Fish and Shellfish Immunology 90 (2019) 20–29

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Identification and bioactivity of a granulocyte colony-stimulating factor b homologue from large yellow croaker (*Larimichthys crocea*)

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ARTICLE INFO

Keywords:
Granulocyte colony-stimulating factor (GCSF)
Large yellow croaker (*Larimichthys crocea*)
Inflammatory response
Bacterial infection
Phagocytosis

ABSTRACT

Granulocyte colony-stimulating factor (GCSF) is a pleiotropic cytokine that plays a key role in regulation of hematopoiesis, innate and adaptive immune responses in mammals. However, bioactivity of GCSF in teleost fish remains largely unknown. In this study, a GCSFb homologue from large yellow croaker (*Larimichthys crocea*) (*Lc*GCSFb) was cloned by RACE-PCR techniques. The open reading frame (ORF) of *Lc*GCSFb is 603 bp long and encoded a protein precursor of 200 amino acids (aa), with a 19-aa signal peptide and a 181-aa mature peptide. In healthy fish, the *Lc*GCSFb was constitutively expressed in all examined tissues, with the highest levels in mucous tissues, such as gills, intestine, and stomach. Its transcripts in head kidney, spleen, gills, intestine and stomach were significantly induced by *Vibrio alginolyticus* challenge. *Lc*GCSFb transcripts were also detected in primary head kidney leukocytes (PKL), primary head kidney macrophages (PKM), primary head kidney granulocytes (PKG) and head kidney cell line (LYCK), and markedly upregulated by inactivated *V. alginolyticus*. These data suggested that *Lc*GCSFb may play a role in immune response against bacterial infection. *In vivo* administration of recombinant *Lc*GCSFb protein (r*Lc*GCSFb) significantly upregulated the expression levels of the inflammatory cytokines (IL-6 and TNFα), and transcription factor C/EBPβ, which is required for proliferation of neutrophils. Furthermore, r*Lc*GCSFb showed an ability to strengthen the phagocytosis of PKL *in vitro*. Taken together, *Lc*GCSFb may be involved in antibacterial immunity via promoting the inflammatory response and the phagocytic activity of leukocytes. To our knowledge, this is the first report on immunoregulatory roles of GCSF in teleost.

1. Introduction

Granulocyte colony-stimulating factor (GCSF) is a pleiotropic cytokine that plays a key role in regulation of hematopoiesis, innate and adaptive immune responses in mammals [1]. It is a glycoprotein characterized for its relatively specific stimulation of the growth and differentiation of neutrophil progenitor cells in semi-solid cultures [2]. GCSF is produced mainly by hematopoietic cells, such as monocytes/macrophages and lymphocytes [3]. Other cells, such as fibroblasts, endothelial cells, astrocytes and bone marrow stromal cells can also produce GCSF following activation by LPS, IL-1 or TNF-α [4–6]. GCSF exerts its effects by binding to a single homodimer receptor, GCSFR [7], which is present on a variety of hematopoietic cells, including myeloid progenitors, mature neutrophils, monocytes, B cells, and T cells [8,9].

In mammals, GCSF has significant roles in regulating the production of inflammatory cytokines and the activities of immune cells [10]. Under normal conditions, the expression levels of GCSF are very low. During inflammatory processes, however, the expression of GCSF can be dramatically induced [11]. Induction of GCSF is an important driver of emergency granulopoiesis [12], which is a specific response to infectious or inflammatory challenge and represents an essential component of the innate immune response. GCSF also has been shown to modulate the function of mature neutrophils, including their survival [13], superoxide release [14], degranulation [15], chemotaxis [16], and phagocytic and bactericidal activities [17]. The studies in mice lacking GCSF showed that GCSF is vital for macrophage phagocytosis and increase of absolute peritoneal macrophage numbers elicited by *L.monocytogenes* [18,19]. Additionally, human GCSF shows modulating effects on the inflammatory response by affecting the expression of cytokines, such as IL-6 and TNFα [13,20,21].

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https://doi.org/10.1016/j.fsi.2019.04.048
Received 6 February 2019; Received in revised form 27 March 2019; Accepted 13 April 2019

Table 1
Primers used in this study.

Primer name	Sequence (5′-3′)
For RACE PCR	
*Lc*GCSFb -5′Outer-R	GCAGCCCCTGGTACAGCTGGCTG
*Lc*GCSFb -5′Inner-R	CGTGCACGGTGGGGATGTCTCTC
*Lc*GCSFb -3′Outer-F	CGTTCAGAGAGGCGGCTGAGCG
*Lc*GCSFb -3′Inner-F	CAGGTGGCGGTCCACCTGACGCT
*Lc*GCSFb-full-F	AGTACATGGGGAAGGAGAATTTACCG
*Lc*GCSFb-full-R	CCTGAAGGCATCCTGAGACATCCTG
For recombinant plasmid construction	
*Lc*GCSFb–F3	CGGAATTCGCTCCCATCAGCTCGTCCGG
*Lc*GCSFb–R3	CCCAAGCTTTTAGCGGGGCCTTTGTGTGGCGG
For Real-time PCR	
*Lc*GCSFb-DistF	CGCCCACACAGCCAACCT
*Lc*GCSFb-DistR	CGCCCACTCGCTCACACA
TNFα-DistF	GGACGATTCTTCGTTTACAG
TNFα-DistR	GTTTGTCACCTCTGTTCAGG
IL-6-DistF	GCTGTTCTCAAGTATGTGGCG
IL-6-DistR	TGTTGTAAATAGTGGGTGTGTCG
C/EBPβ-DistF	CCCGTCAGGTAAAAGCAGGT
C/EBPβ-DistR	ACGCGCTTTTGTAAACGGTC
Actin-DistF	GACCTGACAGACTACCTCATG
Actin-DistR	AGTTGAAGGTGGTCTCGTGGA

Two copies of GCSF, GCSFa and GCSFb, have recently been identified in several fish species, such as fugu (*Takifugu rubipes*), pufferfish (*Tetraodon nigroviridis*), black rockfish (*Sebastes schlegelii*), rock bream (*Oplegnathus fasciatus*) and zebrafish (*Danio rerio*) [22–25]. Teleost GCSFs share similar gene structure and domains to their mammalian counterparts, although they indeed possess low sequence identity [25]. Bacteria and/or LPS stimulation can induce the expression of GCSFs, suggesting their involvement in the immune response against bacterial infection [22,25]. It was found that two zebrafish GCSFs played roles in hematopoietic development and maintenance [23]. Furthermore, zebrafish GCSFb also promoted neutrophil migration to damaged tissue [26]. However, functions of GCSFs in teleost fish remain largely unknown.

In this study, a GCSFb homologue (*Lc*GCSF) was cloned and characterized in large yellow croaker (*Larimichthys crocea*), an economically important marine fish in China [27]. Its expressions in normal or immune-stimulated tissues and primary immune-related cells were investigated. Furthermore, the effects of recombinant *Lc*GCSFb on the expression of genes involved in the proinflammatory response and granulopoiesis, and on the phagocytosis of PKL were also analyzed. Notably, the results revealed, for the first time, that teleost GCSF had the immunoregulatory roles.

