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Granulocyte colony stimulating factor (GCSF) of Japanese flounder (*Paralichthys olivaceus*): Immunoregulatory property and anti-infectious function

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ABSTRACT

Granulocyte colony stimulating factor (GCSF) is a key regulator of neutrophil production, and plays a vital role in immune response of mammals and teleost against pathogen. Sequences of GCSF were identified in several teleost species, however, the function and activity of GCSF in teleost remain largely unknown. In this study, we examined the biological activity and the immunomodulatory property of a GCSF homologue, PoGCSF, from Japanese flounder (*Paralichthys olivaceus*). Structural analysis showed that PoGCSF possesses conserved structural characteristics of GCSF proteins, including a signal peptide and a typical IL-6 domain. The expression of *PoGCSF* was upregulated in a time-dependent manner by extracellular and intracellular bacterial pathogens and viral pathogen. Different expression patterns were exhibited in response to the infection of different types of microbial pathogens in different immune tissues. Recombinant PoGCSF increased the capability of host cells to defense against pathogen infection and enhanced the expression of immune related genes. The knockdown of PoGCSF attenuated the ability of host cells to eliminate pathogenic bacteria. *In vivo* results showed that overexpression of PoGCSF promoted the host defense against invading pathogenic microorganism. Collectively, this study is the first report about the immunoregulatory property and anti-infectious immunity of GCSF in teleost. These findings suggested that PoGCSF serves as an immune-related cytokine and plays an important role in the immune defense system of Japanese flounder.

1. Introduction

Colony-stimulating factors play a central role in mediating the development of pluripotent hematopoietic stem cells of the myeloid lineage [1]. Granulocyte-colony stimulating factor (G-CSF or GCSF), also known as colony-stimulating factor 3, is a glycoprotein that stimulates growth and differentiation of granulocyte precursor cells and production of granulocytes [2–4]. GCSF is mainly produced by bone marrow stromal cells, macrophages, epithelial cells, and endothelial cells [5]. The biological function of GCSF is embodied via its interactions with GCSF receptor (GCSFR). GCSFR expression is distributed widely in various cells including lymphocytes, granulocytes, monocytes, neutrophils, hematopoietic stem cells and so on, suggesting extensive pleiotropy of GCSF functionality [5–7].

GCSF has attracted much attention due to its roles in mediating the proliferation, survival, terminal maturation, functional activation of mammalian neutrophils which form an essential part of the innate immune system and differentiation of neutrophilic granulocytes, monocytes, macrophages and their respective progenitors [8,9]. GCSF has immunoregulatory effects on both innate immune cells and adaptive immune cells, especially T-cells [10–12]. Studies suggested that GCSF may polarize T cells from Th1 to Th2 phenotype by skewing cytokine secretion profile, including increasing interleukin-4 (IL-4) and IL-10 as well as decreasing IL-2 and interferon-γ [12]. GCSF acts as cytokine regulator of immunological cells such as granulocytes [13]. Studies in mammals showed that the cytokine GCSF had a predominant role in regulating the output of neutrophils from the bone marrow [14,15], and enhanced neutrophil function against bacteria [16–18].

In teleost, GCSF was identified in several species, including Japanese flounder (*Paralichthys olivaceus*), fugu (*Takifugu rubipes*),

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pufferfish (*Tetraodon nigroviridis*), zebrafish (*Danio rerio*), black rockfish (*Sebastes schlegelii*), and rock bream (*Oplegnathus fasciatus*) [4,19–21]. Only few functional studies have been performed. In zebrafish, it was found that two GCSFs played roles in hematopoietic development and maintenance [21]. The results of other fish revealed that the expression of GCSF was induced by LPS and pathogen infection, which indicated that GCSF participated in teleost immune response [19,20]. Japanese flounder is an important marine flatfish farmed worldwide, especially in Asian countries such as Japan, Korea, and China. Currently flounder has suffered serious diseases, and the studies of its responses to pathogen infection are limited. In a previous study, Santos et al. reported the cloning of a GCSF sequence (named PoGCSF in this study for convenience) from flounder. They identified the sequence of PoGCSF and found that PoGCSF expression existed in immune-related tissues and was upregulated by stimulation with LPS and ConA/PMA [19]. However, the function and activity of PoGCSF have not been investigated. In this study, expression patterns of *PoGCSF* upon post-pathogen infection were examined, and the immunoregulatory effect and antimicrobial activity of PoGCSF were investigated. This study will be helpful to further understanding of the biological functions of teleost GCSF in innate immunity.

2. Materials and methods

2.1. Fish

Clinically healthy Japanese flounder (average weight 14.4 ± 1.5 g) were purchased from a commercial fish farm in Shandong Province, China, and maintained at 24 °C in aerated seawater. Before experiments, fish were acclimatized in the laboratory for two weeks and verified to be free of pathogens in the liver, head kidney, and spleen, as reported previously [22]. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma-Aldrich Corporation, St. Louis, MO, USA), as reported previously [23].

