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TroCCL4, a CC chemokine of *Trachinotus ovatus*, is involved in the antimicrobial immune response

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ABSTRACT

CC chemokines are a large subfamily of chemokines that play an important role in the innate immune system. To date, several CC chemokines have been identified in fish species; however, the activities and functions of these putative chemokines remain ambiguous in teleosts, especially in the golden pompano, *Trachinotus ovatus*. Here, we characterized CC chemokine ligand 4 from *T. ovatus* (TroCCL4) and studied its functions. TroCCL4 contains a 294 bp open reading frame that encodes a putative peptide comprising 97 amino acids. TroCCL4 shares a high amino acid sequence similarity of 31.11%–78.35% with other CC chemokines sequences in humans and teleosts and has four cysteine residues that are conserved among other CC chemokines. TroCCL4 is also related to the macrophage inflammatory protein (MIP) group of CC chemokines. *TroCCL4* expression was most abundant in immune organs and significantly upregulated in a time-dependent manner following *Edwardsiella tarda* infection. Recombinant TroCCL4 (rTroCCL4) induced the migration of peripheral blood leukocytes and the cellular proliferation of head kidney lymphocytes. In addition, rTroCCL4 inhibited the growth of *Escherichia coli* and *E. tarda*, indicating an antimicrobial function. Furthermore, the results of *in vivo* analysis showed that *TroCCL4* overexpression in *T. ovatus* significantly enhanced macrophage activation; upregulated the gene expression of interleukin 1-β (IL-1β), interleukin 15 (IL15), interferon-induced Mx protein (Mx), tumor necrosis factor α (TNFα), complement C3, and major histocompatibility complex (MHC) class Iα and class IIα; and protected against bacterial infection in fish tissues. In contrast, knockdown of *TroCCL4* expression resulted in increased bacterial dissemination and colonization in fish tissues. Taken together, our results provide evidence indicating that TroCCL4 has the ability to stimulate leukocytes and macrophages and enhance host immunity to defend against bacterial infection.

1. Introduction

Chemokines are a superfamily of chemotactic cytokines that play a key role in mediating leukocyte migration and regulating immune responses and the differentiation of the recruited cells [1,2]. These cytokines belong to the group of small secreted proteins (8–10 kDa) and perform their functions via binding to a subset of seven transmembrane G protein-coupled receptors [3,4]. Chemokines are considered crucial to both innate and adaptive immune responses [5–7]. To date, a large number of chemokines have been reported in mammals and teleosts [8–10].

All members of the chemokine family share a common structural feature: a conserved number of cysteine residues. Structurally, chemokines are classified into five subfamilies, CXC, CC, CX_3C, XC and CX (C indicates cysteine and X indicates other amino acids) based on the number and location of the first two cysteine residues in their sequences [11–13]. These five subfamilies exhibit different disulfide bridges and target cells, and the CC chemokines constitute the largest subfamily [14,15]. In CC chemokines, the first two cysteine residues are adjacent in the N-terminus [15–17]. These CC chemokines can perform chemotactic activity on monocytes/macrophages, T cells, B cells, eosinophils, dendritic cells, mast cells, and natural killer (NK) cells and participate in inflammation and the pathogenesis of several diseases [17,18]. To date, many fish CC chemokines have been reported, such as those in

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Table 1
Primers used in this study.

Primer name	Primer sequence (5′-3′)	Amplification target
B2M-F	AAGTCAGTCCACCCAAGGTTCA	qPCR
B2M-R	GGGATTTCCATTCCGTTCTTCATG	
CN—F1	CTTGCGTTTCTGATAGGCACCTA	Detection of pTroCCL4
CN-R1	TGCGGGCCTCTTCGCTATT	
His-R	GTGGTGGTGGTGGTGGTG	Detection of pTroCCL4
TroCCL4-F1	GATATCGCCACCATGGCTGCACCTCGTCTCACT	TroCCL4 ORF cloning
TroCCL4-F2	GATATCGCCACCATGTTGCGTGGTACTGGCCCA	TroCCL4 ORF cloning (no signal peptide)
TroCCL4-R1	GATATCCAGGTTGGACACCTCTCCTGGT	
TroCCL4-RT-F	TTTGTGCTGATGCTGGCTTTC	qPCR
TroCCL4-RT-R	CCGCTGGCTGGTCTTGATG	
Mx-F	CCAGGAGCATGAGGAAGAGAT	qPCR
Mx-R	AGGTCAATGAGCGTCAGGTCT	
IL1β-F	GGAGACTGTGGAGGACAAGAGC	qPCR
IL1β-R	GCGGGCAGACATGAAGGTG	
TNFα-F	GGCGTCGTTCAGAGTCTCCT	qPCR
TNFα-R	TCCTCCTGGGCAGTGGTTT	
IgM-F	AGAAATGGCTGACTCCTCAATGA	qPCR
IgM-R	CCCGCCTTCCTTTCATAGAG	
CC3L-F	CGCCGCTCCCTCTACATCA	qPCR
CC3L-R	GACAGCCACCCAACCATTACG	
IgD-F	GCAAAGCACCGATACTCTAACC	qPCR
IgD-R	AATATCCAGAAACTAGGCAAGTAAGT	
IL15-F	CGAGACCAGCGAGCGAAAG	qPCR
IL15-R	GACGGAGCATACAGCATAGCATC	
MHC Iα-F	ACTGGACCTTTCATCAGTCAAACCTG	qPCR
MHC Iα-R	GGACGCTTGGCACTCTTCTTTCT	
MHC IIα-F	GGCGTGAAGCCATGAAGGACAT	qPCR
MHC IIα-R	AGACTTTGACGGGAGCAGGGTAGA	
siTroCCL4-P1	GGATCCTAATACGACTCACTATAGGTACTGGCCCAAAGAAAT	Synthesizing siTroCCL4
siTroCCL4-P2	AAATTTCTTTGGGCCAGTACCTATAGTGAGTCGTATTAGGATCC	
siTroCCL4-P3	GGATCCTAATACGACTCACTATAATTTCTTTGGGCCAGTACC	
siTroCCL4-P4	AAGGTACTGGCCCAAAGAAATTATAGTGAGTCGTATTAGGATCC	
siTroCCL4-C—P1	GGATCCTAATACGACTCACTATAGCCACAACGTCTATATCAT	Synthesizing siTroCCL4-C
siTroCCL4-C—P2	AAATGATATAGACGTTGTGGCTATAGTGAGTCGTATTAGGATCC	
siTroCCL4-C—P3	GGATCCTAATACGACTCACTATAATGATATAGACGTTGTGGC	
siTroCCL4-C—P4	AAGCCACAACGTCTATATCATTATAGTGAGTCGTATTAGGATCC	

Notes: The underlined bases indicate the *EcoR* V restriction enzyme sites that were added to the 5′ end of primers.

Oncorhynchus mykiss [17], *Sparus aurata* [8], *Pseudosciaena crocea* [9], *Danio rerio* [10], *Paralichthys olivaceus* [11,12], and *Cynoglossus semilaevis* [13,14]. As reported in previous studies, the fish CC chemokines are divided into seven subgroups based on phylogenetic analyses: the CCL17/22 subgroup, the CCL19/21/25 subgroup, the CCL20 subgroup, the CCL27/28 subgroup, the macrophage inflammatory protein (MIP) subgroup, the monocyte chemotactic protein (MCP) subgroup and the fish cc subgroup [17–19].

