 or rTrx, and bacteria-bound protein was determined by ELISA (A) or Western blot (B). In (B), rCsIL-10 was used as a positive control. Data are the means of three independent assays and presented as means ± SEM. (C) *V. harveyi* was incubated with rCsIL-10 (Ca and Cb) or rTrx (Cd and Ce); the cells were then treated with anti-His antibody and FITC-labeled secondary antibody and stained with DAPI. The cells were observed with a fluorescence microscope. Cc, a merged image of Ca and Cb; Cf, a merged image of Cd and Ce. (D) Tongue sole PBL were incubated with rCsIL-10 (Da to Dc) or rTrx (De to Dg); the cells were then treated with anti-His antibody and FITC-labeled secondary antibody and stained with DAPI and CM-DiI. The cells were observed with a fluorescence microscope. Dd, a merged image of Da to Dc; Dh, a merged image of De to Dg. Bar size, 10 μm.

2.7. Phagocytosis determined by fluorescence activated cell sorting (FACS)

Phagocytosis determined by FACS was performed as reported previously [34]. Briefly, *E. tarda* and *V. harveyi* were cultured as above and resuspended in 100 μg/ml of FITC (Tiangen, Beijing, China). After incubation at 37 °C for 2 h, the cells were collected by centrifugation and washed five times in PBS. The washed cells were resuspended in L-15 medium (Thermo Scientific HyClone, Beijing, China) to 1×10^8 cells/ml. One hundred microliters of FITC-labeled *E. tarda* or *V. harveyi* containing or not containing 16 μg rCsIL-10 or 16 μg rTrx was added to 1 ml PBL (1×10^6 cells) prepared above, and the mixture was incubated in the dark for 2 h. The PBL in the mixture were collected by centrifugation and washed with PBS for three times. To quench extracellular fluorescence, 1 ml 0.125% trypan blue in PBS was added to the cells. The cells were subjected to FACS analysis with a FACSAria II flow cytometer (BD Biosciences, Heidelberg, Germany). The data were analyzed with the FlowJo software Version 7.6.1 (Tree Star Inc., Ashland, OR, USA). The assay was repeated three times.

2.8. Measurement of reactive oxygen species (ROS)

To measure ROS production, the method reported previously [45] was adopted. Briefly, PBL were incubated at 22 °C for 30 min with 10 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Beyotime, Shanghai, China) for ROS quantification. PBL were washed in PBS (5×10^7 cells/ml), treated with LPS (10 μg/ml) at 22 °C for 15 min, and then mixed with rCsIL-10 (16 μg/ml) or rTrx (16 μg/ml) in the presence or absence of *V. harveyi* (5×10^7 CFU/ml) or *E. tarda* (5×10^7 CFU/ml). LPS treatment was to increase the ROS production, because both *E. tarda* and rCsIL-10 inhibited ROS production, so the ROS level would be too low to be detected without LPS pre-stimulation. The control cells were untreated. The cells were seeded in a black 96-well plate (5×10^6 cells/well), and incubated at 22 °C for 2 h, 3 h, and 4 h. After incubation, ROS was measured as relative fluorescence units (RFU) at 488 nm excitation and 525 nm emission using a fluorescence spectrophotometer (Infinite M1000, Tecan, Switzerland).

2.9. Apoptosis assay

Apoptosis determined by FACS was performed as reported previously [46]. Briefly, tongue sole PBL were distributed in 12-well tissue culture plates (5×10^6 cells/well) in L-15 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. PBL were treated with LPS (10 μg/ml) containing or not containing rCsIL-10 (16 μg/ml) or rTrx (16 μg/ml). The cells were incubated at 22 °C for 12 h or 24 h. After incubation, the cells were washed twice with cold PBS. The washed cells were treated with FITC-conjugated annexin V and propidium iodide (PI) by using annexin V-FITC and PI Cell Apoptosis Detection Kit (Majorbio Biotech, Shanghai, China) according to the manufacturer's instructions. The cells were then subjected to flow cytometry using a FACSAria II flow cytometer (BD Biosciences, Heidelberg, Germany). The data were analyzed with the FlowJo software Version 7.6.1 (Tree Star Inc., Ashland, OR, USA). The assay was repeated three times.