2.2. Bacterial and viral strains

The fish pathogen *Edwardsiella tarda* has been reported previously [24]. *Vibrio anguillarum* and fish infectious spleen and kidney necrosis virus (ISKNV) were kindly provided by Doctor Min Zhang of Qingdao Agricultural University and viral proliferation were reported previously [25]. *Escherichia coli* was purchased from Transgene (Beijing, China). Bacteria strains were cultured in Luria-Bertani broth (LB) medium at 37 °C (for *E. coli*) or at 28 °C (all other microbes).

2.3. Sequence analysis

The sequence of PoGCSF was obtained by PCR from flounder head kidney cDNA with primers PoGCSFF1 and PoGCSFR1 (Table S1) based on the sequence reported by Santos et al. [19]. The sequence of PoGCSF was analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART). The calculated molecular mass and theoretical isoelectric point were predicated by EditSeq in DNASTAR software package.

2.4. Quantitative real time reverse transcription-PCR (RT-qPCR) analysis of PoGCSF expression under normal conditions

RT-qPCR analysis of *PoGCSF* expression under normal conditions was determined as follows. Total RNA from the spleen, liver, head kidney, blood, intestine, muscle, gill, and brain of five fish were extracted using the EZNA Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). RNA was digested with DNaseI. One microgram of total RNA was used for cDNA synthesis with RevertAid Fiest Strand cDNA Synthesis Kit (Thermo Scientific, USA). RT-qPCR was performed using a Roche Lightcycler 96 (Switzerland) using the SYBR ExScript RT-qPCR Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) [26]. The PCR reaction was performed in a 20 μl volume containing 10 μl SYBR® premix Ex Taq™, 0.2 μM of each specific primer pairs GCSFRTF/R, and 2 μl diluted cDNA. The PCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 20 s. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified. The expression level of *PoGCSF* was analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with beta-actin as an internal reference [27].

2.5. PoGCSF expression upon bacterial and viral infection

RT-qPCR analysis of *PoGCSF* expression during bacterial infection was performed as reported previously [26]. *V. anguillarum* and *E. tarda* were cultured in LB broth at 28 °C to an optical density of 0.8 at 600 nm. Then, the cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS to a concentration of 5×10^6 CFU (colony forming units)/ml. ISKNV was resuspended in PBS to a concentration of 1×10^6 copies/ml. Flounder were divided randomly into four groups (16 fish per group) and injected intraperitoneally with 50 μl V. *anguillarum, E. tarda,* ISKNV, or PBS. After infection, head kidney, spleen, and liver from three or four fish were taken aseptically at 6 h, 12 h, 24 h, 48 h and 72 h post-infection (hpi) for bacterial infection, and at 1 d, 3 d, 5 d, and 7 d post-infection (dpi) for viral infection. *PoGCSF* expression was determined by RT-qPCR, as described in section 2.4. The experiment was repeated three times.

2.6. Plasmid construction

The primers used in this study were listed in Table S1. To construct pEtPoGCSF, which expresses a His-tagged PoGCSF, the sequence of *PoGCSF* was amplified by PCR with primers PoGCSFF2 and PoGCSFR2, and the PCR products were ligated into pET 258 at the BamH I and Hind III site. To construct pCNPoGCSF, which expresses PoGCSF from the human cytomegalovirus immediate-early promoter, the coding sequence of PoGCSF was amplified with primers PoGCSFF3 and PoGCSFR3, and the PCR products were inserted into the eukaryotic expression vector pCN3 [28] at the EcoR V site.

2.7. Protein expression and purification

Recombinant PoGCSF (rPoGCSF) was purified as follows. *E. coli* BL21 (DE3) was transformed with the plasmids pEtPoGCSF; the transformants were cultured in LB medium at 37 °C to OD_{600} 0.5, and expression of the exogenous protein was induced by adding isopropyl-b-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After growing at 20 °C for an additional 8 h, the cells were harvested by centrifugation, and the His-tagged protein was purified under native conditions using glutathione sepharose columns (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. The purified protein was dialyzed in phosphate-buffered saline (PBS) and concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The protein was analyzed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of purified protein was determined using the Bradford method with bovine serum albumin as the standard.

2.8. Effect of rPoGCSF on bacterial infection

E. tarda was cultured to mid-logarithmic phase, washed, and resuspended in L-15 medium to 5×10^6 CFU/ml. Head kidney lymphocytes (HKLs) were prepared as described as before [29]. HKLs (1×10^5 cells/well) were added with rPoGCSF or PBS, and incubated at 23 °C for