Chemokine CC-motif ligand 4 (CCL4) is a member of the CC chemokine family that was first isolated from the culture medium of lipopolysaccharide (LPS)-activated macrophages and is also known as MIP-1β [20–22]. Mammalian CCL4 has been revealed to be indispensable to the migration, activation, differentiation, and proliferation of immune cells, such as macrophages, NK cells, dendritic cells, lymphocytes, granulocytes and T cells [23,24]. In addition, increasing evidence indicates that some chemokines have antimicrobial activity [25–27]. In fish, studies have shown that CCL4 can recruit macrophages and attract leukocytes [27–30]. To date, the biological functions of CCL4 remain ambiguous in teleosts, and no functional studies have been performed to date on CCL4 in the golden pompano, *Trachinotus ovatus*.

The *T. ovatus* is a commercially harvested marine fish with considerable economic value in China [31]. Disease outbreaks have led to the abuse of antibiotics in *T. ovatus* [32–34]. Immune prophylaxis methods are effective strategies for fighting disease. Many previous studies have provided evidence that CC chemokines potentially possess infection-fighting properties and can be used for treatment strategies against infection in the aquaculture industry [35–38].

In the present study, we cloned and identified a member of the CC chemokine family in *T. ovatus*, which was designated TroCCL4. Furthermore, we studied the tissue distribution pattern of TroCCL4 and its expression pattern following *Edwardsiella tarda* infection. The aim of the current study was to examine the *in vivo* and *in vitro* activity and antibacterial effects of TroCCL4. The results of this study will expand our understanding of the bioactivity of teleost CC chemokine and provide valuable insights into the disease resistance of *T. ovatus*.

2. Materials and methods

2.1. Fish

Trachinotus ovatus (average weight 14 ± 0.8 g) were purchased from a commercial fish farm in Chengmai, Hainan Province, China. The fish were maintained at 26 °C for one week before the experiments to allow them to acclimate to the laboratory environment. Prior to experimentation, fish were randomly selected and euthanized with tricaine methanesulfonate (Sigma-Aldrich, St. Louis, MO, USA) to confirm that they were in fact free of any bacteria as previously reported by Cao [39].

2.2. Pathogen strains and culture conditions

The fish pathogen *E. tarda* PBMM02142 was isolated from one diseased *T. ovatus* which was suffered from skin lesions in the Hainan Province of China and cultured at 28 °C in Luria-Bertani (LB) broth in our lab. *Escherichia coli* DH5α and BL21 (Takara, Dalian, PR China) were cultured in LB broth at 37 °C. Cell cultures were maintained in culture medium containing 25% (v/v) glycerol at −80 °C for long-term

storage.

2.3. Sequence analysis and primers design

The full-length sequence of *T. ovatus CC chemokine ligand 4* (*TroCCL4*) was obtained from our transcriptome database (GenBank accession number: PRJNA505850) and analyzed using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information website using CC chemokine ligand sequences from other species as query sequences. Signal peptide searches and protein structure predictions were performed with SignalP 4.1 and SMART version 4.0 software, respectively. The molecular mass and theoretical isoelectric point (pI) were predicted using EditSeq in the DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Phylogenetic analyses were performed with ClustalX and the neighbor-joining algorithm of MEGA 6.0 [40]. The specific qPCR primers are designed using the Primer 5 software (Table 1) according to the product manual. The specificity of the primer sets was confirmed by the presence of a single band of appropriate size obtained after PCR amplification and the dissociation curve of qPCR peaked to be sharp.

2.4. TroCCL4 expression in T. ovatus tissues under normal physiological conditions

Muscle, heart, brain, liver, head kidney, spleen, gill, skin, stomach, and intestine tissues were aseptically sampled from fifteen different fish. Five equivalent amounts of each tissue were pooled to make one sample, and the samples were then quickly placed into liquid nitrogen for preservation. Total RNA extraction was performed with an RNA Tissue Kit (Omega Biotek, Doraville, GA, USA) and treated with RNase-free *DNase* I (Omega Biotek, Doraville, GA, USA). The quality of the RNA was measured via electrophoresis and a NanoDrop spectrophotometer. Then, cDNA synthesis was performed with reverse transcriptase and 2 μg of total RNA using a cDNA synthesis kit (Takara, Dalian, China). qRT-PCR was carried out on the QuantStudio™ 6 Flex Real-Time PCR System (ABI, Singapore) using the SYBR ExScript qRT-PCR Kit (Promega, Madison, USA). *TroCCL4* was amplified using the primers TroCCL4-F1/R1 (Table 1) and beta-2-microglobulin (B2M) as the internal control as described in our previous study [41].

2.5. TroCCL4 expression in response to bacterial infection

The fish bacterial pathogen *E. tarda* was cultured at an OD_{600} of approximately 0.8 and then resuspended in PBS to a concentration of 10^6 CFU/ml. Fish (60 fish/group) were injected intraperitoneally with 100 μl of *E. tarda* or PBS (as control). Then, the tissues (liver, head kidney, and spleen) from each group were aseptically sampled from fifteen different fish at 6 h, 12 h, 24 h, and 48 h post-infection, with five tissues pooled as one sample as described above. Then, RNA was extracted and cDNA was synthesized. The qRT-PCR analysis was carried out using TroCCL4-RT-F/R primers and the internal control primers B2M-F/R as described in our previous study (Table 1) [41].

2.6. Plasmid construction

To construct the plasmid pEtTroCCL4 expressing His-tag and Trx-tag recombinant TroCCL4 (rTroCCL4), the coding sequence of *TroCCL4* without signal peptide was amplified by PCR with the primers TroCCL4-F2/R1 (Table 1). Briefly, the PCR products were ligated to a T-Simple T-A cloning vector (TransGen Biotech, Beijing, China), and then the recombinant plasmid was digested with *EcoR* V to obtain the TroCCL4 fragment. Backbone plasmid pET-32a(+) (Solarbio, Beijing, China) was digested with *EcoR* V. To construct pEtTroCCL4, the TroCCL4 fragment was linked to pET-32a(+) (Solarbio, Beijing, China) at the *EcoR* V site. Thus, pEtTroCCL4 was constructed, which expressed TroCCL4, His-tag and Trx-tag.

To construct the plasmid pTroCCL4 expressing His-tagged TroCCL4, the primer pair TroCCL4-F1/R1 was used to amplify the whole coding sequence of TroCCL4. Briefly, the PCR products were ligated to a T-Simple T-A cloning vector (TransGen Biotech, Beijing, China), and then the recombinant plasmid was digested with *EcoR* V to obtain the TroCCL4 fragment. Backbone plasmid pCN3 was constructed basing on pCI-neo, which contained a human cytomegalovirus (CMV) immediate-early promoter. Linker L86, linker L87, and a 6 × histidine tag were inserted into the based plasmid [42]. pCN3 was digested with *Sma* I, and the TroCCL4 fragment was ligated into the *Sma* I site of the plasmid pCN3. Thus, pTroCCL4 was constructed.

2.7. Purification of recombinant protein

The recombinant plasmid pEtTroCCL4 and the backbone plasmid pET-32a(+) (Solarbio, Beijing, China) were separately transformed into *E. coli* BL21 (DE3). The positive clones were verified by PCR and cultured in LB broth at 37 °C to an OD_{600} of approximately 0.6. Subsequently, IPTG was added to the medium at a final concentration of 0.1 mM, and culturing continued at 20 °C for an additional 12 h. The culture of the backbone plasmid pET-32a(+) was continued at 37 °C for an additional 5 h. Recombinant protein was purified under native conditions. In brief, the cells were harvested by centrifugation and the pellet was added 10 ml cold lysis buffer and frozen at −80 °C. After thawing at 4 °C, the cells were broken by sonication for 30 min and then the cell debris were removed by centrifugation, while the supernatant were collected to apply on the Ni-NTA agarose column (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions to purify recombinant protein. The purified proteins were analyzed by SDS-PAGE and measured using a Bradford assay with BSA as a standard as previously reported [14].