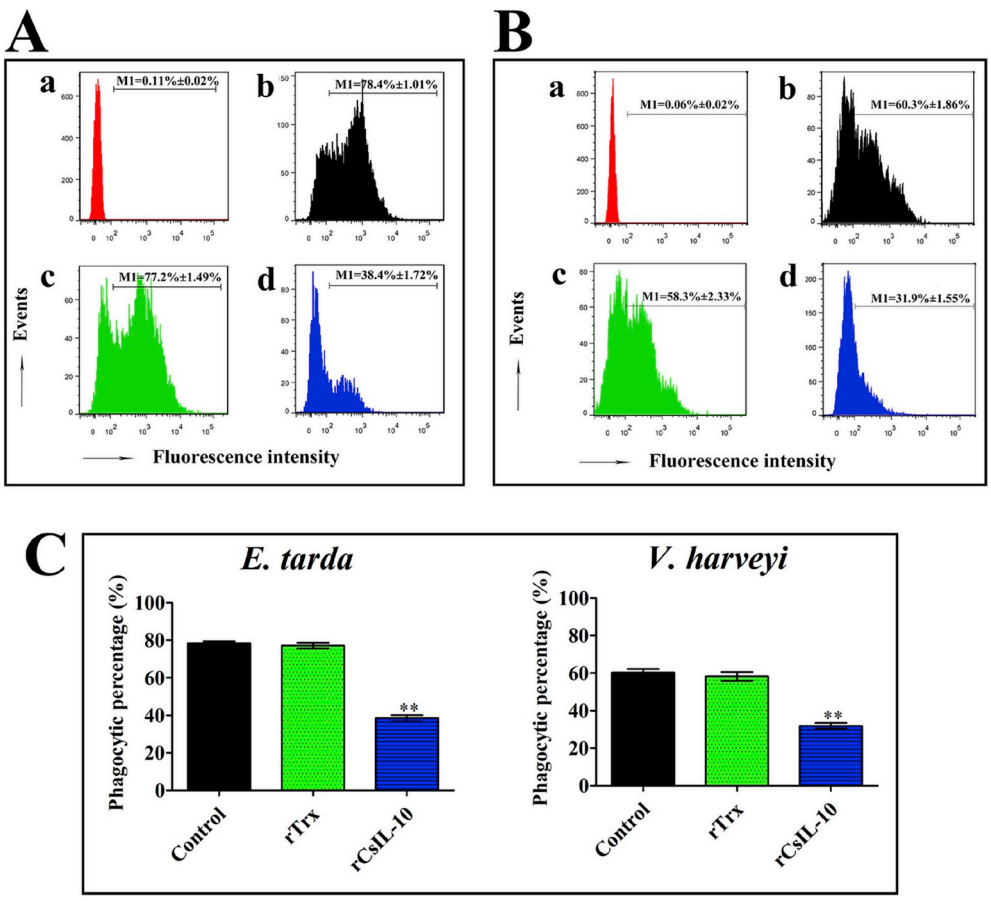

Fig. 4. Effect of rCsIL-10 on phagocytosis in peripheral blood leukocytes (PBL). (A) Tongue sole PBL were infected with FITC-labeled *Edwardsiella tarda* alone (Ab) or in the presence of rCsIL-10 (Ad) or rTrx (Ac). The control cells (Aa) were without any infection or protein treatment. Bacterial phagocytosis was determined by fluorescence activated cell sorting (FACS). (B) PBL were infected with FITC-labeled *Vibrio harveyi* alone (Bb) or in the presence of rCsIL-10 (Bd) or rTrx (Bc). The control cells (Ba) were without any infection or protein treatment. Bacterial phagocytosis was determined by FACS. M1 represents the cellular population with enhanced fluorescence as a result of bacterial uptake. (C) Phagocytic percentages of A and B. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. 3 fish were used in each experiment.

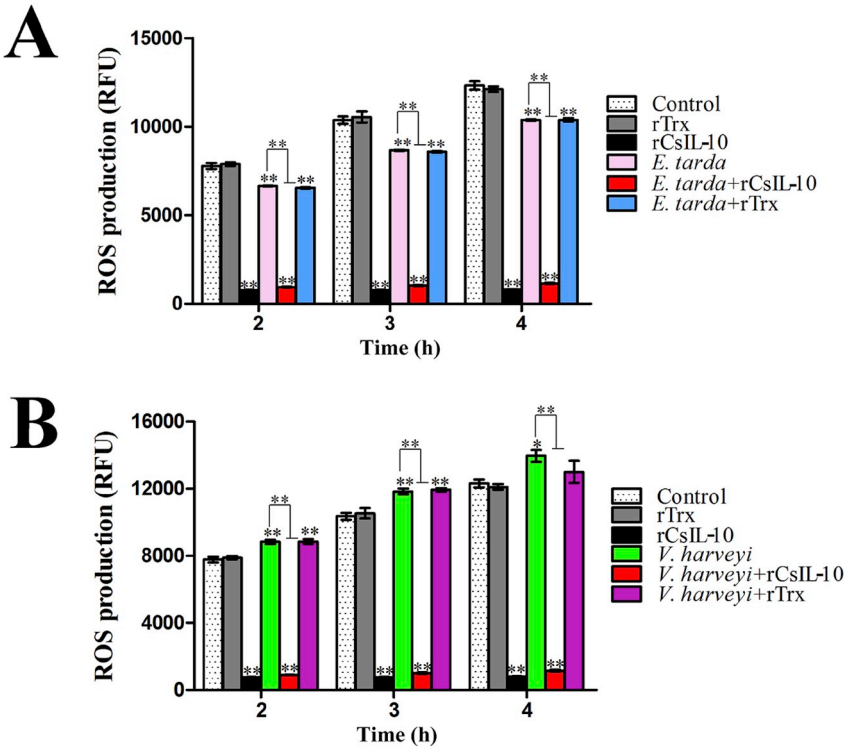

Fig. 5. Effect of rCsIL-10 on the production of reactive oxygen species (ROS) in peripheral blood leukocytes (PBL). Tongue sole PBL were infected with *Edwardsiella tarda* (A) or *Vibrio harveyi* (B) in the presence or absence (control) of rCsIL-10 or rTrx, and ROS was determined at 2 h, 3 h, and 4 h after infection. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. 3 fish were used in each experiment.*$P < 0.05$; **$P < 0.01$.