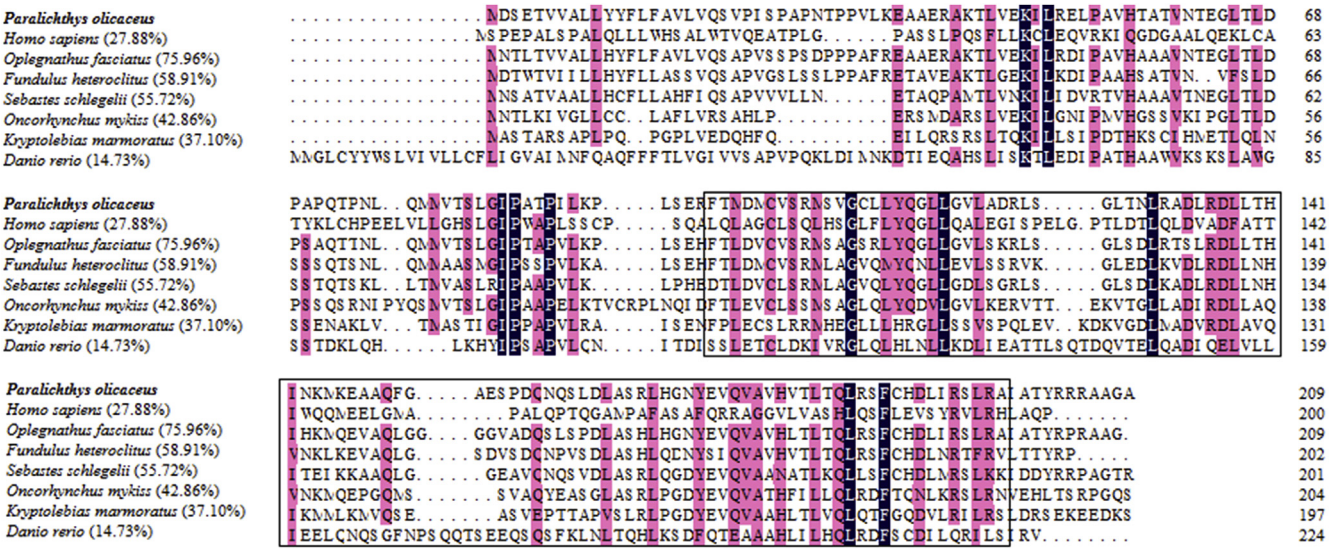

Fig. 1. Multiple sequence alignment of GCSF homologues. The percentage number in the bracket following each species name represents the overall sequence identity between PoGCSF and the specified species. The consensus residues are in dark blue, the residues that are ≥75% identical among the aligned sequences are in pink. The signal sequence is indicated by "-", and the IL-6 domain was in black box. The Gen Bank accession numbers of the aligned sequences are as follows: *Paralichthys olicaceus*, BAE95631; *Oplegnathus fasciatus*, ARP51376.1; *Fundulus heteroclitus*, XP_012708375.1; *Sebastes schlegelii*, BAH56612.1; *Oncorhynchus mykiss*, CAQ42965.1; *Kryptolebias marmoratus*, XP_017273871.1; *Danio rerio*, CAQ64749.1; *Homo sapiens,* AAH33245.1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2 h. After the incubation, the cells were mixed with *E. tarda* (100 μl/well). The plate was incubated at 23 °C for 6 h and cells were washed three times with PBS. The cells were lysed by adding 100 μl of 1% Triton X-100 to each well. The lysate was plated on LB agar plates. The plates were incubated at 28 °C for 24 h, and the colonies that emerged on the plates were counted. The assay was performed three times.

2.9. Effect of rPoGCSF on expression of immune related genes

After incubating with rPoGCSF for 2 h as described above, the HKLs were washed three times with PBS. Total RNA was prepared from the cells with Total RNA Kit I of Omega Bio-tek (Norcross, GA, USA) and used for RT-qPCR analysis of IL-6, IL-10, TNFα, and arginase (ARG) expression as described above. The assay was performed three times.

2.10. PoGCSF knockdown and its effect on bacterial infection

Knockdown of PoGCSF was achieved via injection of synthesized siRNA (*PoGCSF*-Ri). siRNA interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, preventing translation. The siRNA was synthesized with an In vitro Transcription T7 Kit (for siRNA Synthesis) (TaKaRa Biotechnology Co., Ltd., Dalian, China). In brief, two pairs of primers, siPoGCSF-F1/R1 and siPoGCSF-F2/R2 (Table S1), which contained 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 95 °C for 2 min. Then, the templates were allowed to cool to 25 °C during 45 min and maintain 10 min. Next, the two DNA oligonucleotides were used to transcription in vitro at 42 °C for 2 h following the manufacturer's instructions. Afterwards, the DNA template was removed from the separate short RNA strands by digestion with DNase. Finally, the synthesized siRNA was purified following the manufacturer's instructions. The control siRNA (PoGCSF-RiC) was synthesized with two pairs of primers siPoGCSF-CF1 and R1 and siPoGCSF-CF2 and R2 (Table S1) as given above.

Transfection was performed as reported previously [30]. Briefly, HKLs were distributed into two 96-well culture plates (1×10^5 cells/well) in L-15 medium without FBS. Transfection of the cells with PoGCSF-RiC, PoGCSF-Ri, and PBS were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions given by the manufacturer. After transfection for 24 h, plates were replaced with new medium containing 1×10^6 *E. tarda*. The plates were incubated at 23 °C for 2 h and washed three times with PBS. The bacterial number was determined as described above. The plates not treated with *E. tarda* were used to isolate the total RNA as described. The assay was performed three times.