2. Material and methods

2.1. Fish and challenge experiments

Large yellow croakers (21 ± 1.5 cm, 104 ± 13.6 g) were obtained from a mari-culture farm at Ningde city, China. After 7 days of acclimatizing in aerated seawater tanks, the fish were used for the challenge experiments. Two groups of 30 fish each were injected intraperitoneally with poly (I:C) at a dose of 0.2 mg/100 g fish or 1.0×10^8 colony-forming units of live *Vibrio alginolyticus*, respectively. A third group of 30 fish were injected with sterilized PBS at a dose of 0.1 mL/100 g as a control. The head kidney, spleen, gills, intestine and stomach tissues were collected from at least three fish at each time point (3, 6, 12, and 24 h) after stimulation, frozen immediately in liquid nitrogen, and stored at −80 °C for RNA extraction.

2.2. Cloning of full-length cDNA of *Lc*GCSF

Partial cDNA sequence of *Lc*GCSFb was obtained from spleen transcriptomic data [28]. To obtain the full-length cDNA of *Lc*GCSFb, primers for the 5′ and 3′ RACE were designed based on its partial cDNA sequences (Table 1). Then 5′ and 3′ RACE-PCR were performed using 5′ and 3′-Full RACE Kit according to manufacturer's instruction (TaKaRa, China). The resulting PCR product was cloned into a pMD18-T simple vector (TaKaRa), sequenced, and assembled to obtain the full-length cDNA sequence of *Lc*GCSFb. To confirm the integrity of the cDNA sequence, PCR was performed and the resulted PCR products were sequenced.

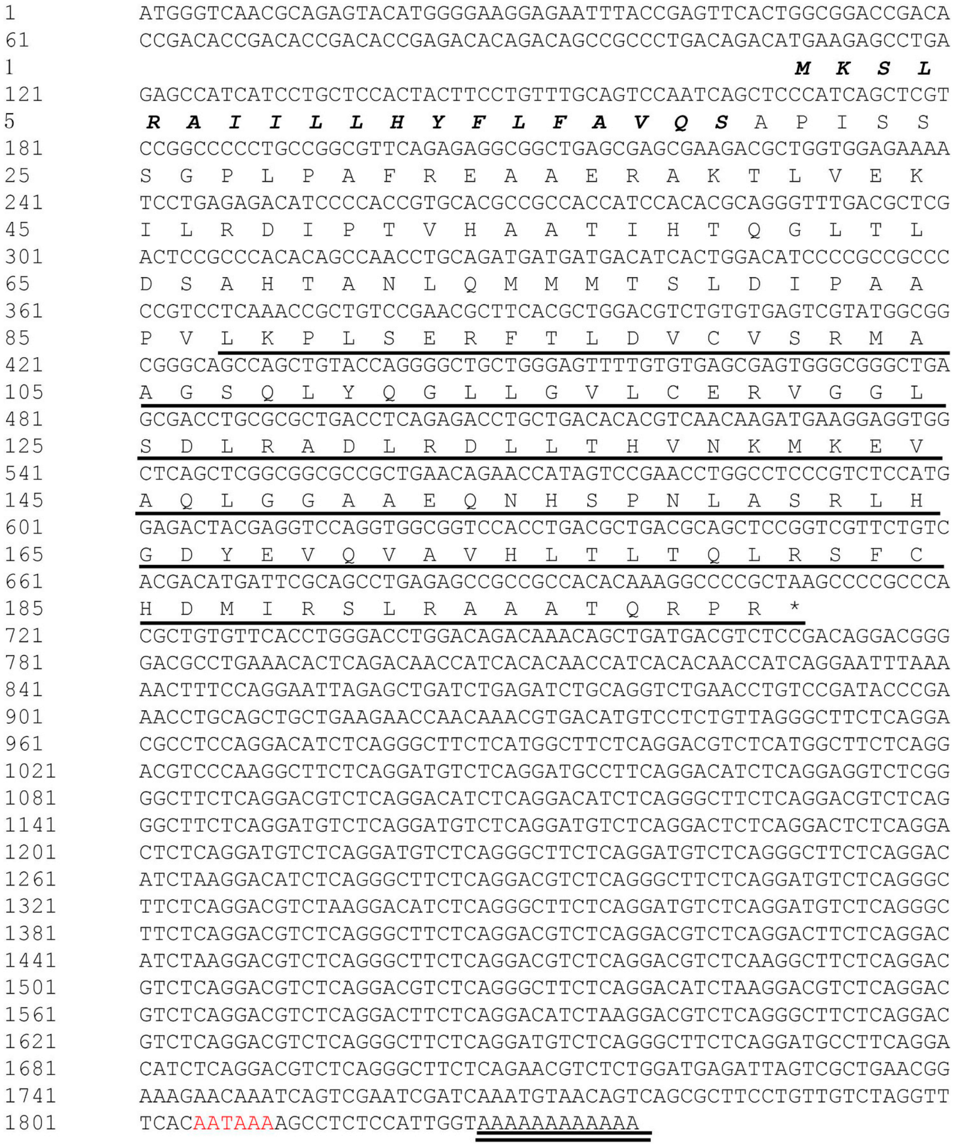
Sequence similarity analysis was performed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Signal peptide prediction was made using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignments were performed with DNAMAN8, and phylogenetic tree was constructed by the Neighbor-Joining and Minimum Evolution methods using the MEGA (version 6) software package. Gene synteny at the *Lc*GCSFb loci in large yellow croaker and other species was analyzed using the NCBI database.

2.3. Primary immune-related cells and cell line

The large yellow croaker head kidney cell line, LYCK, was established and maintained in our own laboratory [29]. The primary head kidney leukocytes (PKL), primary head kidney macrophages (PKM) and primary head kidney granulocytes (PKG) were prepared as reported previously [30–32]. Briefly, head kidney was aseptically collected from large yellow croaker and gently pushed through a 70-μm nylon mesh (BD, USA) to get single cell suspension. To isolate PKL, cell suspension was layered on freshly prepared 34%/51% Percoll (GE, USA) density gradients and centrifuged at 650 × *g* for 30 min at 4 °C. The PKL at gradient interface were collected and re-suspended with completed L-15 medium containing 10% FBS, 100 IU/mL penicillin (Gibco, USA) and 100 mg/mL streptomycin (Sigma, USA), then seeded into a 6-well plate and cultured at 25 °C. To isolate the PKM, the PKL suspension was added to culture dish and incubated at 25 °C for 2 h, then the culture medium was discarded and the attached PKM were cultured with completed L-15 medium at 25 °C. For PKG isolation, the single cell suspension of head kidney was layered over 51% Percoll and centrifuged at 650 × *g* for 30 min at 4 °C. The pellets containing PKG were collected. After the erythrocytes in pellets were cracked by Red Cell Lysis Buffer (TIANGEN, China), the PKG in supernatants were washed twice with L-15 medium, and then cultured at 25 °C. The cells were used for total cell count, viability determination, and Wright's stain as reported previously [32].