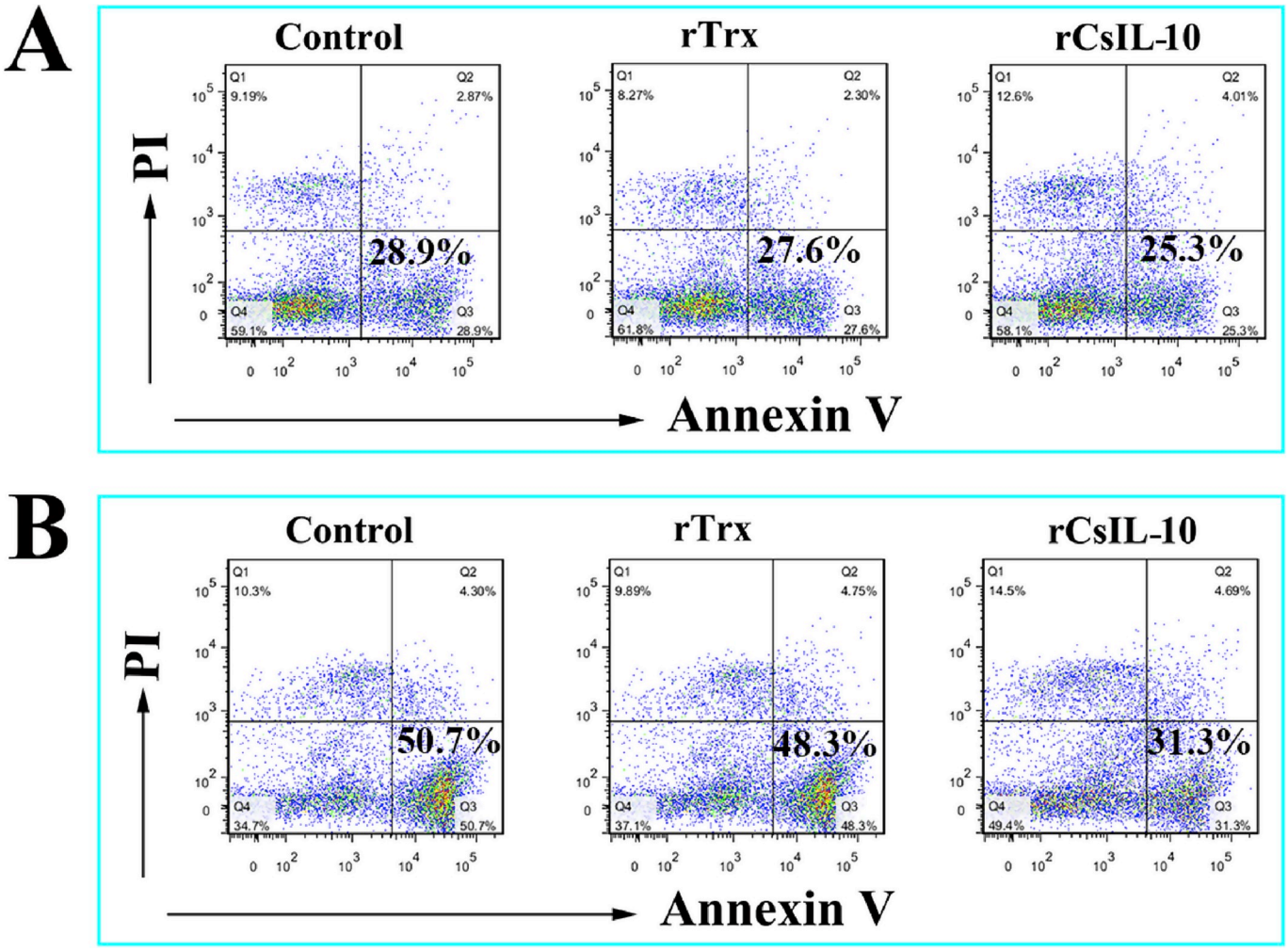

Fig. 6. Effect of rCsIL-10 on the apoptosis of peripheral blood leukocytes (PBL). PBL were treated with LPS in the presence or absence (control) of rCsIL-10 or rTrx for 12 h (A) and 24 h (B). The cells were then stained with annexin V and PI and subjected to fluorescence activated cell sorting. The results are one representative of three independent experiments. 3 fish were used in each experiment.

2.10. In vivo effect of rCsIL-10

In vivo infection was performed as reported previously [34]. Briefly, tongue sole were divided randomly into three groups and infected with *E. tarda* as described above in the presence or absence of 100 μg/ml rCsIL-10 or rTrx. Head kidney, spleen, and blood were taken from the fish (five at each time point) at 12 hpi, 24 hpi, and 48 hpi, and subjected for bacterial recovery analysis as reported previously [34]. To examine the expression of the genes of *nuclear factor-kappa B* (*NF-κB/p65*), *interleukin* (*il*)-*1β, il-8, tumor necrosis factor (tnf)-α*, total RNA was extracted from the spleen of *E. tarda*-infected flounder at 24 hpi as above, and qRT-PCR was used to analyze the expression of the genes as described above. The PCR primers of the genes are listed in Table 1. All experiments were performed at least three times. To examine the effect of bacteria bound rCsIL-10, rCsIL-10 or rTrx was incubated with *E. tarda* at a final concentration of 100 μg/ml at room temperature for 2 h. The bacteria were harvested by centrifugation, washed three times with PBS, and resuspended in PBS to 2 × 107 CFU/ml. Three groups of fish were injected intraperitoneally (i.p.) with 50 μl untreated *E. tarda* or with the above rCsIL-10- or rTrx-treated *E. tarda*. At 12 hpi, 24 hpi, and 48 hpi, head kidney, spleen, and blood were taken from the fish (5 fish/time point) and examined for bacterial recovery by plate count [31]. The experiment was performed in triplicate.

2.11. Csil-10 knockdown

Csil-10 knockdown was performed by small RNA (siRNA) interference as reported previously [31]. To select effective siRNAs for *Csil-10*, the sequences of three siRNA targeting *Csil-10* were designed according to https://www.genscript.com/ssl-bin/app/rnai and inserted into the siRNA expression vector pRNAT-CMV3.1 (GenScript, Piscataway, USA) between BamHI/AlfII sites, resulting in plasmids pCsIL-10-1, pCsIL-10-2, and pCsIL-10si-3. The plasmid pCsIL-10siC, which expresses a scramble siRNA, was constructed similarly. Endotoxin-free plasmid DNA was prepared using Endo-Free plasmid Kit (Omega Bio-Tek, Doraville, USA) and diluted in PBS to 300 μg/ml. Tongue sole were divided randomly into five groups (5 fish/group) and injected intramuscularly (i.m.) on the back with 100 μl (about 2 μg plasmid/g fish) pCsIL-10-1, pCsIL-10-2, pCsIL-10si-3, or pCsIL-10siC; the control group of fish was injected with PBS. At 7 d post-plasmid injection, expression of *Csil-10* in head kidney, spleen, and blood was determined by qRT-PCR as described above. The plasmid showing the strongest inhibitory effect on *Csil-10* expression was re-named pCsIL-10si. The experiment was performed in triplicate. The siRNA sequences expressed by pCsIL-10si and pCsIL-10siC are 5′-CTCTAGCTACACTCAGATG-3′ and 5′-ACTCGCGCTGATCATATAC-3′, respectively.