2.8. Chemotaxis assay

Peripheral blood leukocytes (PBLs) from *T. ovatus* were prepared with Percoll (GE Healthcare, Piscataway, NJ, USA) as described previously [14]. First, PBLs were used rapid Wright-Giemsa stain (Biosharp, Shanghai, China) to count and identify by microscope (Leica DMi8 GER) [43]. Subsequently, PBLs were resuspended in L-15 (Gibco, Grand Island, NY, USA) without fetal calf serum, and the trypan blue dye exclusion method was used to examine the viability of the cells. Chemotaxis assay was carried out in 24-well Costar Transwell plates (Corning Costar Co., Cambridge, MA, USA) as described previously [14]. Briefly, the cells were adjusted to 10^6 (cells/ml) in L-15 medium, and one hundred microliters of target cells was added to the upper Transwell chamber. rTroCCL4, rTrx, or PBS (control) was diluted to various concentrations in L-15 medium. Six hundred microliters of the various dose proteins or PBS was added to the lower Transwell chamber. The Costar Transwell plate was incubated at 26 °C for 40 min. The number of cells that migrated into the lower Transwell chamber was counted with a microscope to calculate the chemotactic index. Meanwhile, the migrated cells were collected for morphology identification using rapid Wright-Giemsa stain. To ensure that chemotaxis was induced by rTroCCL4, the same concentrations of rTroCCL4 were also added to both the upper and lower Transwell chambers as indicated above. Chemotaxis of rTroCCL4 and rTrx protein was expressed as the chemotactic index (CI), which is calculated as CI = No. of treatment group cells/No. of control cells. The assay was performed independently three times.

2.9. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay

MTT assays were performed to analyze the function of rTroCCL4. Briefly, head kidney lymphocytes (HKLs) were observed and counted with rapid Wright-Giemsa stain by microscope. Subsequently, HKLs in L-15 medium were adjusted and added to a 96-well plate with 10^5 cells

per well. The cell suspension was mixed with different concentrations of rTroCCL4, rTrx or PBS (control) at 26 °C for 24 h. The cells in the 96-well plate were observed by the microscope. After that, 20 μl of MTT solution (5 mg/ml) was added to the wells, and the mixture was incubated at 26 °C for 4 h. Then, the mixture was centrifuged (10 min, 1000 rpm), and the supernatants were carefully discarded. Finally, 200 μl of dimethyl sulfoxide (DMSO) was added to the wells. The absorbance was measured with a microplate reader at a wavelength of 490 nm. The results are expressed as proliferation indices, which were calculated as follows: A_{490} of protein-treated cells/A_{490} of control cells. The assay was performed independently three times.

2.10. Antimicrobial activity of rTroCCL4

To examine whether rTroCCL4 had antimicrobial activity, colony counting was performed [44]. Briefly, *E. coli* or *E. tarda* was cultured to an $OD_{600} \approx 0.8$ in LB broth. The bacteria were collected, and the concentrations were adjusted in PBS; then, 100 μl of rTroCCL4, rTrx (150 μg/ml, in PBS) or PBS was incubated with 5×10^4 CFU *E. coli* or *E. tarda* for 4 h, respectively. The samples were then inoculated on plates and incubated for 12 h. The number of bacteria was determined by plate counting.

2.11. TroCCL4 overexpression in fish tissues

The overexpression plasmid pTroCCL4 was constructed. The plasmids were prepared with the EndoFree Plasmid Kit (Tiangen, Dalian, China), and the integrity was checked by agarose gel electrophoresis. The purity was measured spectrophotometrically based on $A_{260/280}$ and $A_{260/230}$ absorbance ratios. The pTroCCL4 and pCN3 plasmid concentrations were adjusted to 150 μg/ml with PBS. After that, the fish were injected intramuscularly with 100 μl of pTroCCL4, pCN3 or PBS (5 fish/group). DNA was prepared using the Tissue DNA Kit (Omega, USA). The primer pair TroCCL4-F1/CN-R1 (Table 1) were used in PCR reactions to detect the plasmids pTroCCL4 or CN−F1/CN-R1 (Table 1) were used in PCR reactions to detect the plasmids pCN3 in tissues. In addition, RNA was extracted and cDNA was synthesized using the RNA extraction and Reverse Transcriptase kit (Takara, Dalian, China). The primer pair TroCCL4-F1/His-R (Table 1) was used to confirm the expression of *TroCCL4* in tissues by RT-PCR as previously reported [45].

2.12. Macrophage activity

2.12.1. Preparation of head kidney (HK) macrophages

T. ovatus (average weight 250 ± 20 g) were injected intramuscularly with pTroCCL4 (100 μg/fish) or with pCN3 (100 μg/fish) as a control [46–49]. After 5 days, the fish were sacrificed, and the head kidneys were collected aseptically. Preparation of HK macrophages (HKMs) was performed. Briefly, HKMs were washed three times with PBS and passed through a sterile metal mesh. The cells were extracted with a 34/51% Percoll (GE Healthcare, Piscataway, NJ, USA) gradient, followed by centrifugation at 800 × *g* for 30 min. After centrifugation, the cells were washed twice and resuspended in L-15 medium containing 0.1% calf serum (Gibco, Grand Island, NY, USA), 1% penicillin and streptomycin (Gibco, Grand Island, NY, USA), and 20 U/ml heparin (Sigma-Aldrich, St. Louis, MO, USA).

2.12.2. Respiratory burst activity and alkaline phosphatase activity

HKMs were added to 96-well microplates (3×10^5 cells/well) and incubated at 26 °C for 5 h. Respiratory burst activity of the HKMs was determined with a nitroblue tetrazolium (NBT, Sigma-Aldrich) assay as described in previous studies [45]. Spectrophotometric results were measured at 630 nm, and KOH/DMSO was used as a blank. Alkaline phosphatase (AKP) activity was measured with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions.

2.13. Immune high expressed expression

Trachinotus ovatus were injected intramuscularly with pTroCCL4 or pCN3 (as a control) as described above. Spleens were taken from the fish at 5 days post-injection. Total RNA was prepared as described above, and a qRT-PCR analysis of the expression of interleukin 1-β (IL-1β), interleukin 15 (IL15), interferon-induced Mx protein (Mx), tumor necrosis factor α (TNFα), complement C3, major histocompatibility complex (MHC) class Iα and class IIα, and immunoglobulin M (IgM) and D (IgD) was performed. The PCR primers for the immune genes are listed in Table 1.

2.14. TroCCL4 overexpression and E. tarda infection

To examine the effects of TroCCL4 against bacterial infection, *T. ovatus* were injected intramuscularly with pTroCCL4 (15 μg/fish), pCN3 (15 μg/fish), or PBS (100 μl/fish). After 5 days, the fish were infected with 100 μl E. *tarda* (10^6 CFU/ml) via intraperitoneal injection. At 6 h, 12 h, and 24 h post-infection, the head kidney, spleen, and liver (5 fish at each time point) were harvested under aseptic conditions. The number of bacteria in the tissue was determined by plate counting. The experiment was repeated three times independently.