2.4. Expression analysis of *Lc*GCSFb gene by real-time PCR

To determine the tissue expression patterns of *Lc*GCSFb, total RNA was extracted from various tissues (including spleen, kidney, intestine, gills, heart, liver, skin, stomach, blood and brain) or 2×10^6 of each immune-related cell type (PKL, PLM and PKG) from at least three fish collected above, and then used for cDNA synthesis as described previously [33]. Real-time PCR was performed with gene-specific primer sets (Table 1) on the Mastercycler epgradient realplex4 (Eppendorf, Germany) using SYBR® Premix ExTaq II ™ (TaKaRa). The expression levels of *Lc*GCSFb were normalized by the reference gene *Lc*β-actin using the $2^{-\Delta\Delta CT}$ method. To further understand the modulation of *Lc*GCSFb expression after stimulation with poly (I:C) and *V. alginolyticus*, total RNA was extracted from head kidney, spleen, gills, intestine and stomach at each time points post-induction above. Real-time PCR was then performed using the conditions described above. The relative expression levels of *Lc*GCSFb were normalized by *Lc*β-actin and expressed as fold changes by comparing the normalized gene expression level of poly (I:C)- and *V. alginolyticus*-injected fish with that of the PBS-injected fish at the same time point. For *Lc*GCSFb induction in cells, 1×10^6 of LYCK, PKL, PKM and PKG were treated with 1×10^5 inactivated *V. alginolyticus* respectively, then harvested at 0.5, 1, 2, 4 and 8 h post-treatment for total RNA extraction. The control was treated



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1      ATGGGTCAACGCAGAGTACATGGGGAAGGAGAATTTACCGAGTTCACTGGCGGACCGACA
61     CCGACACCGACACCGACACCGAGACACAGACAGCCGCCCTGACAGACATGAAGAGCCTGA
1                                                     M  K  S  L
121    GAGCCATCATCCTGCTCCACTACTTCCTGTTTGCAGTCCAATCAGCTCCCATCAGCTCGT
5      R  A  I  I  L  L  H  Y  F  L  F  A  V  Q  S  A  P  I  S  S
181    CCGGCCCCCTGCCGGCGTTCAGAGAGGCGGCTGAGCGAGCGAAGACGCTGGTGGAGAAAA
25     S  G  P  L  P  A  F  R  E  A  A  E  R  A  K  T  L  V  E  K
241    TCCTGAGAGACATCCCCACCGTGCACGCCGCCACCATCCACACGCAGGGTTTGACGCTCG
45     I  L  R  D  I  P  T  V  H  A  A  T  I  H  T  Q  G  L  T  L
301    ACTCCGCCCACACAGCCAACCTGCAGATGATGATGACATCACTGGACATCCCCGCCGCCC
65     D  S  A  H  T  A  N  L  Q  M  M  M  T  S  L  D  I  P  A  A
361    CCGTCCTCAAACCGCTGTCCGAACGCTTCACGCTGGACGTCTGTGTGAGTCGTATGGCGG
85     P  V  L  K  P  L  S  E  R  F  T  L  D  V  C  V  S  R  M  A
421    CGGGCAGCCAGCTGTACCAGGGGCTGCTGGGAGTTTTGTGTGAGCGAGTGGGCGGGCTGA
105    A  G  S  Q  L  Y  Q  G  L  L  G  V  L  C  E  R  V  G  G  L
481    GCGACCTGCGCGCTGACCTCAGAGACCTGCTGACACACGTCAACAAGATGAAGGAGGTGG
125    S  D  L  R  A  D  L  R  D  L  L  T  H  V  N  K  M  K  E  V
541    CTCAGCTCGGCGGCGCCGCTGAACAGAACCATAGTCCGAACCTGGCCTCCCGTCTCCATG
145    A  Q  L  G  G  A  A  E  Q  N  H  S  P  N  L  A  S  R  L  H
601    GAGACTACGAGGTCCAGGTGGCGGTCCACCTGACGCTGACGCAGCTCCGGTCGTTCTGTC
165    G  D  Y  E  V  Q  V  A  V  H  L  T  L  T  Q  L  R  S  F  C
661    ACGACATGATTCGCAGCCTGAGAGCCGCCGCCACACAAAGGCCCCGCTAAGCCCCGCCCA
185    H  D  M  I  R  S  L  R  A  A  A  T  Q  R  P  R  *
721    CGCTGTGTTCACCTGGGACCTGGACAGACAAACAGCTGATGACGTCTCCGACAGGACGGG
781    GACGCCTGAAACACTCAGACAACCATCACACAACCATCACACAACCATCAGGAATTTAAA
841    AACTTTCCAGGAATTAGAGCTGATCTGAGATCTGCAGGTCTGAACCTGTCCGATACCCGA
901    AACCTGCAGCTGCTGAAGAACCAACAAACGTGACATGTCCTCTGTTAGGGCTTCTCAGGA
961    CGCCTCCAGGACATCTCAGGGCTTCTCATGGCTTCTCAGGACGTCTCATGGCTTCTCAGG
1021   ACGTCCCAAGGCTTCTCAGGATGTCTCAGGATGCCTTCAGGACATCTCAGGAGGTCTCGG
1081   GGCTTCTCAGGACGTCTCAGGACATCTCAGGACATCTCAGGGCTTCTCAGGACGTCTCAG
1141   GGCTTCTCAGGATGTCTCAGGATGTCTCAGGATGTCTCAGGACTCTCAGGACTCTCAGGA
1201   CTCTCAGGATGTCTCAGGATGTCTCAGGGCTTCTCAGGATGTCTCAGGGCTTCTCAGGAC
1261   ATCTAAGGACATCTCAGGGCTTCTCAGGACGTCTCAGGGCTTCTCAGGATGTCTCAGGGC
1321   TTCTCAGGACGTCTAAGGACATCTCAGGGCTTCTCAGGATGTCTCAGGATGTCTCAGGGC
1381   TTCTCAGGACGTCTCAGGGCTTCTCAGGACGTCTCAGGACGTCTCAGGACTTCTCAGGAC
1441   ATCTAAGGACGTCTCAGGGCTTCTCAGGACGTCTCAGGACGTCTCAAGGCTTCTCAGGAC
1501   GTCTCAGGACGTCTCAGGACGTCTCAGGGCTTCTCAGGACATCTAAGGACGTCTCAGGAC
1561   GTCTCAGGACGTCTCAGGACTTCTCAGGACATCTAAGGACGTCTCAGGGCTTCTCAGGAC
1621   GTCTCAGGACGTCTCAGGGCTTCTCAGGATGTCTCAGGGCTTCTCAGGATGCCTTCAGGA
1681   CATCTCAGGACGTCTCAGGGCTTCTCAGAACGTCTCTGGATGAGATTAGTCGCTGAACGG
1741   AAAGAACAAATCAGTCGAATCGATCAAATGTAACAGTCAGCGCTTCCTGTTGTCTAGGTT
1801   TCACAATAAAAGCCTCTCCATTGGTAAAAAAAAAAAA
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Fig. 1. Nucleotide and deduced amino acid sequences of *Lc*GCSFb cDNA.