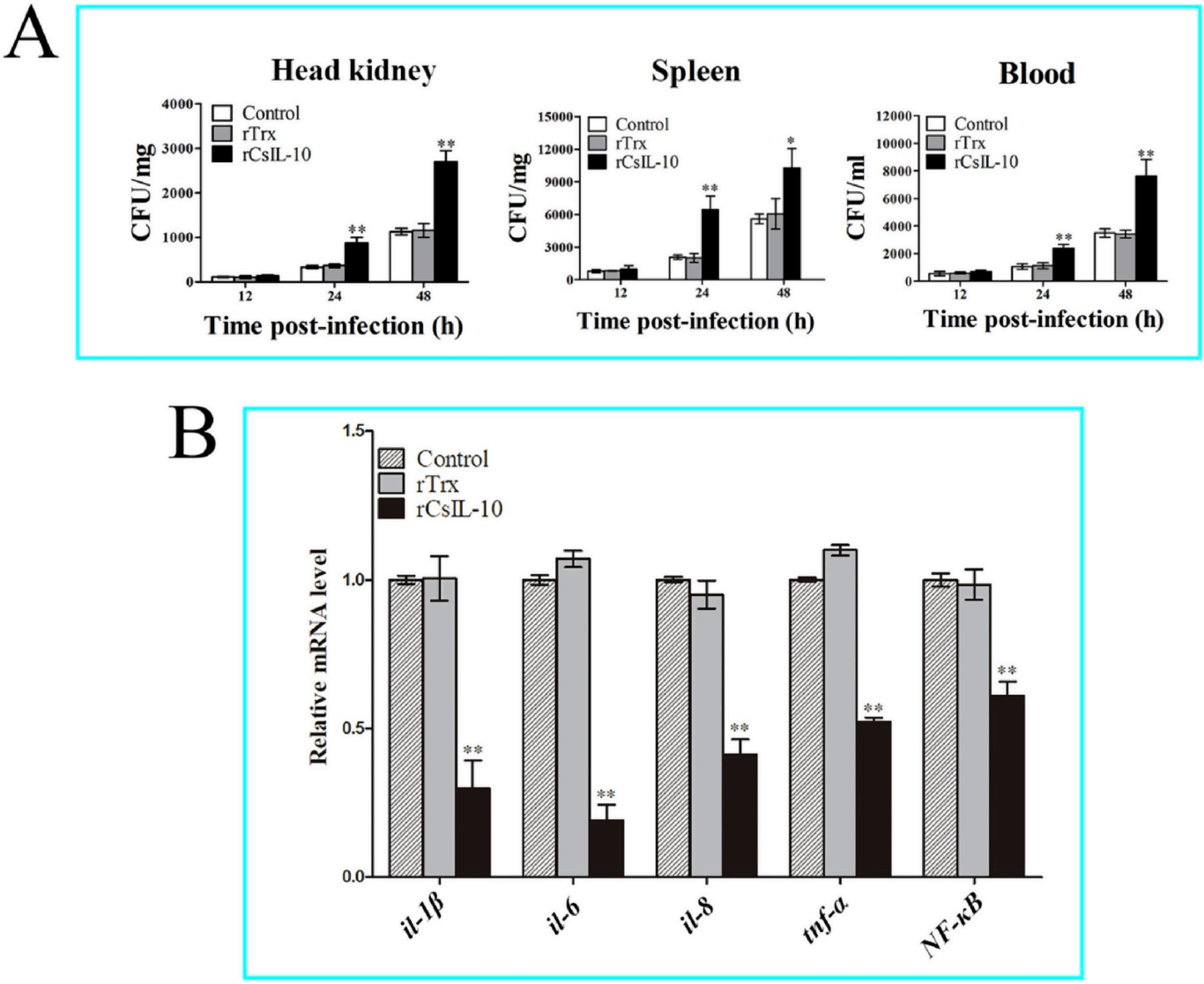

Fig. 7. *In vivo* effect of rCsIL-10 on bacterial infection and immune gene expression. Tongue sole were infected with *Edwardsiella tarda* in the presence or absence (control) of rCsIL-10 or rTrx. Bacterial numbers in head kidney, spleen, and blood at 12 h, 24 h, and 48 h post-infection (hpi) were determined (A), and immune gene expression in spleen at 24 hpi was determined by quantitative real time RT-PCR (B). Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. 45 fish were used in each experiment. *$P < 0.05$; **$P < 0.01$.

2.12. Effect of CsIL-10 knockdown on bacterial infection

Tongue sole were divided randomly into three groups (N = 15) and administered with pCsIL-10si, pCsIL-10C, or PBS as above. At 7 d post-plasmid administration, the fish were infected as above with 50 μl E. *tarda* (2×10^7 CFU/ml). At 12 hpi, 24 hpi, and 48 hpi, head kidney, spleen, and blood were taken from the fish (5 fish/time point) and examined for bacterial recovery by plate count as reported previously [31]. The experiment was performed in triplicate.

2.13. Statistical analysis

All experiments were performed in triplicate or independently for three times, and statistical analyses were carried out with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with one-way analysis of variance (ANOVA), and statistical significance was defined as $P < 0.05$.

3. Results

3.1. Sequence characteristics of CsIL-10

CsIL-10 is composed of 186 amino acid residues and has a calculated molecular mass of 21.5 kDa and a pI of 6.08. CsIL-10 shares 46.3–71.7% overall sequence identities with the IL-10 of teleost *Danio rerio*, *Ctenopharyngodon idella*, *Cyprinus carpio*, *Oncorhynchus mykiss*, *Takifugu rubripes*, *Paralichthys olivaceus*, and *Scophthalmus maximus* (Fig. 1A). CsIL-10 contains an N-terminal signal peptide sequence (residues 1 to 22), an IL-10 family signature motif (KAMGELDLLFNYIE) and four conserved cysteine residues (Cys-26, 80, 129 and 135) (Fig. 1A). Phylogenetic analysis showed that teleost IL-10 formed a clade that was distinctly separated from that formed by other vertebrate and viral IL-10 (Fig. 1B). Within the fish clade, CsIL-10 formed a group with the IL-10 of *Paralichthys olivaceus* and *Scophthalmus maximus*.

3.2. Expression of CsIL-10 in fish tissues

qRT-PCR analysis showed that constitutive *Csil-10* expression

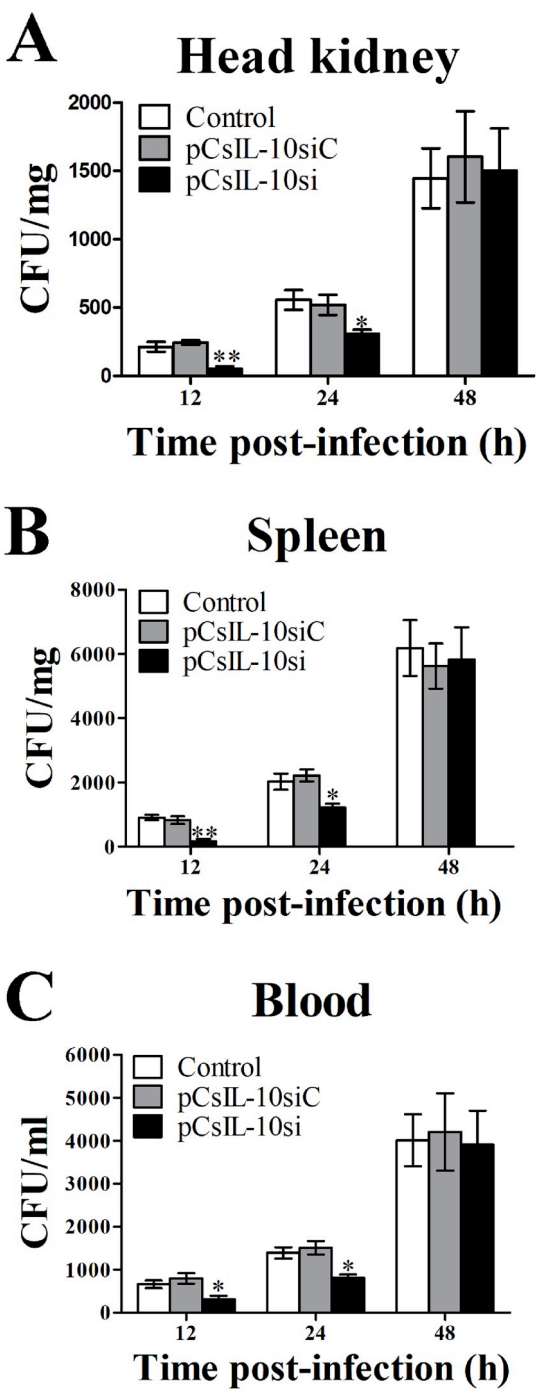

Fig. 8. Effect of *Csil-10* knockdown on bacterial infection. Tongue sole pre-administered with or without (control) pCsIL-10si and pCsIL-10siC were infected with *Edwardsiella tarda*, and bacterial numbers in head kidney (A), spleen (B), and blood (C) were determined at various time points. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. 45 fish were used in each experiment. $^{*}P < 0.05$; $^{**}P < 0.01$.

occurred, in increasing order, in the intestine, head kidney, blood, liver, spleen, heart, gill, muscle, and brain of tongue sole, with highly expression level in muscle and brain, followed by gill and heart. Lowest levels were observed in intestine. (Fig. S1). When tongue sole were infected with the bacterial pathogen *E. tarda*, *Csil-10* expression was significantly upregulated at 6 hpi in head kidney, at 12 hpi, 24 hpi, and 48 hpi in spleen, and at 24 hpi in blood (Fig. 2A). When the fish were infected with the bacterial pathogen *V. harveyi*, *Csil-10* expression was significantly induced at 6 hpi in head kidney, at 6 hpi and 12 hpi in spleen, and at 12 hpi, 24 hpi, and 48 hpi in blood (Fig. 2B).