2.11. In vivo effect of PoGCSF overexpression on bacterial infection

pCNPoGCSF and pCN3 were diluted in PBS to 200 μg/ml. Flounder were divided randomly into three groups (30 fish per group) and injected intramuscularly (i.m.) with 50 μl of pCNPoGCSF, pCN3, or PBS, respectively. At 5 d after plasmid administration, the fish were infected with *E. tarda* as described above. Head kidney and spleen were taken under aseptic conditions at 24 h and 48 h post-infection. Bacterial number in the tissues was determined by plate count [31]. Briefly, the tissue was homogenized in PBS and the homogenate was diluted serially, then the dilutions were plated on LB agar plates. The plates were incubated at 28 °C for 24 h, and the colonies that appeared on the plates were enumerated. The experiment was performed three times.

2.12. Statistical analysis

All statistical analyses were performed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with analysis of variance (ANOVA), and statistical significance was defined as $P < 0.05$.

3. Results

3.1. Sequence analysis and structure characteristics of PoGCSF

The cDNA sequence of the gene *PoGCSF* contains 633 bp ORF which codes 210 amino acid residues with a calculated molecular mass of 20.67 kDa and a theoretical pI of 9.30. PoGCSF contains a signal peptide sequence (residues 1 to 21) and an IL-6 domain (residues 98 to 197). The multiple sequence alignment showed that PoGCSF shares

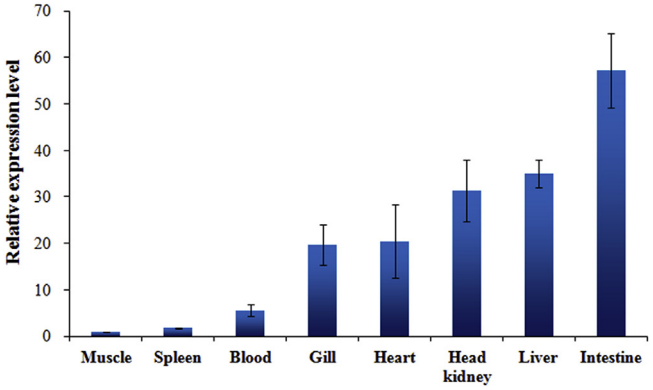

Fig. 2. *PoGCSF* expression in flounder tissues. *PoGCSF* expression in the muscle, spleen, blood, gill, heart, head kidney, liver, and intestine of flounder were determined by quantitative real time RT-PCR. For convenience of comparison, the expression level in muscle was set as 1. Data are the means of three independent assays and presented as means ± SEM (N = 3). N represents the number of times the experiment was performed.

high overall amino acid sequence identities with GCSF homologues of *O. fasciatus* (75.96%) and *Fundulus heteroclitus* (58.91%), moderate overall identities with GCSF homologues of *Oncorhynchus mykiss* (42.86%) and *Kryptolebias marmoratus* (37.10%), and low overall identity with GCSF homologues of *D. rerio* (14.73%). The identities of GCSF homologues between *P. olivaceus* and *Homo sapiens* is 27.88% (Fig. 1). The three-dimensional structure predicted by SWISS-MODEL indicated that the PoGCSF is mainly composed of α-helices (Fig. S1). The phylogenetic analysis was performed using the neighbor-joining (NJ) method of MEGA 7 program and the result showed that GCSF of *P. olicaceus* is closest with that of *Lates calcarifers*, which means they had a closer evolution position. (Fig. S2).

3.2. Expression of PoGCSF under normal physiological condition

RT-qPCR was carried out to examine the expression profile of *PoGCSF* in different tissues of flounder under normal physiological condition. The result showed that *PoGCSF* expression distributed in all the examined tissues, the highest three tissues are intestine, liver, and head kidney, followed by heart, gill, and blood, and the lowest two tissues are spleen and muscle (Fig. 2).

3.3. Expression profiles of PoGCSF upon experimental infection with bacterial and virus pathogens

To examine the expression patterns of the *PoGCSF* upon fish pathogen infection, flounder were challenged experimentally with *V. anguillarum*, *E. tarda*, and ISKNV. Total RNA was extracted from the tissues at different time and cDNA was synthesized. Then the expression of *PoGCSF* was determined by RT-qPCR. The results showed that expression patterns of *PoGCSF* in a manner depended on the nature of the pathogen, tissue type, and infection time. Specifically, upon the infection of *E. tarda*, *PoGCSF* expression in head kidney was significantly upregulated at all examined time points and peaked at 24 hpi (7.74-fold), then the expression level declined gradually. *PoGCSF* expression in spleen was significantly increased at all examined time points and peaked at 12 hpi (41.42-fold), then the expression level declined gradually. The expression of *PoGCSF* in liver was similar to that in head kidney with maximum induction was detected at 24 hpi (83.42-fold), except a low induction value appeared at 12 hpi (Fig. 3).