The predicted signal peptide is shown in bold and italicized. A conserved IL-6 homologue domain is underlined. A typical poly(A) signal (AAAAAA) is double underlined. Stop codon TAA is indicated with an asterisk. A typical polyadenylation signal (AATAAA) is in red. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2

The GCSF protein sequences used for structure and phylogenetic analysis.

Species	GCSF (identity, %)	NCBI Accession number
	Entire sequence	
Larimichthys crocea	100	
Homo sapiens	25.15	AAH33245.1
Bos Taurus	23.93	NP_776453.1
Mus musculus	22.56	EDL16160.1
Gallus gallus	21.34	AAA48694.1
Numida meleagris	24.39	XP_021233905
Meleagris gallopavo	21.34	XP_003213980
Danio rerio GCSFa	29.07	NP_001138714.1
Danio rerio GCSFb	34.36	NP_001137226.1
Takifugu rubripes GCSFa	36.36	XP_003961515.1
Takifugu rubripes GCSFb	59.44	XP_003965085.1
Tetraodon nigroviridis GCSFa	37.06	CAG04394.1
Tetraodon nigroviridis GCSFb	51.69	XP_003965085
Fundulus heteroclitus GCSFb	62.92	XP_012708375
Paralichthys olivaceus GCSFb	71.82	XP_019961936

with an equal volume of PBS. Each experiment was repeated three times.

2.5. Expression and purification of recombinant LcGCSFb protein

To investigate the biological function of *Lc*GCSFb, a *Lc*GCSFb fragment excluding the signal peptide was amplified with the primer set of *Lc*GCSFb–F3 and –R3, and then inserted into the *Eco*R I/*Hin*d III digested vector pET-His. The resulting recombinant plasmid pET-His-*Lc*GCSFb was transformed into *E. coli* Rosetta competent cells (Novagen). Recombinant *Lc*GCSFb protein (r*Lc*GCSFb) was expressed by 0.1 mM IPTG induction at 16 °C overnight and SDS-PAGE was performed for expression analysis of recombinant protein. The r*Lc*GCSFb was purified by Ni-NTA nitrilotriacetic acid (NI-NTA) affinity chromatography under nature conditions using GE Purification System (Invitrogen, U.S.A.). The protein was then dialyzed against PBS at 4 °C. The resulting proteins were quantitated using Bradford protein quantitation assay by Nanodrop 1000 (Thermo Fisher Scientific, USA), filtered with a sterile 0.2 μm filter and stored at −80 °C. In parallel,

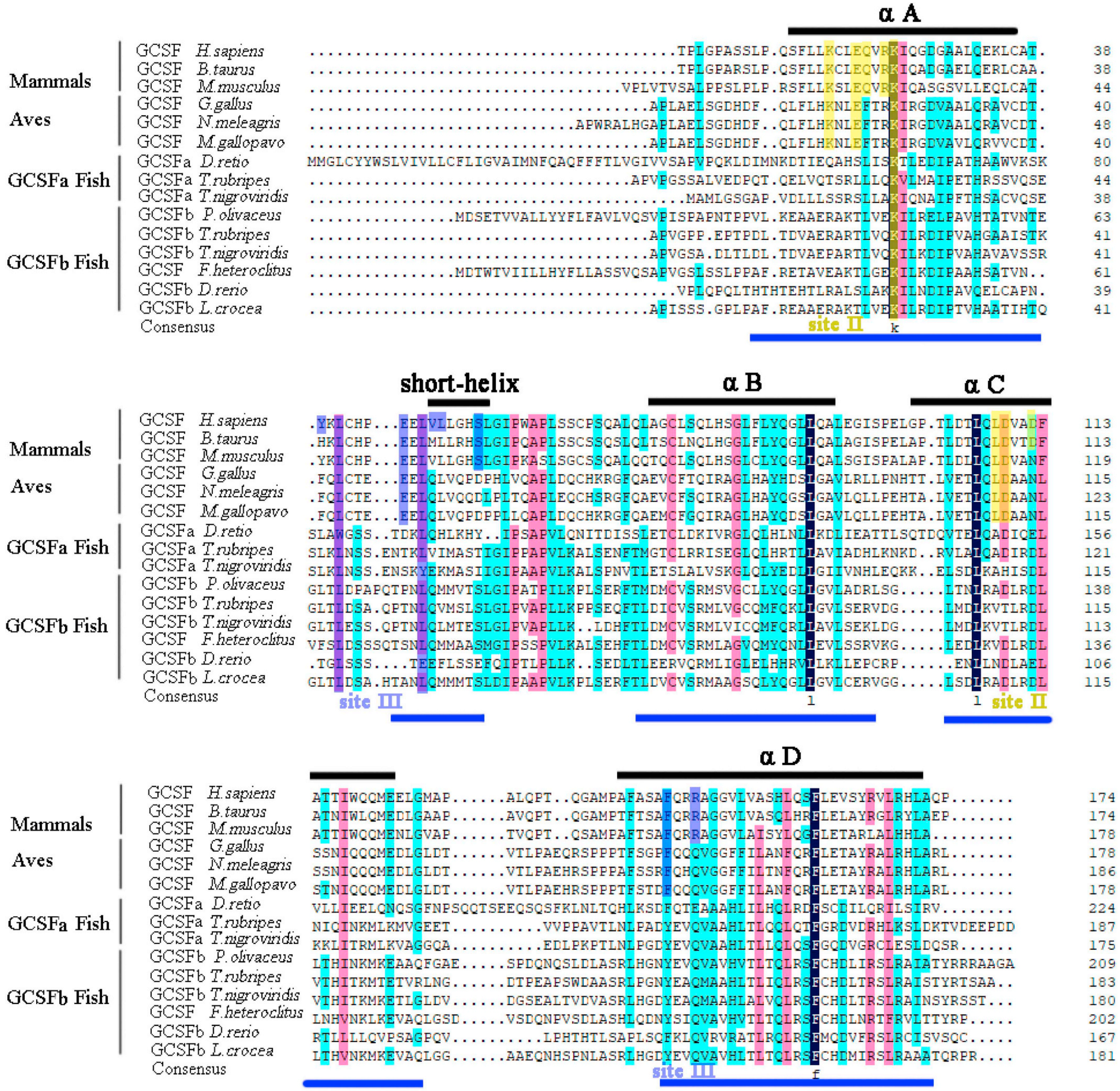

Fig. 2. Multiple amino acid sequence alignment of GCSF from different species.