2.15. TroCCL4 knockdown and E. tarda infection

Knockdown of TroCCL4 was achieved via injection of synthesized siRNA (siTroCCL4) *in vivo*. siRNA interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, preventing translation. The siRNA was synthesized with a T7 RiboMAX™ Express RNAi System (Promega, Madison, USA) according to the manual and as described in a previous study [50]. In brief, two pairs of primers, siTroCCL4-P1 and P2 and siTroCCL4-P3 and P4 (Table 1), containing the target sequence plus the T7 RNA polymerase promoter sequence and 6 extra nucleotides upstream of the minimal promoter sequence were designed to obtain two DNA oligonucleotides after incubation at 90–95 °C for 3–5 min. Then, the templates were allowed to cool slowly to room temperature. Next, the two DNA oligonucleotides were used to separately synthesize either the sense strand RNA or the antisense strand RNA templates at 37 °C for 2 h. Afterwards, the DNA template was removed from the separate short RNA strands by digestion with *DNase*, and then the two RNA strands were mixed to synthesize the siRNA. Finally, the synthesized siRNA was purified following the manufacturer's instructions. The control siRNA (siTroCCL4-C) was synthesized with two pairs of primers, siTroCCL4-C−P1 and P2 and siTroCCL4-C−P3 and P4 (Table 1), as described above.

Trachinotus ovatus were injected intramuscularly with 100 μl of siTroCCL4 (approximately 20 μg), siTroCCL4-C (approximately 20 μg), or PBS. TroCCL4-RT-F/R primers (Table 1) were used to verify the expression of *TroCCL4* in tissues by qRT-PCR as previously reported [39]. A total of 12 h after the first injection with siRNA, fish were infected with 100 μl of *E. tarda* (10^6 CFU/ml) via intraperitoneal injection. At 6 h, 12 h, and 24 h post-infection, the head kidney, spleen, and liver (5 fish at each time point) were harvested aseptically. Assessment of the bacterial loads was conducted as described above. The experiment was repeated three times independently.

2.16. Statistical analysis

Statistical analyses were performed using SPSS 17.0 software (IBM-SPSS. Inc., Chicago, IL, USA). The data were analyzed with GraphPad Prism 5, and statistical significance was defined as a probability (p) value < 0.05.

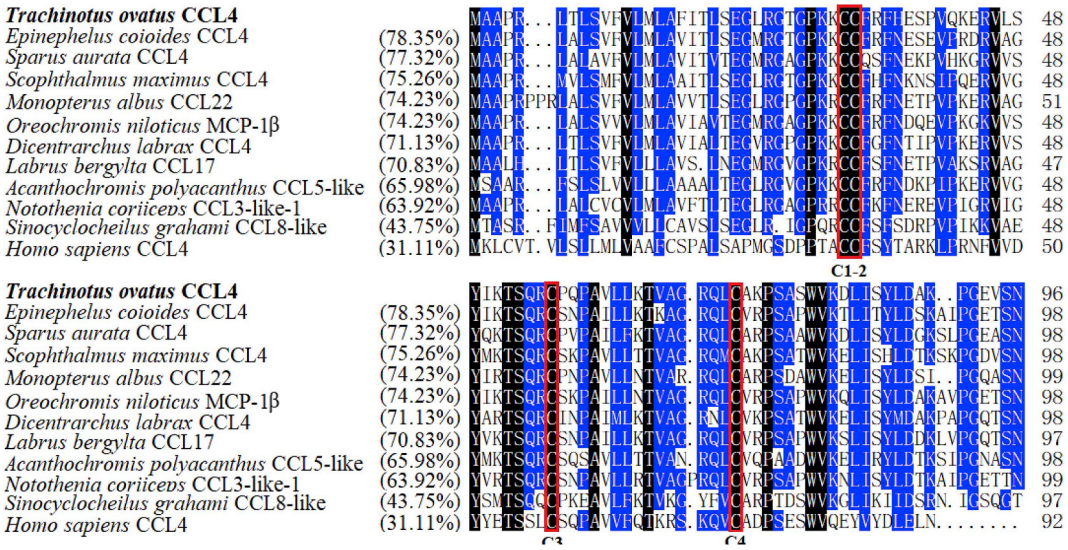

Fig. 1. Alignment of the predicted amino acid sequences of TroCCL4 homologues. Numbers in brackets indicate overall sequence identities between TroCCL4 and the compared sequences. The conserved cysteine residues are boxed. The consensus residues are in balck and the residues that are ≥75% identical among the aligned sequences are in blue. The GenBank accession numbers of the aligned sequences are as follows: *Epinephelus coioides*, AFN58329.1; *Sparus aurata*, CAO78735.1; *Scophthalmus maximus*, AWP07929.1; *Monopterus albus*, XP_020441352.1; *Oreochromis niloticus*, XP_003439451.2; *Dicentrarchus labrax*, CAM32187.1; *Labrus bergylta*, XP_020508427.1; *Acanthochromis polyacanthus*, XP_022077334.1; *Notothenia coriiceps*, XP_010783000.1; *Sinocyclocheilus grahami*, XP_016133073.1; *Homo sapiens*, CAG46916.1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Sequence and homology analysis of TroCCL4

The open reading frame of TroCCL4 (GenBank accession no. MK069501) was 294 bp long and encoded a putative peptide comprising 97 amino acids and a signal peptide with 23 amino acids. TroCCL4 harbored four conserved cysteine residues, similar to other CC chemokines (Fig. 1). The putative molecular mass was 10.6 kD, and the theoretical isoelectric point was 9.699. BLAST analysis showed that TroCCL4 shared high similarity in amino acid sequence with the CCL4 sequences of *Epinephelus coioides* (78.35%), *S. aurata* (77.32%), and *Scophthalmus maximus* (75.26%) and 31.11% identity with the CCL4 sequence of *Homo sapiens* (Fig. 1). The phylogenetic tree was constructed with 124 sequences of the CC chemokines from teleosts and mammals. A phylogenetic analysis showed that TroCCL4 clusters with the MIP subgroup (Fig. 2).

3.2. Expression of TroCCL4 in fish tissues under normal conditions

The expression profiles of *TroCCL4* under normal conditions were examined via qRT-PCR. *TroCCL4* was expressed in all examined tissues and high expressed (more than 5-fold greater than that in the tissue with the lowest expression) in the spleen (~11.8-fold greater), liver (~7.6-fold greater), skin (~7.1-fold greater), head kidney (~6.2-fold greater), gill (~5.6-fold greater), and brain (~5.5-fold greater). The expression of TroCCL4 was lowest in the heart (set as 1) (Fig. 3).

3.3. Expression of TroCCL4 in fish tissues following bacterial infection

qRT-PCR analysis showed that following *E. tarda* infection, the expression of *TroCCL4* was significantly upregulated at 24 h, and 48 h post-infection in the spleen, liver, and head kidney. Peak induction (~113.6-fold increase) in the spleen occurred at 48 h post infection (Fig. 4A). Similarly, the maximum expression level of *TroCCL4* in the liver was ~140.0-fold greater than control levels at 48 h post-infection, and that in the head kidney was ~30.4-fold greater than control levels at 48 h post-infection (Fig. 4B and C).

3.4. rTroCCL4 chemotactic activity and effect on cellular proliferation

rTroCCL4 and rTrx were purified using nickel nitrilotriacetic acid columns, and SDS-PAGE showed that the purified proteins matched the predicted molecular masses (Fig. S1). Before the chemotactic activity experiment, PBLs were observed with rapid Wright-Giemsa stain by microscope (Fig. 5A). A chemotactic activity analysis revealed that the migration of *T. ovatus* PBLs was dependent on the dose of the rTroCCL4 protein (Fig. 5B). In contrast, no PBLs migrated with rTrx. In addition, no PBLs migrated when rTroCCL4 was present in both the upper and lower Transwell chambers. Meanwhile, under microscope, the number of migrated cells induced by rTroCCL4 (40 μg/ml) was significantly more than the number of migrated cells induced by rTrx or PBS (Fig. 5C, D and E). These results indicated that PBLs migration was induced by rTroCCL4 chemotaxis.