When infected by *V. anguillarum*, *PoGCSF* expressions in three tissues were all significantly induced at all examined time points except 48 and 72 hpi in liver. Maximum induction in head kidney, spleen, and liver was 11.61-fold (12 hpi), 38.80-fold (24 hpi), 262.96-fold (6 hpi), respectively. Compared to *E. tarda* infection, *V. anguillarum* infection caused higher induction and maximum induction arose earlier (Fig. 3).

During the infection of ISKNV, *PoGCSF* expressions in three tissues were all significantly enhanced at all examined time points. In head kidney, the highest expression of *PoGCSF* arose at 1 dpi (19.01-fold), then the expression declined gradually. However, in spleen and liver, the expression level of *PoGCSF* increased gradually from 1 dpi to 7 dpi, and maximum induction was 63.18- and 40.60-fold, respectively, at 7 dpi (Fig. 3). Compared to *E. tarda* and *V. anguillarum*, maximum induction difference in three tissues caused by ISKNV infection was smallest. These results indicated that *PoGCSF* could participate in the immune defense response of flounder to pathogens infection.

3.4. In vitro potential of rPoGCSF against bacterial infection and its effect on immune-related genes expression

Since PoGCSF involved in defense against pathogen, we want to know whether PoGCSF have any effect on immune cell activity. To detect this opinion, rPoGCSF was purified using nickel nitrilotriacetic acid columns, and SDS-PAGE showed that the purified proteins appeared as a single band with the predicted molecular masses (Fig. S3). We investigated the effect of rPoGCSF on host cell defense against bacterial pathogen. After incubating with PBS or rPoGCSF, HK lymphocytes (HKLs) were infected by *E. tarda*, then the bacteria infected with the host cells were enumerated. The results showed that numbers of *E. tarda* recovered from cells treated by rPoGCSF ($10 \pm 1.2 \times 10^3$ CFU) were enormously lower than those of control ($5.2 \pm 1.8 \times 10^4$ CFU), which indicated that rPoGCSF played a role in defense against *E. tarda* infection.

It is known that GCSF plays a role in regulation expressions of immune-related genes [32]. We speculated the effect of rPoGCSF on bacterial infection partly owing to its immunoregulatory function. To determine whether rPoGCSF has any effect on the expressions of cytokines such as IL-6, IL-10, TNFα, and ARG, RT-qPCR was performed after HKLs treated by rPoGCSF. The results showed that rPoGCSF enhanced the expressions of IL-6, IL-10, and TNFα, but not ARG expression. We also found that the expression of *PoGCSF* was enormously induced by rPoGCSF (Fig. 4). These results suggested that PoGCSF possessed the immunoregulatory properties.

3.5. PoGCSF knockdown and its effect on antibacterial in HKLs

Since, as observed above, rPoGCSF enhanced cells resistance to pathogen infection, we further examined the effect of *PoGCSF* knockdown on bacterial invasion. For this purpose, *PoGCSF*-Ri and *PoGCSF*-RiC (RNAi control) were synthetized and transfected into HKLs. The expression of PoGCSF was determined by RT-qPCR and the results showed that in *PoGCSF*-Ri-administered cells, the expression *PoGCSF* was significantly reduced compared to that in the control cells. The expression of *PoGCSF* in *PoGCSF*-RiC-administered cells was comparable to that in PBS-administered cells (NC) (Fig. 5A).

To examine the effect of *PoGCSF* knockdown on bacterial infection, HKLs treated with *PoGCSF*-Ri or *PoGCSF*-RiC were infected with *E. tarda*, and bacterial numbers were determined at 6 h post-infection. The results showed that cells administered with *PoGCSF*-Ri exhibited significantly increased bacterial amounts compared to control cells, whereas cells administered with *PoGCSF*-RiC exhibited comparative bacterial amounts to those in the control cell (Fig. 5B).

3.6. In vivo potential of PoGCSF overexpression against bacterial infection

With the above in vitro observations, we further investigated the in *vivo* effect of PoGCSF on host defense against bacterial pathogen. For this purpose, the eukaryotic expression plasmid pPoGCSF was constructed, which constitutively expresses PoGCSF. Flounder were injected with pPoGCSF or the control plasmid pCN3, which is well