The alignment was performed using DNAMAN version 8.0. The predicted signal peptide sequences have been removed. Residues are shaded by sequence identity (green: 50%, pink: 75%, black: 100%). The predicted secondary structures (four α-helix αA, αB, αC, αD and a short-helix) of hGCSF and *Lc*GCSFb are indicated by black horizontal line and blue baseline, respectively. The residues concerned with ligand–receptor binding at II and III are colored by yellow and blue, respectively. Whilst "." denotes that there is no amino acid in this place. The GenBank accession numbers of GCSF amino acid sequences used here are listed in Table 2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

recombinant thioredoxin (rTrx) was expressed and purified in the same system, and used as a control protein in subsequent experiments.

2.6. Bioactivity analysis of rLcGCSFb proteins in vivo

For the bioactivity study, a group of 30 healthy fish was injected intraperitoneally with purified r*Lc*GCSFb at a dose of 10 μg/100 g fish. Another group of 30 healthy fish was injected with the purified rTrx proteins at the same dose as control. The head kidney and blood were collected from six fish at each time points after injection (3, 6, 12, 24, and 48 h). Total RNA was extracted from these tissues and used for expression analysis of genes involved in inflammatory response and granulopoiesis, including *Lc*GCSFb, IL-6 (KKF25177.1), TNFα (EU689001), and C/EBPβ (XM_010732057). Real-time PCR was performed with the respective gene-specific primers (Table 1).

2.7. Phagocytosis assay in vitro

In vitro phagocytosis assay was performed as previously described [34]. Briefly, PKL grown in 24-well plates (1×10^6 cells/well) were

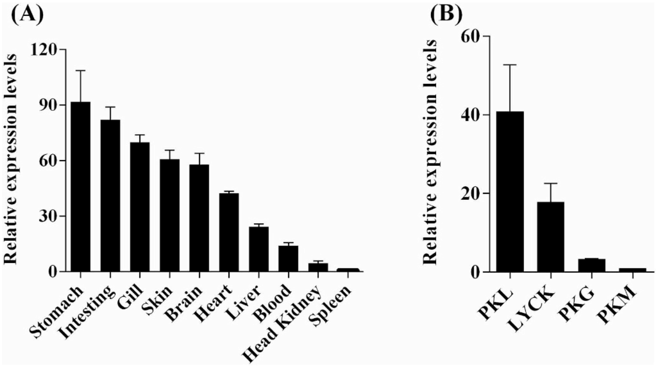

Fig. 3. Expression of *Lc*GCSFb in different tissues (A) and cells (B).
Expression levels of *Lc*GCSFb gene in different tissues, immune-related cells including PKM, PKG and PKL, and a fibroblast lineage LYCK were investigated. The expression levels of *Lc*GCSFb were normalized by *Lc*β-actin and expressed as the ratio to the gene expression levels in the spleen (A) or in the PKM (B). The tissues or cells were ordered according to the relative expression levels from the highest to the lowest. Deviation bars represent the standard errors of the mean (± SEM).

incubated with *Lc*GCSFb with a final concentration of 10, 100, and 1000 ng/mL for 24 h. The rTrx at a final concentration of 1000 ng/mL was used as a control. One μL red-fluorescent microspheres (580/605, Thermo Fisher) were added, and the cells continued to be incubated for 1 h. These cells were then harvested and suspended in FACS buffer. The cells were examined using FACSCaliber flow cytometer and data were analyzed using FlowJo software.

2.8. Statistical analysis

All data were analyzed using GraphPad Prism 6 software and expressed as the standard error of the mean (SEM) of three repeated experiments. The data were subjected to analysis of one way ANOVA by using IBM SPSS Statistics 19, and p-values of < 0.05 were considered to be statistically significant.

3. Results

3.1. Characterization of LcGCSFb

The full-length cDNA of the *Lc*GCSFb (**MH996462**) is 1837 nucleotides (nt) in length, including a 94-nt 5′-untranslated region and a