To determine whether rTroCCL4 had any effect on the cellular proliferation of HKLs, rTroCCL4 or rTrx was incubated with lymphocytes. Before the MTT experiment, HKLs were observed with rapid Wright-Giemsa stain by microscope (Fig. 6A). The MTT assay results showed that the interaction of rTroCCL4 with lymphocyte cells enhanced cell proliferation in a dose-dependent manner (Fig. 6B). Meanwhile, under microscope, the number of cells induced by rTroCCL4 (40 μg/ml) was significantly more than the number of cells induced by rTrx or PBS in 96-well microplates (Fig. 6C, D and E).

3.5. Antimicrobial activity of rTroCCL4

To examine whether rTroCCL4 had antimicrobial activity, rTroCCL4, rTrx or PBS was incubated with *E. coli* or *E. tarda* bacteria for 4 h. The results of plate counting showed that rTroCCL4 significantly inhibited the growth of *E. coli* or *E. tarda* compared with that of the control group (PBS) (Fig. 7). In contrast, the number of bacteria incubated with rTrx was comparable to that in the control group (Fig. 7).

3.6. Effect of TroCCL4 overexpression on the immune response of T. ovatus

3.6.1. Overexpression of TroCCL4

TroCCL4 was overexpressed by injecting *T. ovatus* with pTroCCL4, which expresses His-tagged TroCCL4, or the control plasmid pCN3. At 5

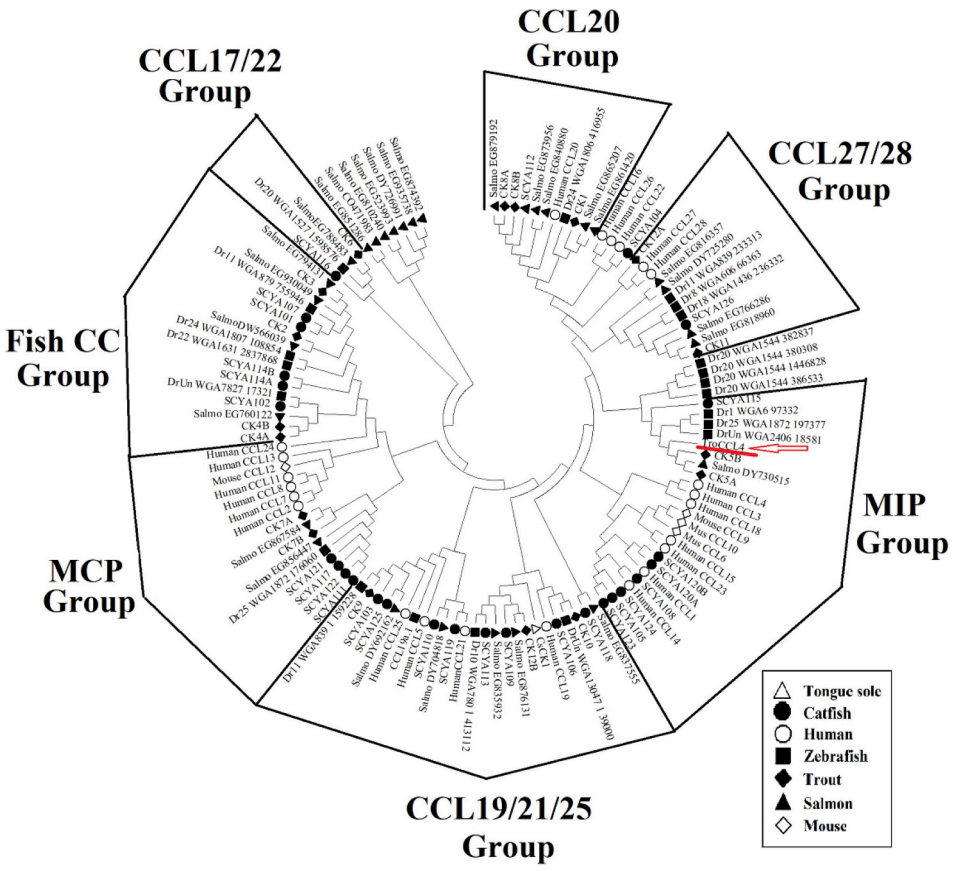

Fig. 2. Phylogenetic analysis of TroCCL4. The phylogenetic tree was drawn from a ClustalW-generated multiple sequence alignment of 124 CC chemokines using the neighbor joining method of the MEGA 6.0 package. The chemokine sequences and phylogenetic groups were named based on a previous report [19]. Zebrafish (Dr-), catfish (SCYA-), trout (CK—), salmon (Salmo), human, mouse, and tongue sole (Cs—) are donated using the shape and color as shown in the legend. Data were analyzed using p-distance, with gaps removed by pairwise deletion. The topological stability of the tree was evaluated by 1000 bootstrap replications. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

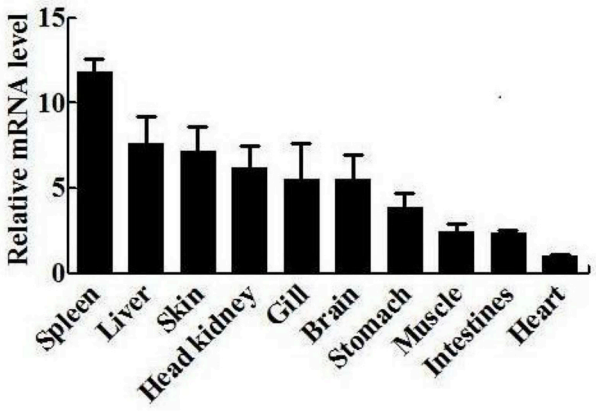

Fig. 3. *TroCCL4* expression in fish tissues under normal physiological conditions. *TroCCL4* expression levels in spleen, liver, skin, head kidney, gill, brain, stomach, muscle, intestine, and heart from *T. ovatus* were determined using qRT-PCR. The expression level of *TroCCL4* in the heart was set as 1. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed.

days post administration, the RT-PCR detected mRNA specific to pTroCCL4-encoded *TroCCL4* in spleen of pTroCCL4-administered fish but not in the tissues of pCN3-administered fish (Fig. S2). These results suggested that the *TroCCL4* gene was successfully expressed *in vivo* by the plasmid pTroCCL4.

3.6.2. Effect of TroCCL4 overexpression on macrophage activation

An immune response analysis showed that the macrophages from the fish administered pTroCCL4 exhibited significantly enhanced respiratory burst (RB) activity and AKP activity compared to those from the fish administered pCN3 (Fig. 8A and B).

3.6.3. Effect of TroCCL4 overexpression on immune-related gene expression

To examine the effect of TroCCL4 overexpression on immune-related gene expression, the expression of several immune genes, including IL-1β, IL15, Mx, TNFα, C3, MHC Iα, MHC IIα, IgM, and IgD, were analyzed via qRT-PCR. The results showed that compared with the fish administered pCN3, the fish injected with pTroCCL4 exhibited significantly enhanced expression of IL-1β, IL15, Mx, TNFα, C3, MHC Iα, and MHC IIα (Fig. 8C).

3.7. Effect of TroCCL4 overexpression on bacterial infection

To examine the effect of TroCCL4 overexpression on bacterial infection, fish were infected with *E. tarda* at 5 days post-plasmid administration, and bacterial loads in the spleen, liver, and head kidney tissues were determined at 6 h, 12 h, and 24 h post-infection. The results revealed a significantly lower number of bacteria in the three examined tissues from the pTroCCL4-administered fish than in the tissues from the control fish (PBS). In contrast, the number of bacteria in the tissues from the pCN3-administered fish was comparable to that in the tissues from the control fish (Fig. 9).