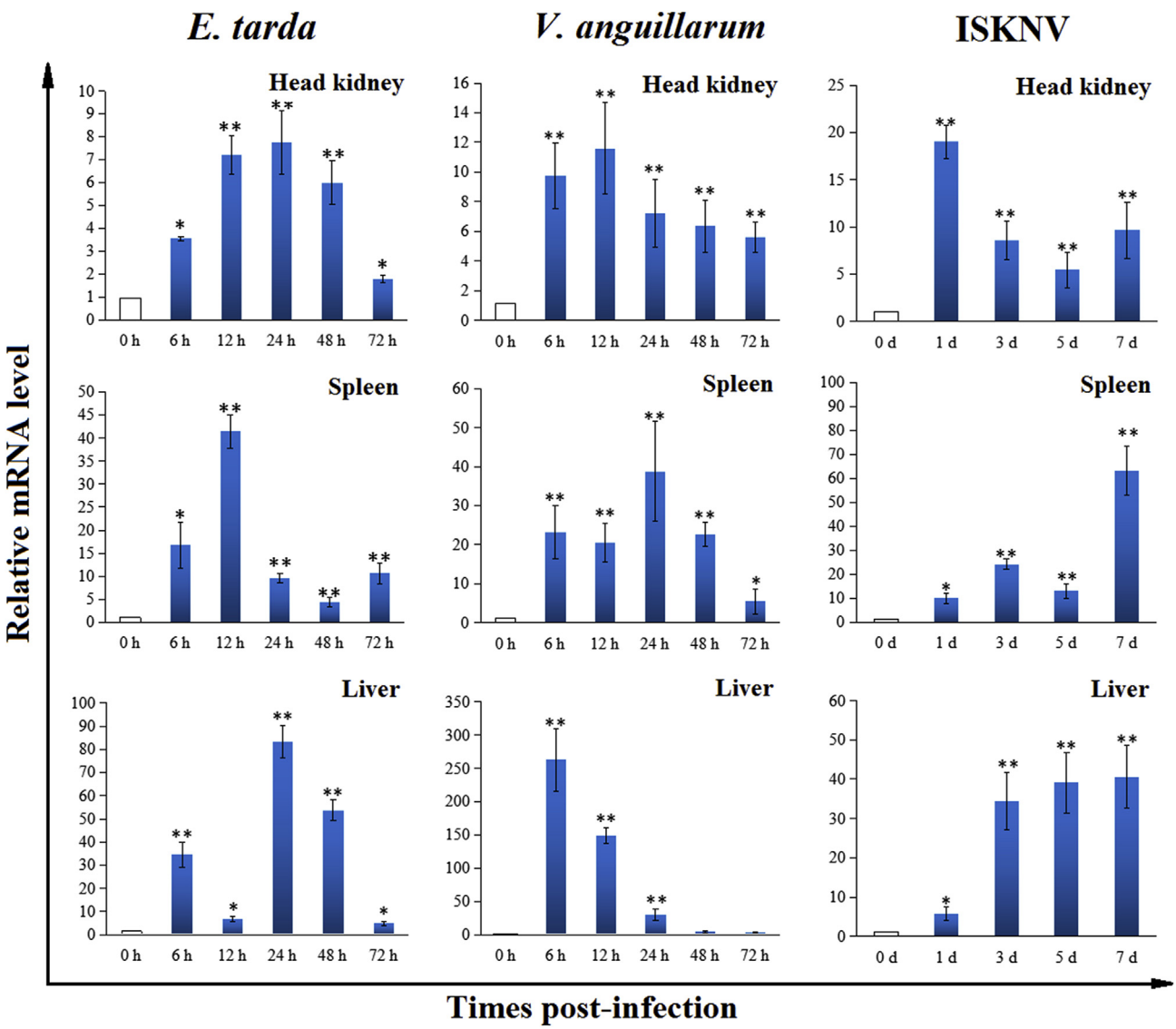

Fig. 3. *PoGCSF* expression in response to pathogens challenge. Japanese flounder were infected with the intracellular bacterial pathogen *Edwardsiella tarda*, the extracellular bacterial pathogen *Vibrio anguillarum*, the viral pathogen fish infectious spleen and kidney necrosis virus (ISKNV), or PBS (as the control). After infection, head kidney, spleen, and liver were taken aseptically at 6 h, 12 h, 24 h, 48 h and 72 h post-infection (hpi) for bacterial infection, and at 1 d, 3 d, 5 d, and 7 d post-infection (dpi) for viral infection. The *PoGCSF* expressions in three tissues were determined by RT-qPCR at various time points. In each case, the expression level at 0 h was set as 1. Values are shown as mean ± SEM (N = 3). N represents the number of times the experiment was performed. *, $P < 0.05$, **, $P < 0.01$.

verified that pCN3 and recombinant plasmids based pCN3 can exist steadily and express in flounder and other species of fish [33–36]. Then the fish were infected with *E. tarda* at 5 d post-plasmid injection, and bacterial amounts in kidney and spleen were determined at 24 h and 48 h post-infection. The results showed that in kidney and spleen, the bacterial numbers in pPoGCSF-administered fish were significantly lower than those in the control fish at both time points, respectively, whereas the bacterial numbers in pCN3-administered fish were comparable to those in the control fish (Fig. 6A). Consistently, the mortality of fish administered with pPoGCSF was significantly lower than that of fish administered with pCN3 or PBS (Fig. 6B).

4. Discussion

GCSF is an important cytokine with pleiotropic roles such as proliferation, survival, and differentiation of immunological cells including neutrophilic granulocytes, monocytes, macrophages and their respective progenitors, and participates extensively in antimicrobial immune response [8,12,37]. GCSF is wildly distributed in both mammals and teleost. Although sequence of GCSF of Japanese flounder was cloned as early as 2006 [19], its function has been unknown. In this study, we characterized the GCSF homologue, PoGCSF, from Japanese flounder, and examined its expression and biological properties. Structural analysis showed that PoGCSF possesses conserved structural characteristics of GCSF proteins, including a signal peptide and a typical IL-6 domain, which is a common characteristic of GCSF family members and is involved in mediation of acute phase response such as microbial infection [13]. The amino acid sequence of PoGCSF shares moderate identity (37%–75%) with GCSF homologues of teleost fish. A phylogenetic analysis indicated that PoGCSF is clustered with the GCSF proteins from *L. calcarifer*. The high sequence identity, together with the conserved GCSF structural features, demonstrated that PoGCSF is a member of teleost GCSF family.