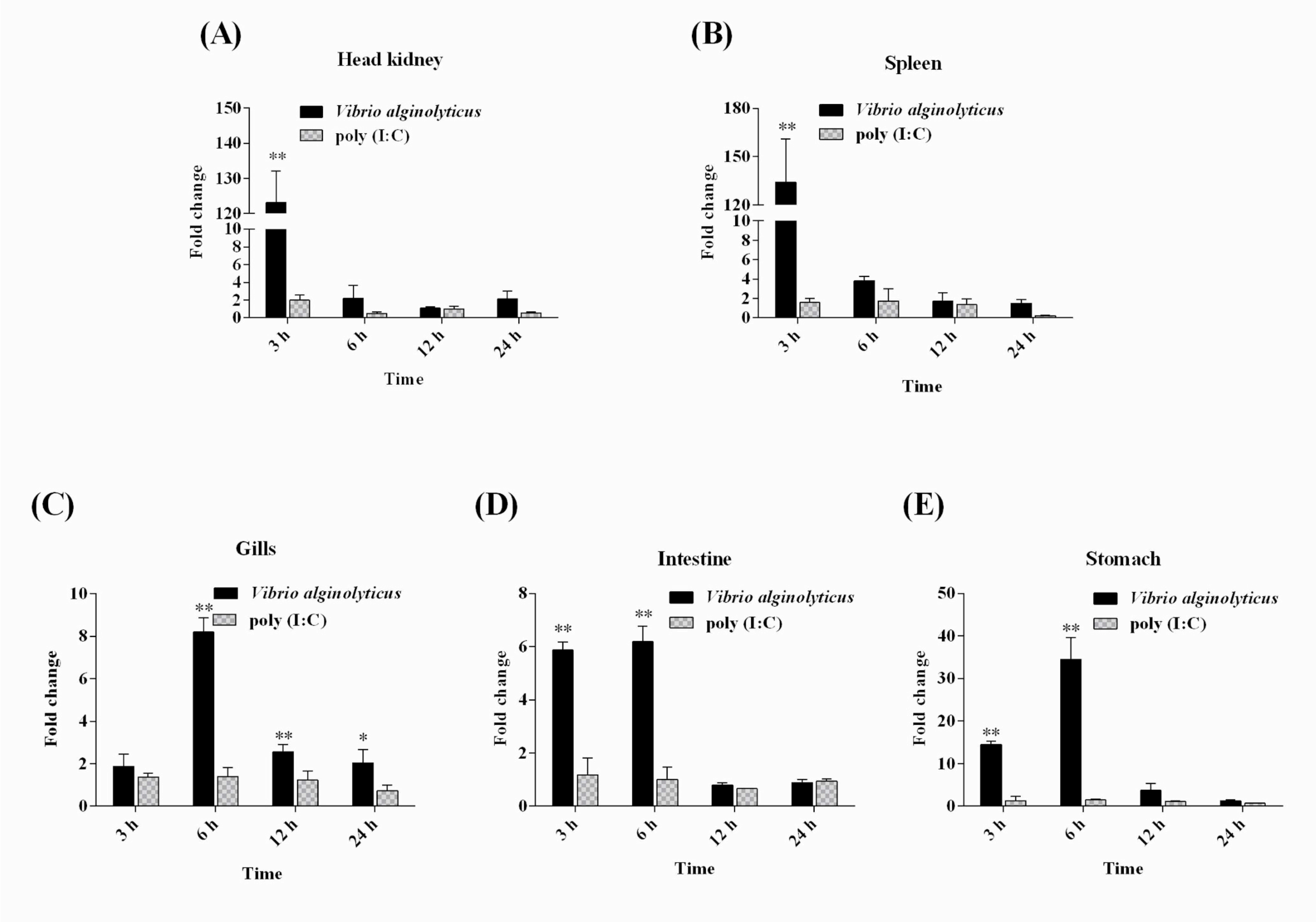

Fig. 4. Expression analysis of *Lc*GCSFb gene after immune stimulation.
*Lc*GCSFb gene expression in head kidney (A), spleen (B), gills (C), intestine (D) and stomach (E) after stimulation with poly (I:C) or *V. alginolyticus* was examined by real-time PCR. These tissues were collected from at least three fish at each time point (3, 6, 12 and 24 h) after induction. The relative expression level of *Lc*GCSFb gene were normalized by *Lc*β-actin, and expressed as fold changes relative to the expression levels in PBS-injected fish. All data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (± SEM) of three independent experiments. Statistically significant differences were indicated with asterisks where $*p < 0.05$ and $**p < 0.01$.

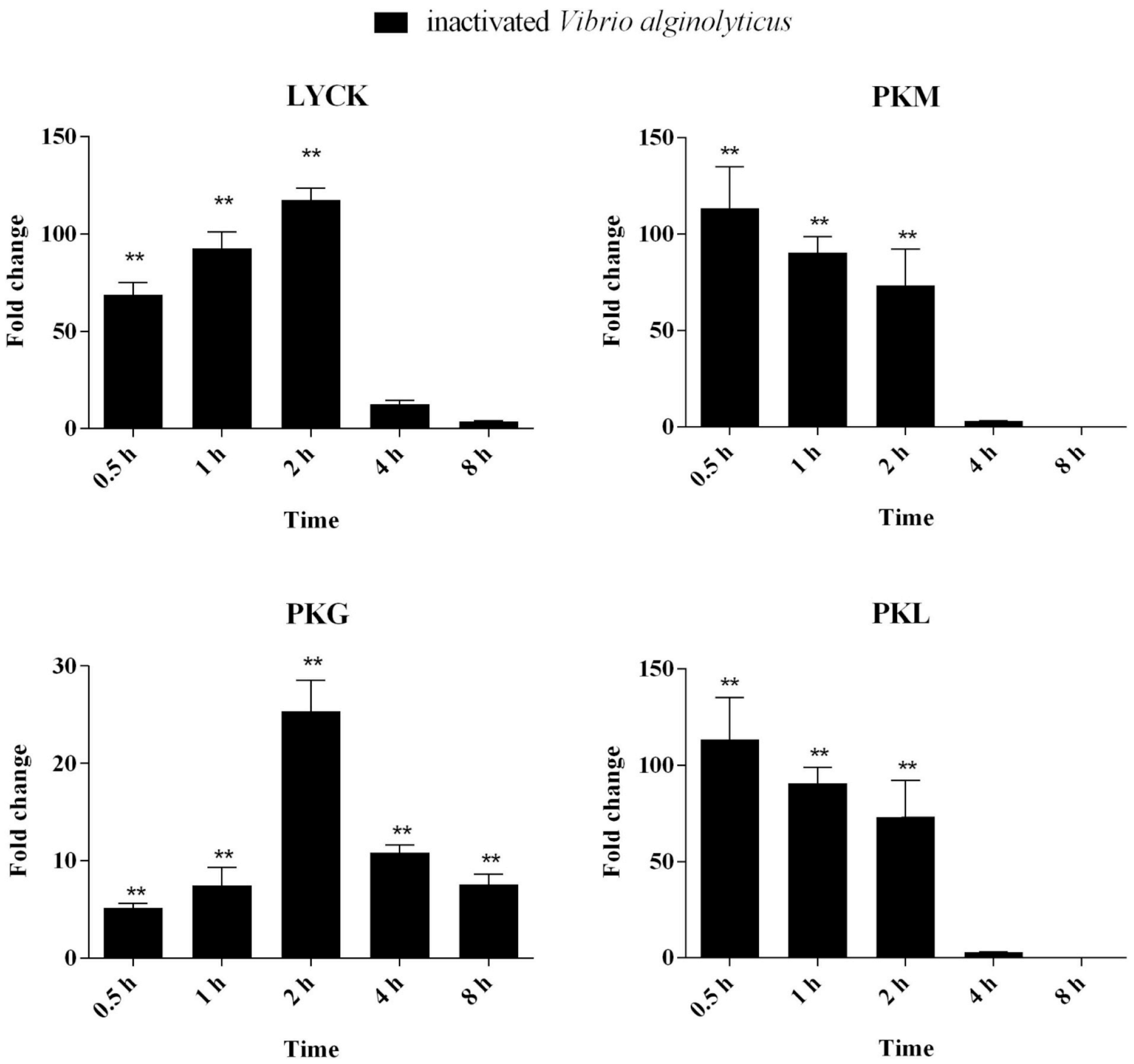

Fig. 5. Expression analysis of *Lc*GCSFb gene in LYCK, PKM, PKG and PKL challenged by inactivated *V. alginolyticus*.
PKM, PKL and PKG were isolated from three different fish, and cultured for 2 h before stimulation. 1×10^6 cells from each cell type including LYCK were treated and harvested at different times, and total RNA was extracted for real-time PCR. The relative expression levels of *Lc*GCSFb were normalized by *Lc*β-actin and expressed as fold change compared with the control at each time point. Error bars represent SED of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$.

1150-nt 3′-untranslated region. A typical polyadenylation signal (AATAAA) is located at 15-nt upstream of the poly(A) tail (Fig. 1). The deduced *Lc*GCSFb protein (**QAT79252**) is composed of 200 amino acids (aa) with a 19-aa signal peptide and a 181-aa mature peptide (Fig. 1). An IL-6 domain of 125-aa, which is conserved in fish homologues, is also observed. Homology comparisons showed the *Lc*GCSFb had highest sequence identity of 71.82% with *Paralichthys olivaceu* GCSF, followed by 62.92–29.07% identity with other known fish GCSF homologues, but a low sequence identity of 21.34–25.15% with its mammalian and avian counterparts (Table 2).

The deduced *Lc*GCSF protein shares a similar secondary structure with human GCSF (Fig. 2) (four-α-helix bundle with a left-handed twist) [35], although they have a low sequence identity (Table 1). Syntenic analysis showed that PMSD3 gene in the vicinity of *Lc*GCSFb locus was overall coincident with that in the vicinity of mammal, Aves, amphibian GCSF and zebrafish GCSFb (Supplementary Fig. 1A). The phylogenetic tree based on the deduced amino acid sequences of GCSF family members showed that *Lc*GCSFb clustered with other fish GCSFb homologues (Supplementary Fig. 1B). These conservative features suggest that the gene identified here encodes a GCSFb homologue in large yellow croaker.