3.8. TroCCL4 knockdown and its effect on bacterial infection

3.8.1. TroCCL4 knockdown

To better understand the effect of TroCCL4 on antibacterial immunity, TroCCL4 was knocked down via RNA interference. Briefly, fish were injected with siTroCCL4, which was synthesized with the T7 RiboMAX™ Express RNAi System. *TroCCL4* expression in the spleen was determined by qRT-PCR at 12 h post-plasmid administration. The results showed that *TroCCL4* expression in the spleen of fish administered siTroCCL4 was significantly reduced compared to that in the spleen of

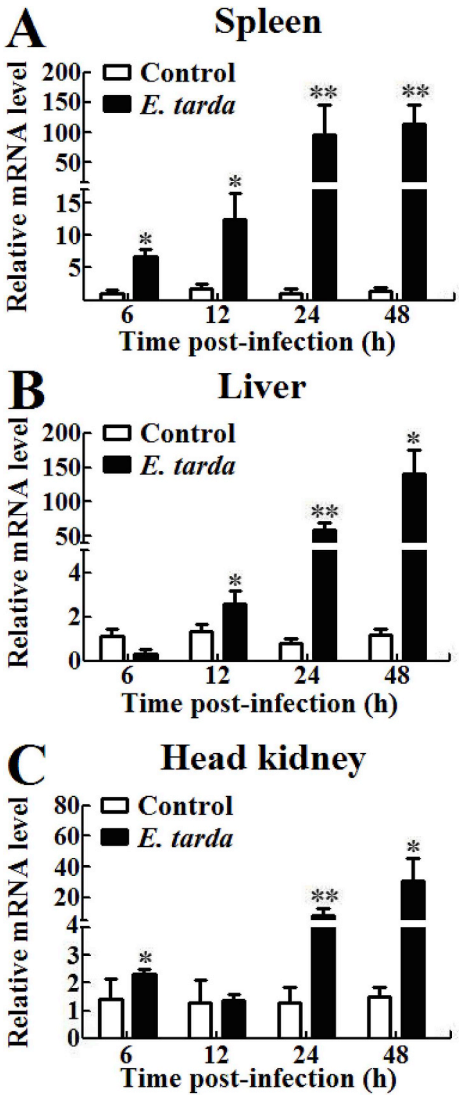

Fig. 4. *TroCCL4* expression during bacterial infection. *Trachinotus ovatus* were infected with or without (control) *Edwardsiella tarda*, and *TroCCL4* expression in spleen (A), liver (B), and head kidney (C) was determined by qRT-PCR at various time points. In each case, the expression level of the control fish at 6 h was set as 1. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. *P < 0.05, **P < 0.01.

the control fish (PBS), whereas *TroCCL4* expression in the spleen of *T. ovatus* administered siTroCCL4-C was comparable to that in the spleen of the control fish (Fig. S3).

3.8.2. Effect of TroCCL4 knockdown on bacterial infection

Fish administered siTroCCL4, siTroCCL4-C, or PBS (as control) were infected with *E. tarda,* and the bacterial numbers in spleen, liver, and head kidney tissues were determined at 6 h, 12 h, and 24 h post-infection. The results showed that the number of bacteria in the fish administered siTroCCL4 was significantly higher than that in the control fish at all time points, whereas the number of bacteria in the fish administered siTroCCL4-C was comparable to that in the control fish (Fig. 10).

4. Discussion

Chemokines are small chemotactic cytokines that have been proven to play key roles in cell migration and activation, particularly under inflammatory conditions, and to act as a bridge between innate and adaptive responses [1–4]. To date, a large number of CC chemokines have been identified in teleosts [6–14]. Nevertheless, only a limited number of studies have addressed the immunological roles and biological functions of CC chemokines in teleosts. In the present study, we characterized the *TroCCL4* from *T. ovatus* and explored its functions.

Sequence analysis results demonstrated that four cysteine residues in TroCCL4 are completely conserved compared with other known CCL4 sequences of mammals and fish, and these four cysteine residues can form two intramolecular disulfide bridges that serve to stabilize the molecule [21,28–30,51]. The homology alignment results revealed that TroCCL4 presents the highest amino acid sequence identity with CCL4 from *E. coioides*. To further understand the genetic relatedness of TroCCL4 to other known CC chemokines, a phylogenetic tree was constructed based on amino acid sequences with which CC chemokines have been classified into seven groups [19,52,53]. The phylogenetic analysis results showed that TroCCL4 was phylogenetically clustered in the MIP group. Our findings suggest that TroCCL4 was a member of the MIP group.

Chemokines were categorized into two subfamilies based on their function: homeostatic chemokines and inflammatory chemokines [6,54]. Homeostatic chemokines were usually expressed in specific tissues and cells and mediated the migration of hematopoietic precursor cells and other types of cells, whereas the expression of inflammatory chemokines was exclusively triggered under pathological conditions, such as infection and inflammation [2,54]. As more immune functions have been found, some CC chemokines have been considered to have a dual role [8,40]. In the present study, TroCCL4 was expressed in all ten examined tissues, especially in immune-related organs. Similarly, in *Megalobrama amblycephala*, *CCL4* was more highly expressed in the liver, intestine, spleen and gill than in other tissues [30]. In *Plecoglossus altivelis*, higher expression of *CCL4-like* was observed in the liver, spleen, and head kidney than in other tissues [51]. This ubiquitous expression profile has also been found in *E. coioides* [28], *P. olivaceus* [29], and *Rachycentron canadum* [55]. Most studies have revealed that CCL4 is ubiquitously expressed in various tissues in many teleosts. When fish were infected with *E. tarda*, *TroCCL4* expression was significantly increased in the spleen, liver, and head kidney. Many previous studies have found the same expression pattern of CCL4 after bacterial, LPS, or poly (I:C) stimulation [28,56,57]. In mammals, CCL4 was described as an inflammatory or inducible chemokine and was elicited following some types of stimulation [56]. The results in teleosts indicate that TroCCL4 may have both homeostatic and inflammatory functions and may be involved in the antibacterial immune response of *T. ovatus*.

CCL4 binds to its major receptors, initiates the migration of immune cells and dendritic cell maturation, and enhances lymphocyte, granulocyte and T cell activation, differentiation, and proliferation [22–24]. In teleosts, a few fish chemokines have been reported to induce the migration of leukocytes. For example, gCCL4 in orange-spotted grouper [28], RbCC1 in rock bream [26], SmCCL19 in turbot [21], CK1 and CK6 in rainbow trout [58,59], SCYA104 and JFCCL3 in Japanese flounder [29,60], and CsCCL17 and CsCCL21 in tongue sole [14,40] possess chemotactic activity toward leukocytes. Similarly, in the current study, we found that rTroCCL4 was able to induce the migration of PBLs from *T. ovatus*, and this effect was dose-dependent. In addition, we found that rTroCCL4 significantly elevated the proliferative activity of HKLs from *T. ovatus*. These results indicate that similar to mammalian CCL4, rTroCCL4 may have an impact on the migration and proliferation of some immune cells. In addition, increasing evidence indicates that some chemokines have antimicrobial activity [25–27]. For example, Hieshima et al. demonstrated that human and mouse CCL28 has potent antimicrobial activity against *Candida albicans*, gram-negative bacteria,