3.3. Purification and analysis of rCsIL-10

To facilitate functional study, rCsIL-10 was purified from *E. coli* as a recombinant protein (Fig. S2A). Native gel electrophoresis showed that rCsIL-10 exhibited a molecular weight of approximately 40 kDa, which is twice of that of the monomeric CsIL-10 (Fig. S2B). Consistently, rCsIL-10 cross-linked with DSS also formed a protein complex of 40 kDa (Fig. S2C). These results indicated that rCsIL-10 existed in the form of a dimer, which is consistent with the three-dimensional structure of CsIL-10 (Fig. S2D).

3.4. Detection of CsIL-10 in PBL culture supernatant

To examine whether CsIL-10 was secreted by PBL, the supernatant and whole-cell proteins of LPS-stimulated PBL culture were subjected to immunoblot analysis. The results showed that CsIL-10 was detected in both supernatant and whole-cell by anti-rCsIL-10 antibody (Fig. S3). Since β-actin was detected only in the whole-cell preparation but not in the supernatant, the CsIL-10 detected in the culture supernatant was not due to contamination with cytoplasmic proteins.

3.5. Interaction of rCsIL-10 with bacteria and PBL

ELISA showed that when rCsIL-10 or rTrx, which was purified under the same condition as rCsIL-10, was incubated with Gram-negative bacteria and Gram-positive bacteria, i.e., *E. tarda*, *P. fluorescens*, *V. harveyi*, *S. iniae*, *B. subtilis*, *S. aureus*, and *M. luteus*, rCsIL-10 was able to bind to all these bacteria in a dose-dependent manner (Fig. 3A). In contrast, rTrx did not bind to any of the bacteria. Relatively high and comparable bindings were observed with *E. tarda*, *P. fluorescens*, *V. harveyi*, *B. subtilis*, and *M. luteus*, while relatively low bindings were observed with *S. iniae* and *S. aureus*. The ELISA results were confirmed by Western blot (Fig. 3B). Consistently, fluorescence microscopy detected binding of rCsIL-10 to *V. harveyi* (Fig. 3C). Growth analysis showed that the presence of rCsIL-10 had no significant effect on the growth of either of the above seven bacteria (data not shown). Fluorescence microscopy also revealed that, following incubation with tongue sole PBL, rCsIL-10 was detected on the surface of the cells, suggesting binding of rCsIL-10 to PBL (Fig. 3D).

3.6. Effect of rCsIL-10 on PBL phagocytosis, ROS production, and apoptosis

FACS analysis revealed that when tongue sole PBL were infected with *E. tarda* in the presence of rCsIL-10, the intracellular bacterial load was significantly ($P < 0.01$) lower than that in untreated PBL or in PBL treated with rTrx (Fig. 4A and C). Similar results were obtained in PBL infected with *V. harveyi* in the presence of rCsIL-10 (Fig. 4B and C).To examine the effect of rCsIL-10 on the ROS production of PBL, LPS was firstly added to the cells to initiate a moderate ROS production. The cells were then infected with or without bacteria, and ROS production was determined. The results showed that compared to uninfected cells, PBL infected with *E. tarda* exhibited significantly reduced ROS levels at 2 h, 3 h, and 4 h of infection, and the presence of rCsIL-10 further reduced the production of ROS to significant extents (Fig. 5A). In contrast, PBL infected with *V. harveyi* exhibited significantly increased ROS production at all time points, but the presence of rCsIL-10 significantly reduced the ROS levels (Fig. 5B). FACS analysis indicated that compared to PBL treated with LPS in the absence of rCsIL-10, PBL treated with LPS in the presence of rCsIL-10 exhibited no apparent change in apoptosis at 12 h post treatment, but showed a significant reduction of apoptosis at 24 h post-treatment (Fig. 6).

3.7. In vivo effect of rCsIL-10 on bacterial infection and immune gene expression

To examine the *in vivo* effect of rCsIL-10 on bacterial infection, tongue sole were infected with *E. tarda* in the presence or absence of rCsIL-10 or rTrx, and bacterial invasion into head kidney, spleen, and blood at 12 hpi, 24 hpi, and 48 hpi was determined by plate count. The results showed that for all three tissues and at 24 hpi and 48 hpi, the bacterial numbers in fish treated with rCsIL-10 were significantly higher than those in the control fish or in the fish treated with rTrx (Fig. 7A). qRT-PCR analysis showed that compared to the control fish, fish infected with *E. tarda* in the presence of rCsIL-10 exhibited significantly decreased expression in spleen in the genes involved in inflammation, i.e. *il-1β*, *il-6*, *il-8*, and *tnf-α*, as well as *NF-κB* (Fig. 7B). To examine whether bacteria bound rCsIL-10 would have a similar effect on bacterial infection, tongue sole were infected with rCsIL-10- or rTrx-bound *E. tarda*, and bacterial invasion into head kidney, spleen, and blood at 12 hpi, 24 hpi, and 48 hpi was determined by plate count. The results showed that in fish infected with rCsIL-10 bound *E. tarda*, the bacterial recoveries were significantly higher in all three tissues at 24 hpi and in head kidney and spleen at 48 hpi (Fig. S4).

3.8. Effect of CsIL-10 knockdown on bacterial infection

To further examine the *in vivo* effect of CsIL-10, *Csil-10* was knocked down by administering into tongue sole the plasmid pCsIL-10si, which expresses a *Csil-10*-specific siRNA. As a control, fish were also administered with the plasmid pCsIL-10siC, which expresses a *Csil-10*-nonspecific siRNA. qRT-PCR analysis showed that at 7 d post-plasmid administration, *Csil*-10 mRNA levels in the head kidney, spleen, and blood of pCsIL-10si-administered fish were significantly ($P < 0.01$) reduced compared to that in the control fish, whereas *Csil-10* expression levels in pCsIL-10siC-administered fish were similar to that in the control fish (Fig. S5). To examine the effect of *Csil-10* knockdown on bacterial infection, pCsIL-10si- and pCsIL-10siC-treated tongue sole were infected with *E. tarda*, and bacterial recoveries from head kidney, spleen, and blood were determined at 12 hpi, 24 hpi, and 48 hpi. The results showed that in all examined tissues, pCsIL-10si-treated fish exhibited significantly lower bacterial recoveries than the control fish at 12 hpi and 24 hpi (Fig. 8). No apparent difference in bacterial recovery between pCsIL-10siC-treated fish and control fish was observed.