3.2. Expression analysis of LcGCSFb mRNA in tissues and immue-related cells

Tissue expression showed that the *Lc*GCSFb gene was ubiquitously expressed in all tissues examined (Fig. 3A), including head kidney, intestine, liver, gills, skin, spleen, brain, heart and blood, with the highest expression levels detected in mucous tissues, such as gills, intestine and stomach. Challenge of *V. alginolyticus* resulted in a significant upregulation of *Lc*GCSFb expression in head kidney and spleen in initial hours, reaching the peak at 3 h post stimulation (123-fold increase in head

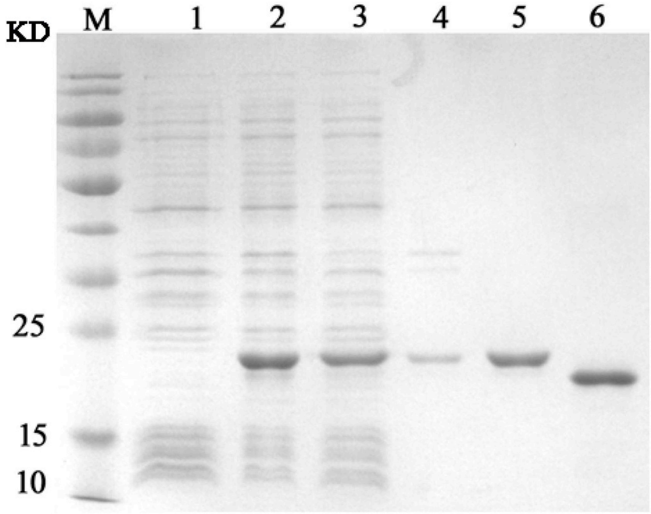

Fig. 6. Expression and purification of r*Lc*GCSFb.
M: standard protein molecular weight; Lane 1: pET-his/Rosetta; Lane 2: pET-his-*Lc*GCSFb/Rosetta, total bacterial lysates; Lane 3: pET-his-*Lc*GCSFb/Rosetta, soluble supernatant; lane 4: pET-his-*Lc*GCSFb/Rosetta, precipitation; lane 5: purified r*Lc*GCSFb; Lane 6: purified rTrx.

kidney and 134-fold increase in spleen) and followed by a rapid recovery at 6 h (Fig. 4A and B). The *Lc*GCSFb transcripts in gills, intestine and stomach after challenge were also increased, although the fold change is relatively low (Fig. 4C, D and 4E). However, *Lc*GCSFb expression levels in these three tissues were not significantly altered after injection with poly (I:C) ($p > 0.05$) (Fig. 4).

As shown in Fig. 3B, *Lc*GCSFb was constitutively expressed in PKL, LYCK, PKG and PKM, with the higher expression levels in LYCK and PKL and the lower levels in PKM. *Lc*GCSFb transcripts were quickly upregulated by inactivated *V. alginolyticus*, and reached the peak levels at 2 h, 1 h, 2 h and 0.5 h post-treatment, with 117.38-, 314.95-, 25.35- and 113.5-fold increases in the LYCK, PKL, PKG and PKM, respectively (Fig. 5).

3.3. The bioactivity analysis of rLcGCSFb

To investigate the regulatory effects of *Lc*GCSFb on the downstream genes involved in the proinflammatory response and granulopoiesis, r*Lc*GCSFb was produced (Fig. 6) and used to stimulate large yellow croaker. After r*Lc*GCSFb stimulation, the expression levels of cytokines TNFα and IL-6 as well as transcription factor C/EBPβ were increased in head kidney and blood (Fig. 7A, B and D). The expression of *Lc*GCSFb was also induced by itself (Fig. 7C). These results suggested that *Lc*GCSFb possessed the immunoregulatory properties.

It is known that GCSF is a key regulator of the activities of immune cells such as neutrophils [36]. To determine whether r*Lc*GCSFb have an effect on the activity of PKL, phagocytosis assay was performed. The results revealed that the phagocytic activity of PKL after treatment by r*Lc*GCSFb (at a final concentration of 10, 100, 1000 ng/mL) was gradually increased (Fig. 8). The phagocytic activity of PKL was augmented to 35.1% by r*Lc*GCSFb at the final concentration of 1000 ng/mL.

4. Discussion

In this study, a *GCSFb* homologue (*Lc*GCSF) from large yellow croaker was characterized. *Lc*GCSFb, present on chromosome 8 [37], shows a high degree of synteny across vertebrate phyla with *PSMD3* adjacent. Structural analysis showed that *Lc*GCSFb possesses conserved structural characteristics of GCSF proteins, including one typical IL-6 domain, which may be involved in the inflammatory response in fish [38]. However, four cysteine residues (except Cys^{74}) responsible for the formation of two disulfide bonds in mammalian GCSF are absent in the teleost GCSF (Fig. 2). The two disulfide bonds in recombinant human GCSF (rhGCSF), Cys^{36}-Cys^{42} and Cys^{64}-Cys^{74}, are both required for its activity, because rhGCSF lacking one or two disulfide bonds exhibits lower biological activity [39]. The deficiency of these conserved cysteine residues in teleost GCSFs suggests that the disulfide bonds may not be required for their bioactivity. In mammals, the receptor recognition of GCSF is achieved through the major (site II) and the minor (site III) sites [40,41] (Fig. 2). However, most conserved amino acids are replaced by other amino acids in teleost. The changes in major interacting sites suggest that the specific sites of ligand–receptor binding in teleost fish may be different from those in mammals, and also indicate that GCSF in teleost may possess different bioactivity from that in mammals. In agreement with this assumption, GCSF signaling in zebrafish could induce hematopoietic stem cell specification and expansion, which have not been described in mammalian GCSF [23].

*Lc*GCSFb mRNA was ubiquitously expressed in various tissues, with the highest expression levels in gills, stomach and intestine (Fig. 1A). Zebrafish GCSFb was also expressed highly in gills and skin [23]. The gills, skin, stomach and intestine are mucosal tissues and forms the first barrier between fish and the external environment containing pathogenic microorganisms. The high expression levels of fish GCSFb in these mucosal tissues suggest that it may be involved in the defense of these tissues against pathogenic invasion. During *V. alginolyticus* infection, the transcript levels of *Lc*GCSFb were significantly increased in head kidney, spleen, gills, intestine and stomach during initial hours (Fig. 4). Similarly, the induction of GCSF by bacteria and/or LPS stimulation was observed in other fish species. *E. tarda* and *S. iniae* challenge caused upregulation of rock bream GCSFb in kidney and spleen [22]. Japanese flounder GCSF was also modulated in kidney after LPS and ConA/PMA stimulation [25]. These findings therefore supported the potential roles in antibacterial immunity of fish GCSFb.