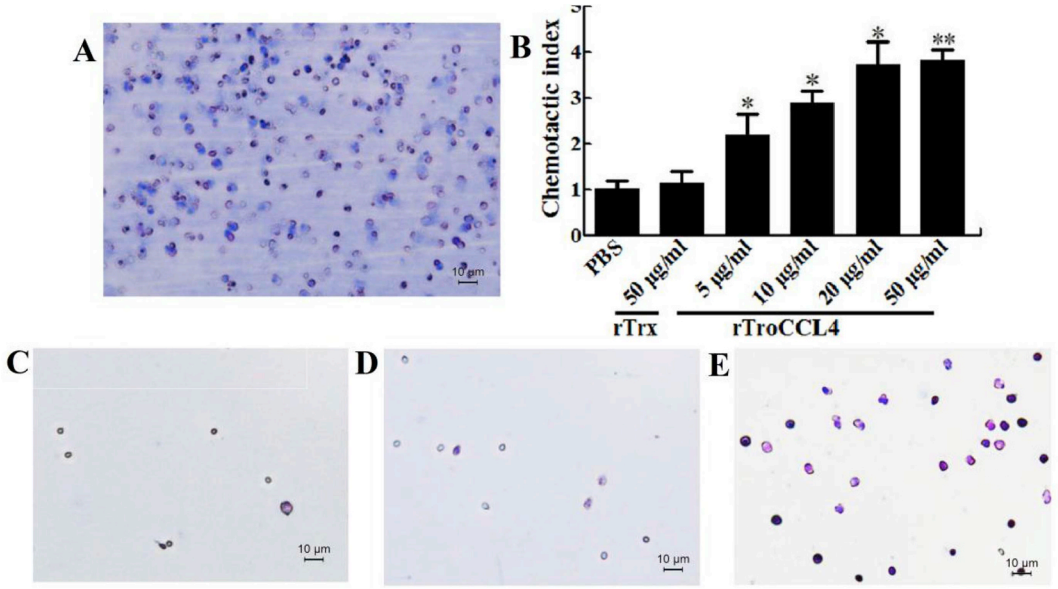

Fig. 5. Chemotactic activity of rTroCCL4 on peripheral blood cells (PBLs). The PBLs were extracted from *T. ovatus* and stained by Wright-Giemsa (A). The chemotactic activity of rTroCCL4 or rTrx with various concentrations against PBLs was determined using Transwell migration assay. Chemotactic index was presented. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. *P < 0.05; **P < 0.01 (B). The migrated PBLs induced by PBS were stained by Wright-Giemsa (C). The migrated PBLs induced by 50 μg/mL rTrx were stained by Wright-Giemsa (D). The migrated PBLs induced by 50 μg/mL rTroCCL4 were stained by Wright-Giemsa (E).

and gram-positive bacteria [25]. Consistent with those findings, we found that rTroCCL4 could significantly inhibit the growth of *E. coli* and *E. tarda*, suggesting that rTroCCL4 exhibits antimicrobial activity.

Previous studies in mammals and teleosts have shown that CCL4 can attract dendritic cells, macrophages, monocytes, natural killer cells, and T cells to the site of inflammation and activate them and enhance the secretion of a variety of cytokines, such as IL-6, IL-1β, and TNFα [40,61–64]. In the present study, the results of *in vivo* analysis showed that the HKMs from *T. ovatus* treated with the plasmid pTroCCL4, which expressed TroCCL4, had significantly higher respiratory burst and AKP activity than the control, suggesting that TroCCL4 overexpression evoked macrophage activation. Furthermore, overexpression of TroCCL4 enhanced the expression of IL-1β, IL15, Mx, TNFα, complement C3, and MHC Iα and IIα. Consistent with our results, previous

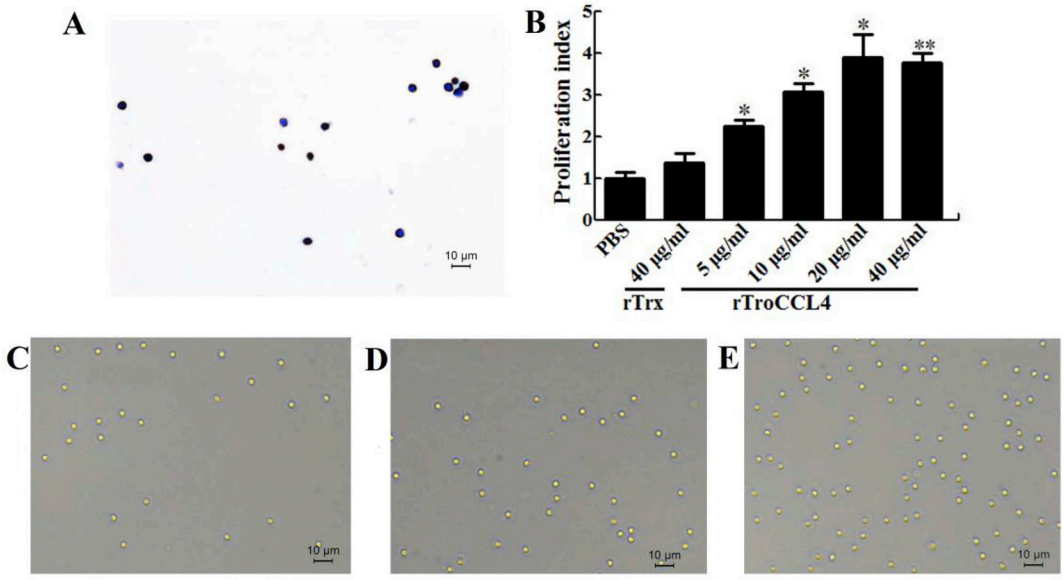

Fig. 6. Effect of rTroCCL4 on the proliferation of lymphocytes. The extracted lymphocytes were stained by Wright-Giemsa staining (Biosharp, Shanghai, China) from the head kidney (HKLs) in *T. ovatus* (A). The lymphocytes were incubated with different concentrations of rTroCCL4, rTrx or PBS (control), and cellular proliferation was determined by MTT assay. Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. *P < 0.05; **P < 0.01 (B). The lymphocytes were observed by microscope after incubated with PBS for 24 h in 96-well plate (C). The lymphocytes were observed by microscope after incubated with 40 μg/mL rTrx for 24 h in 96-well plate (D). The lymphocytes were observed by microscope after incubated with 40 μg/mL rTroCCL4 for 24 h in 96-well plate (E).

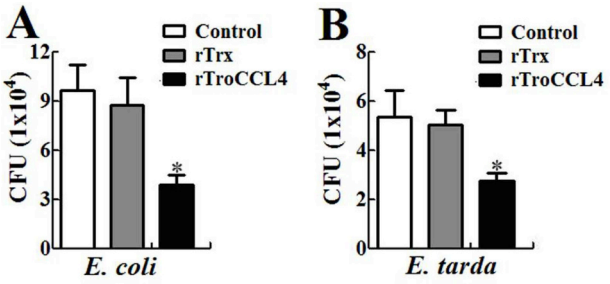

Fig. 7. Antibacterial activity of rTroCCL4. Bacterial growth was evaluated using solid culture bacterial growth inhibition test of *Escherichia coli* and *Edwardsiella tarda* with PBS (control), rTrx or rTroCCL4. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. *P < 0.05, **P < 0.01.