4. Discussion

In this study, we examined the expression and immune effect of tongue sole IL-10, CsIL-10. CsIL-10 was classified as an IL-10 family member based on its possession of the typical IL-10 signature sequence and the conserved cysteine residues essential for maintaining IL-10 structure. In mammals, *il-10* can be expressed on almost all leukocyte subtypes and non-immune cells [10]. In teleost, the constitutive expression of *il-10* varies among species, but head kidney, gut and gill showed high expression in most investigated fish [15,16,18–21,23–26]. In our study, the *Csil-10* was detected ubiquitously in all examined tissues of healthy fish, with relatively higher levels occurring in brain and muscle, and low levels occurring in intestine, which was different from other teleost *il-10* expression profiles. In fish, *il-10* expression was stimulated by bacteria, virus, and LPS [15,16,18–21,23–26]. Similarly, in our study, *Csil-10* expression was detected ubiquitously in the major tissues of tongue sole and was upregulated by *E. tarda* and *V. harveyi* infection. These results indicate that *Csil-10* is involved in host immune response during bacterial invasion.

Biochemical and crystal structure analyses of mammalian and viral rIL-10 revealed that this cytokine forms and functions as a homodimer [47]. In our study, CsIL-10 was predicted to form a homodimeric structure similar to that of mammalian IL-10. Consistently, rCsIL-10 was found to exist in a dimeric form. Cellular studies showed that in mammals, IL-10 was secreted to the extracellular milieu by various cells treated with pathogen [6,10]. In our study, we found that CsIL-10 was detected in the culture medium of LPS-treated PBL, indicating extracellular production of CsIL-10 by PBL under stimulation.

In mammals, IL-10 is known to bind to its cognate membrane-bound receptor and form ligand-receptor complexes [8,48]. In fish, the function of IL-10 receptor is essentially unknown. However, there are evidences indicating the existence of IL-10 ligand-receptor system in zebrafish and goldfish [49,50]. In our study, we found that was able to bind to PBL, suggesting an interaction of rCsIL-10 with membrane-bound receptors on PBL. Interestingly, rCsIL-10 could also bind to bacterial strains of different species, possibly through some molecules existing commonly in Gram-negative and Gram-positive bacteria. Although *in vitro* rCsIL-10-bound and unbound *E. tarda* exhibited no difference in growth, rCsIL-10 bound *E. tarda* showed significantly enhanced dissemination in fish tissues, indicating that bacteria-bound rCsIL-10 also possessed biological functions.

Phagocytosis is an important cellular immune response for pathogen elimination and is accompanied by an oxidative burst with an increase in the production of free radicals [51]. Reports have indicated that mouse IL-10 could suppress macrophage phagocytosis, and human IL-10 could directly inhibit neutrophil-dependent phagocytosis and downregulate the production of reactive oxygen intermediates [52–54]. In teleost, rIL-10 of cyprinid fish could inhibit the oxygen and nitrogen radical production in phagocytes [18,22,29]. Similarly, we found that rCsIL-10 markedly decreased the phagocytic capacity of PBL against bacterial pathogens and caused a prominent reduction in ROS production. These results indicated that rCsIL-10 negatively regulated PBL activation.

Previous studies showed that human IL-10 prevented apoptosis induced by lymphocyte activation and LPS stimulation, and the presence of neutralizing anti-IL-10 antibody enhanced apoptosis [55,56]. Similarly, IL-10 prevented apoptosis of murine macrophages infected with *Salmonella choleraesuis* [57]. However, the effect of fish IL-10 on apoptosis was unclear. In the present study, we found that rCsIL-10 significantly reduced the apoptosis of PBL, which was in agreement with the negative effect of rCsIL-10 on ROS production in PBL, as in apoptosis, the induction of death is accompanied with ROS generation in different cell systems [58].

In humans, excessive expression of IL-10 is associated with persistent and chronic infection [59]. In mice, IL-10 inhibits macrophage antimicrobial activity, and neutralization of IL-10 reduced the infection of *Klebsiella pneumonia* and *M. avium* and enhanced expression of proinflammatory cytokines [13,60]. In fish, IL-10 has been reported to weaken the resistance of zebrafish against *Mycobacterium marinum* infection and suppress the bactericidal activity of amberjack leukocytes [17,30]. In this study, we found that the presence of rCsIL-10 significantly enhanced *E. tarda* dissemination in fish tissues, and knockdown of CsIL-10 in tongue sole significantly reduced *E. tarda* infection. Furthermore, rCsIL-10 treatment significantly reduced the expression of proinflammatory cytokines and *NF-κB*, which is in agreement with the observed negative effects of mammalian and fish IL-10 on proinflammatory cytokines [11,22,49]. It is likely that the enhancing effect of rCsIL-10 on *E. tarda* invasion is due to the inhibitory effect of rCsIL-10 on bacteria-induced proinflammatory response and activation of phagocytes.

In conclusion, this study demonstrates that CsIL-10 is a cytokine that was able to interact with PBL and inhibit phagocytic activity and apoptosis. CsIL-10 could also interact with bacteria of both Gram-negative and Gram-positive nature, though the biological significance is not clear. CsIL-10 negatively regulated the expression of proinflammatory cytokines and could significantly affect the dissemination of bacterial pathogens in host tissues. These results provide new insights into the functionality of teleost IL-10 in host immune defense.

Acknowledgements

This work was supported by the grants from the National Natural Science Foundation of China (31802329), the Shandong Major Science and Technology Innovation Project (2018SDKJ0302-2), China Postdoctoral Science Foundation (2018M632733), and the Taishan Scholar Program of Shandong Province.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.023.