As mentioned above, GCSF is involved in antimicrobial immune, and its expression has been considered to be induced by infection. For example, in human, GCSF expression level was enhanced by pathogen infection and by several inflammatory stimuli [38,39]. Similar results were observed in fish. Expression of the black rockfish GCSF-1

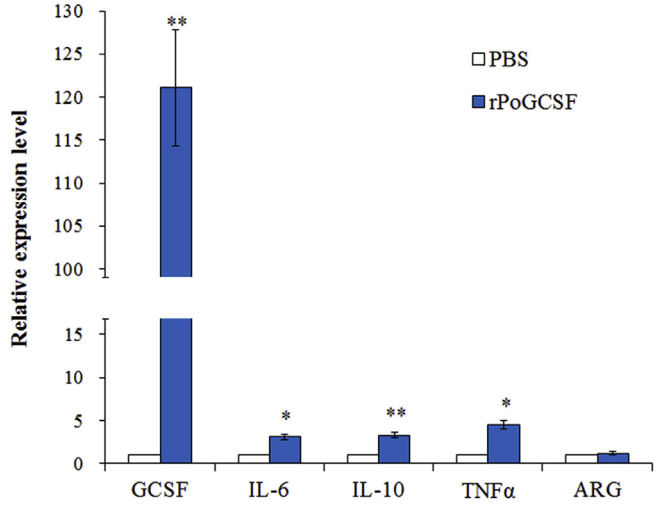

Fig. 4. Effect of rPoGCSF on the expression of immune related cytokines. Head kidney lymphocytes (HKLs) were incubated with PBS or rPoGCSF for 2 h, then HKLs were wash three times with PBS. Total RNA was prepared from the cells, and used for RT-qPCR analysis of PoGCSF, IL-6, IL-10, TNFα, and ARG expression. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. *, $P < 0.05$, **, $P < 0.01$.

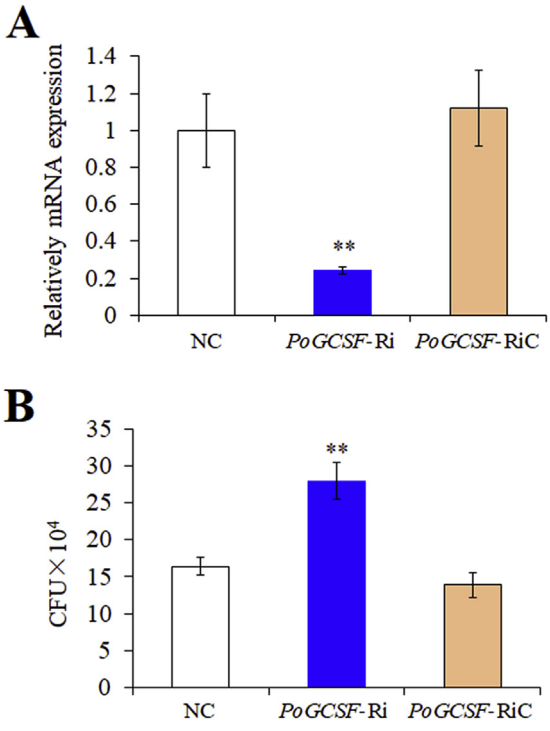

Fig. 5. Effect of *PoGCSF* knockdown on bacterial infection. PoGCSF-Ri and PoGCSF-RiC (RNAi control) were synthetized and transfected into Japanese flounder head kidney lymphocytes (HKLs), the expression of *PoGCSF* was determined by RT-qPCR (A). After transfecting, the HKLs were infected with *Edwardsiella tarda* for 6 h, and the amounts of bacteria were determined (B). NC, the cells treated with PBS. Values are shown as means ± SEM (N = 3). N, the number of times the experiment was performed. **, $P < 0.01$.