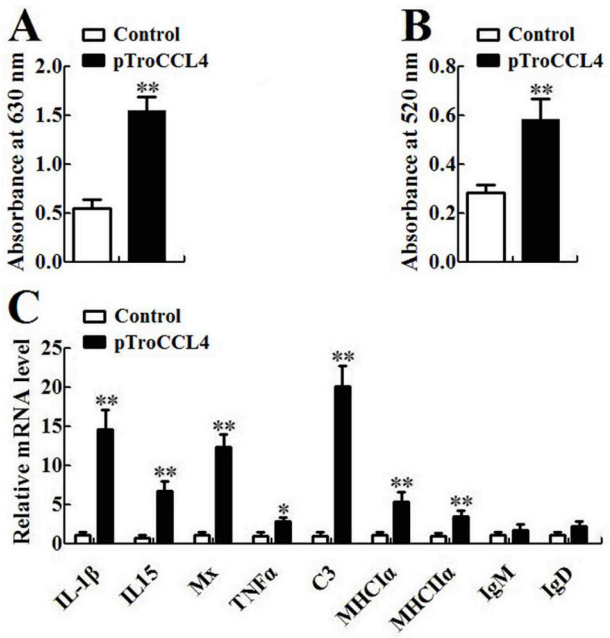

Fig. 8. Effect of *TroCCL4* overexpression on immune response. Macrophages from *T. ovatus* administered with pTroCCL4 and pCN3 (control) were examined for absorbance of respiratory burst at OD_{630} (A) and absorbance of alkaline phosphatase at OD_{520} (B). Expression of immune genes in spleen was determined by qRT-PCR, with the mRNA level of the control fish administered with PBS as 1 (C). Data are expressed as means ± SEM (N = 3). N, the number of times the experiment was performed. *P < 0.05, **P < 0.01.

studies showed that rCsCCL21 of tongue sole increased the expression levels of IL-1β, IL-6, IL-8 and TNFα; rSmCCL19 of turbot enhanced the expression of IL-1β, IL-8, TNFα, Mx, IL-22, and MHC IIα; rCK6 of rainbow trout upregulated Mx and type I INF; and gCCL4 of grouper induced the expression of TNFα1, TNFα2, T-β, and MX [28,36,40,65]. Thus, rTroCCL4 is likely able to enhance macrophage activation and induce inflammation in *T. ovatus*.

In related mammalian studies, chemokines were found to induce nonspecific immunity immediately in order to prevent bacterial infection *in vivo* [64,66,67]. Using mice as a model, Matsukawa et al. found that CCL2 participated in recruiting neutrophils and could effectively activate and mobilized peritoneal macrophages to protect the host against Mycobacterium [68,69]. In contrast, studies with CCL2-deficient mice demonstrated that CCL2 was essential for the migration of monocytes [70]. In our study, when TroCCL4 was overexpressed with pTroCCL4, we found that following *E. tarda* infection, the bacterial

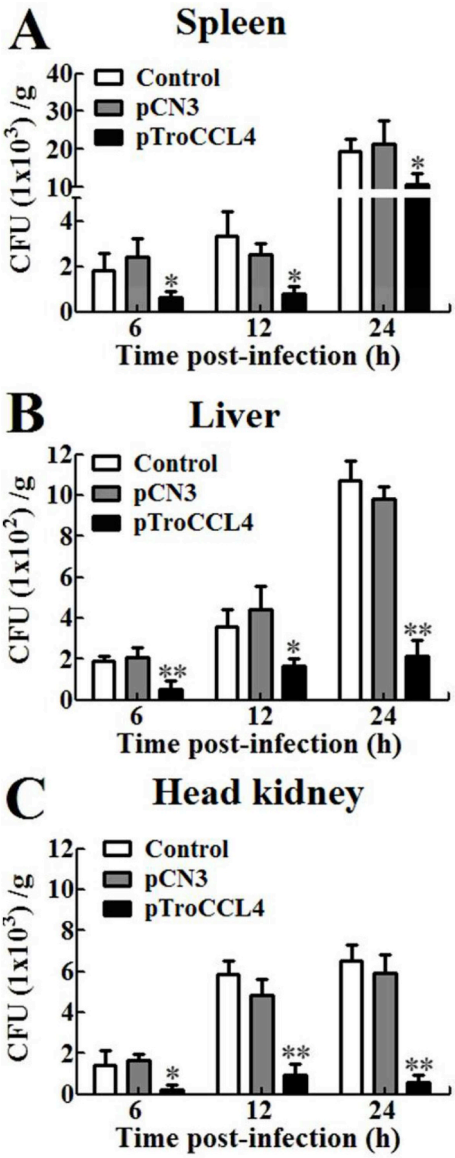

Fig. 9. Effect of *TroCCL4* overexpression on bacterial infection. *Trachinotus ovatus* administered with pTroCCL4, pCN3, or PBS (control) were infected with *Edwardsiella tarda*, and the amount of bacteria in spleen (A), liver (B), and head kidney (C) was determined at different time points. Data are expressed as means ± SEM (N = 3). N, the number of times the experiment was performed. *P < 0.05, **P < 0.01.

loads in pTroCCL4-administered fish were significantly lower than those in the control fish. In line with these results, inhibition of TroCCL4 expression with siTroCCL4 following *E. tarda* infection enhanced bacterial invasion into the spleen, liver, and head kidney. These results regarding the overexpression or inhibition of TroCCL4 expression *in vivo* further support the role of TroCCL4 in the antibacterial immune response of *T. ovatus*.

In conclusion, the results of our *in vivo* and *in vitro* studies demonstrate that TroCCL4 induces PBLs migration, promotes lymphocyte proliferation, inhibits bacterial growth, stimulates macrophage activation, and enhances host resistance to bacterial infection. Taken together, these results indicate that TroCCL4 may be involved in antibacterial immunity.

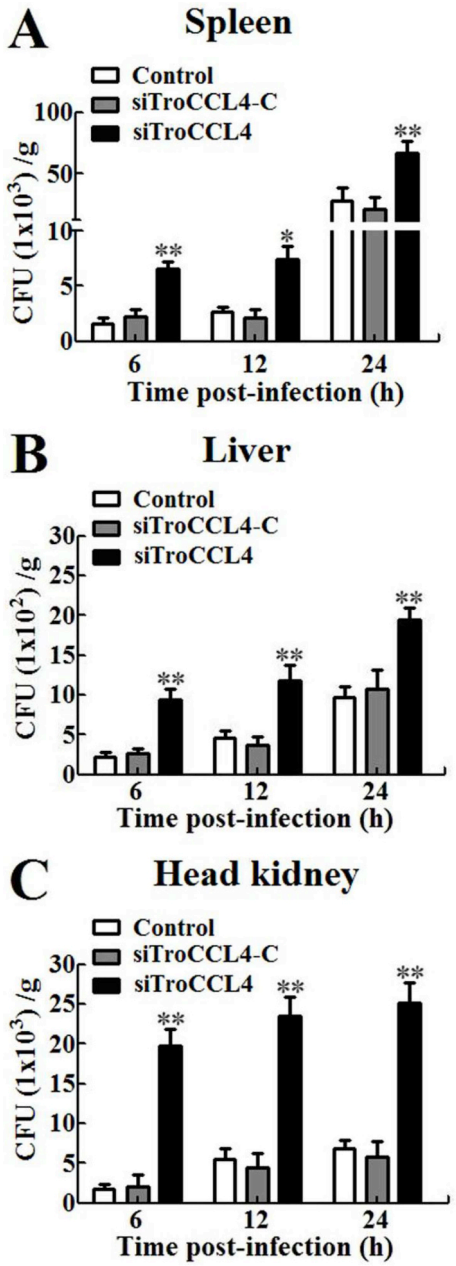

Fig. 10. Effect of *TroCCL4* knockdown on bacterial infection. *Trachinotus ovatus* administered with siTroCCL4, siTroCCL4-C, or PBS (control) were infected with *Edwardsiella tarda*, and the amount of bacteria in spleen (A), liver (B), and kidney (C) was determined at different time points. Data are expressed as the mean ± SEM (N = 3). N, the number of times the experiment was performed. *P < 0.05, **P < 0.01.

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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.2018.11.080.

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