References

[1] K.W. Moore, A. O'garra, W.M.R. De, P. Vieira, T.R. Mosmann, Interleukin-10, Annu. Rev. Immunol. 11 (1993) 165–190.
[2] H. Groux, F. Cottrez, The complex role of interleukin-10 in autoimmunity, J. Autoimmun. 20 (2003) 281–285.
[3] C.J. Secombes, T. Wang, S. Bird, The interleukins of fish, Dev. Comp. Immunol. 35 (2011) 1336–1345.
[4] S.V. Kotenko, S. Saccani, L.S. Izotova, O.V. Mirochnitchenko, S. Pestka, Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10), Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 1695–1700.
[5] J.V. Spencer, K.M. Lockridge, P.A. Barry, G. Lin, M. Tsang, M.E.T. Penfold, et al., Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10, J. Virol. 76 (2002) 1285–1292.
[6] D.F. Fiorentino, M.W. Bond, T.R. Mosmann, Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones, J. Exp. Med. 170 (1989) 2081–2095.
[7] K.N. Couper, D.G. Blount, E.M. Riley, IL-10: the master regulator of immunity to infection, J. Immunol. 180 (2008) 5771–5777.
[8] J.C. Tan, S. Braun, H. Rong, R. Digiacomo, E. Dolphin, S. Baldwin, et al., Characterization of recombinant extracellular domain of human interleukin-10 receptor, J. Biol. Chem. 270 (1995) 12906–12911.
[9] K.W. Moore, R.D.W. Malefyt, R.L. Coffman, A. O'garra, interleukin-10 and the interleukin-10 receptor, Annu. Rev. Immunol. 19 (2003) 683–765.
[10] M.C. Piazzon, G. Lutfalla, M. Forlenza, IL10, a tale of an evolutionarily conserved cytokine across vertebrates, Crit. Rev. Immunol. 36 (2016) 99.
[11] S. Mocellin, F. Marincola, C.R. Rossi, D. Nitti, M. Lise, The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle, Cytokine Growth Factor Rev. 15 (2004) 61–76.
[12] D.M. Rennick, M.M. Fort, N.J. Davidson, Studies with IL-$10^{-/-}$ mice: an overview, J. Leukoc. Biol. 61 (1997) 389–396.
[13] L.E. Bermudez, J. Champsi, Infection with *Mycobacterium avium* induces production of interleukin-10 (IL-10), and administration of anti-IL-10 antibody is associated with enhanced resistance to infection in mice, Infect. Immun. 61 (1993) 3093–3097.
[14] J. Zou, M.S. Clark, C.J. Secombes, Characterisation, expression and promoter analysis of an interleukin 10 homologue in the puffer fish, *Fugu rubripes*, Immunogenetics. 55 (2003) 325–335.
[15] Y. Inoue, S. Kamota, K. Ito, Y. Yoshiura, M. Ototake, T. Moritomo, et al., Molecular cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*) interleukin-10 cDNAs, Fish Shellfish Immunol. 18 (2005) 335–344.
[16] D.C. Zhang, Y.Q. Shao, Y.Q. Huang, S.G. Jiang, Cloning, characterization and expression analysis of interleukin-10 from the zebrafish (*Danio rerion*), J. Biochem. Mol. Biol. 38 (2005) 571–576.
[17] S.K.E. Harjula, M.J.T. Ojanen, S. Taavitsainen, M. Nykter, M. Rämet, Interleukin 10 mutant zebrafish have an enhanced interferon gamma response and improved survival against a *Mycobacterium marinum* infection, Sci. Rep. 8 (2018) 10360.
[18] L. Grayfer, J.W. Hodgkinson, S.J. Hitchen, M. Belosevic, Characterization and functional analysis of goldfish (*Carassius auratus* L.) interleukin-10, Mol. Immunol. 48 (2011) 563–571.
[19] He Wei, Mu Yang, Taiqiang Zhao, et al., Functional expression and characterization of grass carp IL-10: an;essential mediator of TGF-beta 1 immune regulation in peripheral blood;lymphocytes, Mol. Immunol. 53 (2013) 313–320.
[20] H. Wei, L. Yin, S. Feng, X. Wang, K. Yang, A. Zhang, et al., Dual-parallel inhibition of IL-10 and TGF-β1 controls LPS-induced inflammatory response via NF-κB signaling in grass carp monocytes/macrophages, Fish Shellfish Immunol. 44 (2015) 445–452.
[21] B. Swain, M. Samanta, M. Basu, P. Panda, B.R. Sahoo, N.K. Maiti, et al., Molecular characterization, inductive expression and mechanism of interleukin-10 gene induction in the Indian major carp, catla (*Catla catla*), Aquacult. Res. 43 (2012) 897–907.
[22] S. Karan, P. Dash, H. Kaushik, P.K. Sahoo, L.C. Garg, A. Dixit, Structural and functional characterization of recombinant interleukin-10 from Indian major carp *Labeo rohita*, J. Immunol. Res. 2016 (2016) 1–11.
[23] F. Buonocore, E. Randelli, S. Bird, C.J. Secombes, A. Facchiano, S. Costantini, et al., Interleukin-10 expression by real-time PCR and homology modelling analysis in the European sea bass (*Dicentrarchus labrax* L.), Aquaculture 270 (2007) 512–522.
[24] R.D. Pinto, D.S. Nascimento, M.I. Reis, V.A. Do, N.M. Dos Santos, Molecular characterization, 3D modelling and expression analysis of sea bass (*Dicentrarchus labrax* L.) interleukin-10, Mol. Immunol. 44 (2007) 2056–2065.
[25] B.H. Nam, J.Y. Moon, E.H. Park, Y.O. Kim, D.G. Kim, H.J. Kong, et al., Conserved gene structure and function of interleukin-10 in teleost fish, J. Anim. Vet. Adv. (2014) 774–782.
[26] H.J. Huo, S.N. Chen, L. Li, P. Nie, Functional characterization of IL-10 and its receptor subunits in a perciform fish, the Mandarin fish, *Siniperca chuatsi*, Dev. Comp. Immunol. 97 (2019) 64–75.
[27] R. Savan, D. Igawa, M. Sakai, Cloning, characterization and expression analysis of interleukin-10 from the common carp, *Cyprinus carpio* L, Eur. J. Biochem. 270 (2003) 4647–4654.
[28] M. Seppola, A.N. Larsen, K. Steiro, B. Robertsen, I. Jensen, Characterisation and expression analysis of the interleukin genes, IL-1beta, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.), Mol. Immunol. 45 (2008) 887–897.
[29] M.C. Piazzon, H.F. Savelkoul, D. Pietretti, G.F. Wiegertjes, M. Forlenza, Carp Il10 has anti-inflammatory activities on phagocytes, promotes proliferation of memory T cells, and regulates B cell differentiation and antibody secretion, J. Immunol. 194 (2015) 187–199.
[30] M. Matsumoto, M.T. Amer, K. Araki, A. Nishitani, K. Hayashi, Y. Takeuchi, et al., Amberjack *Seriola dumerili* interleukin-10 negatively suppresses host cell-mediated immunity, Fish. Sci. 84 (2018) 857–867.
[31] M.F. Li, J. Li, L. Sun, CsMAP34, a teleost MAP with dual role: a promoter of MASP-assisted complement activation and a regulator of immune cell activity, Sci. Rep. 6 (2016) 39287.
[32] Z.X. Zhou, B.C. Zhang, L. Sun, Poly (I: C) induces antiviral immune responses in Japanese flounder (*Paralichthys olivaceus*) that require TLR3 and MDA5 and is negatively regulated by Myd88, PLoS One 9 (2014) e112918.
[33] Y. Sun, L. Sun, CsBAFF, a teleost B cell activating factor, promotes pathogen-induced innate immunity and vaccine-induced adaptive immunity, PLoS One 10 (2015) e0136015.
[34] T. Wang, L. Sun, CsSAP, a teleost serum amyloid P component, interacts with bacteria, promotes phagocytosis, and enhances host resistance against bacterial and viral infection, Dev. Comp. Immunol. 55 (2016) 12–20.
[35] K. Sun, W.W. Zhang, J.H. Hou, L. Sun, Immunoprotective analysis of VhhP2, a *Vibrio harveyi* vaccine candidate, Vaccine 27 (2009) 2733–2740.
[36] Z.J. Zhou, B.G. Sun, L. Sun, *Edwardsiella tarda* Sip1: a serum-induced zinc metalloprotease that is essential to serum resistance and host infection, Vet. Microbiol. 177 (2015) 332–340.
[37] H. Long, C. Chen, J. Zhang, L. Sun, Antibacterial and antiviral properties of tongue sole (*Cynoglossus semilaevis*) high mobility group B2 protein are largely independent on the acidic C-terminal domain, Fish Shellfish Immunol. 37 (2014) 66–74.
[38] W.W. Zhang, L. Sun, Cloning, characterization, and molecular application of a beta-agarase gene from Vibrio sp. strain V134, Appl. Environ. Microbiol. 73 (2007) 2825–2831.
[39] B.C. Zhang, L. Sun, Tongue sole (*Cynoglossus semilaevis*) prothymosin alpha: cytokine-like activities associated with the intact protein and the C-terminal region that lead to antiviral immunity via Myd88-dependent and-independent pathways respectively, Dev. Comp. Immunol. 53 (2015) 96–104.
[40] V. Méchin, C. Damerval, M. Zivy, Total Protein Extraction with TCA-Acetone. Plant Proteomics, Springer, 2007, pp. 1–8.
[41] X.P. Li, L. Sun, A teleost complement factor Ba possesses antimicrobial activity and inhibits bacterial infection in fish, Dev. Comp. Immunol. 71 (2017) 49–58.
[42] H.R. Wang, Y.H. Hu, W.W. Zhang, L. Sun, Construction of an attenuated Pseudomonas fluorescens strain and evaluation of its potential as a cross-protective vaccine, Vaccine 27 (2009) 4047–4055.
[43] B.C. Zhang, J. Zhang, L. Sun, *Streptococcus iniae* SF1: complete genome sequence, proteomic profile, and immunoprotective antigens, PLoS One 9 (2014) e91324.
[44] X.P. Li, L. Sun, Toll-like receptor 2 of tongue sole *Cynoglossus semilaevis*: signaling pathway and involvement in bacterial infection, Fish Shellfish Immunol. 51 (2016) 321–328.
[45] M.L. Zhao, H. Chi, L. Sun, Neutrophil extracellular traps of Cynoglossus semilaevis: production characteristics and antibacterial effect, Front. Immunol. 8 (2017) 290.
[46] Z.X. Zhou, L. Sun, Immune effects of R848: evidences that suggest an essential role of TLR7/8-induced, Myd88-and NF-κB-dependent signaling in the antiviral immunity of Japanese flounder (*Paralichthys olivaceus*), Dev. Comp. Immunol. 49 (2015) 113–120.
[47] L. Bortesi, M. Rossato, F. Schuster, N. Raven, J. Stadlmann, L. Avesani, et al., Viral and murine interleukin-10 are correctly processed and retain their biological activity when produced in tobacco, BMC Biotechnol. 9 (2009) 22.
[48] Y. Liu, R. De Waal Malefyt, F. Briere, C. Parham, J.M. Bridon, J. Banchereau, et al., The EBV IL-10 homologue is a selective agonist with impaired binding to the IL-10 receptor, J. Immunol. 158 (1997) 604–613.
[49] L. Grayfer, M. Belosevic, Identification and molecular characterization of the interleukin-10 receptor 1 of the zebrafish (*Danio rerio*) and the goldfish (*Carassius auratus* L.), Dev. Comp. Immunol. 36 (2012) 408–417.
[50] H. Wei, X. Wang, A. Zhang, L. Du, H. Zhou, Identification of grass carp IL-10 receptor subunits: functional evidence for IL-10 signaling in teleost immunity, Dev. Comp. Immunol. 45 (2014) 259–268.
[51] K. Drábiková, V. Jančinová, R. Nosáľ, E. Danihelová, Human blood platelets, PMN leukocytes and their interactions *in vitro*. Responses to selective and non-selective stimuli, Gen. Physiol. Biophys. 19 (2000) 393–404.
[52] C. Bogdan, Y. Vodovotz, C. Nathan, Macrophage deactivation by interleukin 10, J. Exp. Med. 174 (1991) 1549–1555.
[53] I.P. Oswald, T.A. Wynn, A. Sher, S.L. James, Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced