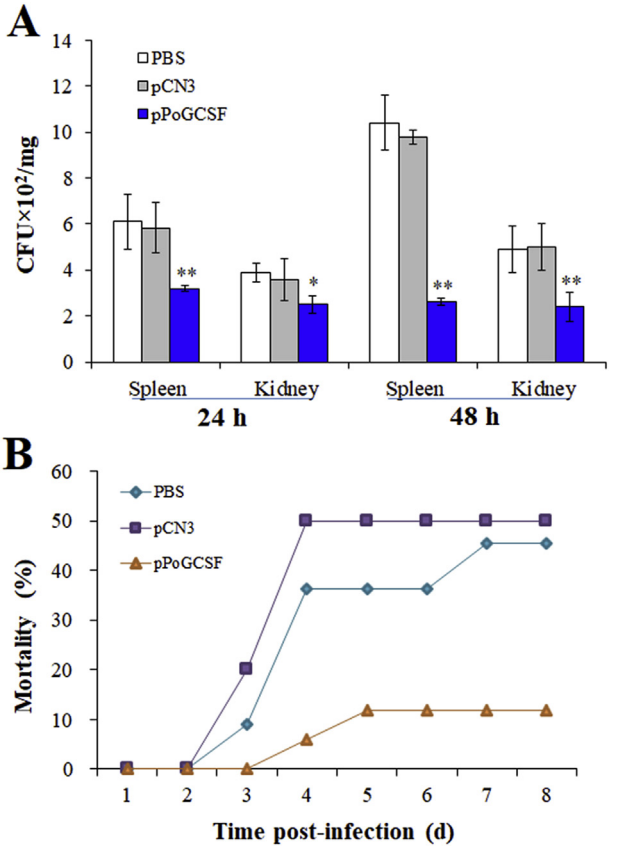

Fig. 6. Effect of PoGCSF overexpression on bacterial infection. Flounder were administered with pPoGCSF, the control plasmid pCN3, or PBS (control). After 5 days post-plasmid administration, the fish were infected with *E. tarda*. Bacterial amounts in kidney and spleen were determined at 24 h and 48 h post-infection (A), and accumulated mortality were monitored for a period of 15 days (only 8 days are shown since no more deaths occurred after 8 days) (B). Data are presented as means ± SEM (N = 3). N, the number of times the experiment was performed. *, $P < 0.05$; **, $P < 0.01$.

homologue was induced in peripheral blood leukocytes after stimulation with LPS, Con A/PMA, or Poly I:C [20]. In rock bream, a significant induction of RbGCSF-1 and RbGCSF-2 were observed after the challenge with *Streptococcus iniae* in kidney, spleen, and gills, whereas *E. tarda* infection induced their expressions only in kidney [4]. However, red seabream iridovirus caused induction of RbGCSF-1 transcription only in gills during initial hours [4]. In our study, the expressions of *PoGCSF* in head kidney, spleen, and liver were significantly induced by the extracellular bacterial pathogen *V. anguillarum*, the intracellular bacterial pathogen *E. tarda*, and the viral pathogen ISKNV. However, there were some different among three pathogens and among three different tissues. The maximum induction of *PoGCSF* by *V. anguillarum* occurred earlier than other two pathogens. Maximum induction difference in three tissues caused by ISKNV infection is smallest, and that caused by *V. anguillarum* is largest. In liver, the highest expression of *PoGCSF* was induced by *V. anguillarum*. In spleen and head kidney, the maximum expression of *PoGCSF* was both induced by ISKNV. These results indicated that *PoGCSF* could participate in the immune defense response of flounder to pathogen infection, and exhibit different expression patterns in response to the infection of different microbial pathogens.

Studies showed GCSF played an important role in resistance against pathogen. For example, studies in mammalian models indicate that recombinant GCSF (rGCSF) not only elevated blood neutrophil counts [40], but also enhanced neutrophil function against bacteria [41]. GCSF also played a critical role in suppressing the load of *Listeria monocytogenes* in spleen and liver [42]. Another report showed that GCSF played important and specific roles in the regulation of immune cells response following *Pseudomonas aeruginosa* infection [43]. Currently, there is no functional report of GCSF about immune defense against pathogenic infection in teleost. Similarly with the results in mammalian, in flounder, we found rPoGCSF enhanced host cells resistance to *E.*

tarda infection. We also found rPoGCSF induced expression of immune-related factors, which suggested GCSF in teleost also possessed the immunoregulatory properties. To further clarify its function, knockdown of *PoGCSF* was conducted and the results showed that following exposure to *E. tarda* infection, HKLs with *PoGCSF* knockdown displayed more bacteria than the control cells. Consistent with the in vitro results, in vivo experiment showed that following *E. tarda* infection, PoGCSF overexpressing flounder exhibited significantly lower bacterial amounts compared to the control fish. These results, together with those of the expressional analysis, indicated a positive role of PoGCSF in host immunity against pathogen infection. The biological effects of PoGCSF are likely mediated via its interactions with PoGCSF receptor, which was found in flounder but its function remains unknown. The PoGCSF/PoGCSF receptor pathway perhaps could active immune cells such as T cells and induce the expression of cytokines, just as the findings in human [5].

In conclusion, we reported for the first time the immunological function of teleost GCSF homologue, PoGCSF, from Japanese flounder. The expressions of *PoGCSF* were significantly upregulated by extracellular and intracellular bacterial pathogens and viral pathogen. Different expression patterns were exhibited in response to the infection of different microbial pathogens in different immune tissues. rPoGCSF enhanced host cells resistance to pathogen infection and regulated the expression of immune-related genes. The knockdown of *PoGCSF* attenuated the ability of host cells to eliminate pathogenic bacteria, which was sustained by the in vivo result that overexpression of PoGCSF promoted the host defense against invading pathogenic microorganism. These findings add new insights into the biological function of teleost GCSF.

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Appendix A. Supplementary data

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