activation, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 8676–8680.
[54] L.L. Laichalk, J.M. Danforth, T.J. Standiford, Interleukin-10 inhibits neutrophil phagocytic and bactericidal activity, FEMS Immunol. Med. Microbiol. 15 (1996) 181–187.
[55] R. Bingisser, C. Stey, M. Weller, P. Groscurth, E. Russi, K. Frei, Apoptosis in human alveolar macrophages is induced by endotoxin and is modulated by cytokines, Am. J. Resp. Cell Mol. 15 (1996) 64–70.
[56] S. Cohen, J. Crawley, M. Kahan, M. Feldmann, B. Foxwell, Interleukin-10 rescues T cells from apoptotic cell death: association with an upregulation of Bcl-2, Immunology 92 (1997) 1–5.
[57] T. Arai, K. Hiromatsu, H. Nishimura, Y. Kimura, N. Kobayashi, H. Ishida, et al., Endogenous interleukin 10 prevents apoptosis in macrophages during Salmonella infection, Biochem. Biophys. Res. Commun. 213 (1995) 600–607.
[58] H.U. Simon, A. Haj-Yehia, F. Levi-Schaffer, Role of reactive oxygen species (ROS) in apoptosis induction, Apoptosis 5 (2000) 415–418.
[59] P.A. Sieling, J.S. Abrams, M. Yamamura, P. Salgame, B.R. Bloom, T.H. Rea, et al., Immunosuppressive roles for IL-10 and IL-4 in human infection. In vitro modulation of T cell responses in leprosy, J. Immunol. 150 (1993) 5501.
[60] M.J. Greenberger, R.M. Strieter, S.L. Kunkel, J.M. Danforth, R.E. Goodman, T.J. Standiford, Neutralization of IL-10 increases survival in a murine model of *Klebsiella pneumonia*, J. Immunol. 155 (1995) 722–729.