In mammals, GCSF is primarily produced by monocytes/macrophages and lymphocytes and its production can be induced in other cell types, such as endothelial cells and fibroblasts, by inflammatory stimuli [4]. Here, we found that *Lc*GCSFb was expressed in several immune-related cells, such as PKL, PKM, and PKG, and in LYCK cells, a fibroblast lineage (Fig. 3B), suggesting that fish GCSFb might be produced in a wide variety of cell types. Furthermore, *Lc*GCSFb expression in these cells was quickly upregulated by inactivated *V. alginolyticus* (Fig. 5), which was consistent with the results found in black rockfish, where increased levels of GCSFb mRNA were observed in peripheral blood leukocytes treated with LPS [24]. Therefore, the quick upregulation of fish GCSFb in these cells by bacteria or LPS further confirmed its important role in antibacterial immunity.

It is known that GCSF possesses immunomodulating effects on innate immune responses, especially on expression of inflammatory cytokines [13,20,21] and the activities of immune cells [5,13,42]. In mammals, GCSF can have a proinflammatory or antiinflammatory role in inflammatory diseases, depending on the context. For example, GCSF may act as an anti-inflammatory cytokine and be beneficial in some patients with inflammatory bowel disease [43]. In contrast, GCSF can exacerbate acute lung injury and intensify inflammation in patients with rheumatoid arthritis [44,45]. Currently, few data are available regarding the effects of GCSF on innate immune response in teleosts. In this study, our results showed that r*Lc*GCSFb induced the expression of proinflammatory cytokines (IL-6 and TNFα), suggesting that GCSFb in teleost fish promoted the inflammatory response. Transcription factor

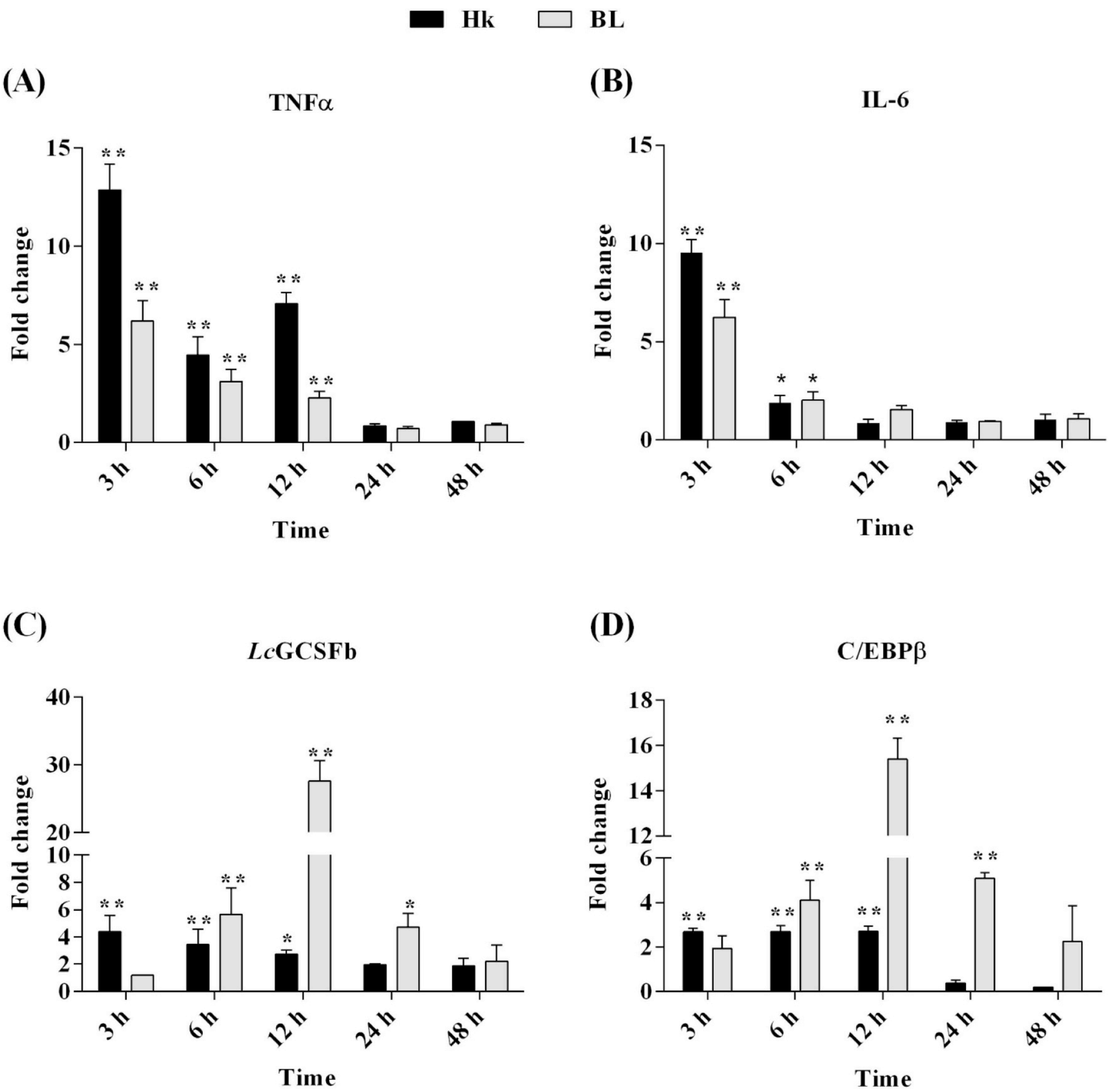

Fig. 7. Expression analysis of cytokines and transcription factor in head kidney and blood of r*Lc*GCSFb-stimulated large yellow croakers.
The expression levels of TNFα (A), IL-6 (B), *Lc*GCSFb (C) and C/EBPβ (D) in head kidney and blood at each time points (3, 6, 12, 24 and 48 h) of six r*Lc*GCSFb-stimulated large yellow croaker were detected by real-time PCR. The relative expression levels of the above genes were normalized by β-actin, and expressed as fold changes by comparing the normalized expression level in the r*Lc*GCSF treated-tissues with that in the rTrx treated tissues at the same time point. All data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (± SEM) of three independent experiments. $*p < 0.05$, $**p < 0.01$.

C/EBPβ, which is a key transcription factor of emergency granulopoiesis [46,47], was also upregulated by r*Lc*GCSFb, suggesting that fish GCSFb may participate in the proliferation of neutrophil during bacterial infection and subsequently promote the host defense against bacteria. In mammals, GCSF has positive effects on neutrophil and monocyte/macrophage activities, including phagocytosis, thus leading to enhanced antimicrobial activity [42,48]. Enhancement of phagocytic activity of PKL by r*Lc*GCSFb further demonstrated that fish GCSF may play a role in innate immune response against pathogenic infection.

In conclusion, we have characterized a GCSFb homologue from large yellow croaker. It was constitutively expressed in all examined tissues and cells. Its expression levels in immune-related tissues and cells could be markedly induced by *V. alginolyticus*, suggesting a role in antibacterial immunity. Furthermore, the r*Lc*GCSFb not only induced the expression of the inflammatory cytokines and granulopoiesis-related transcription factor, but also enhanced the phagocytosis of PKL, indicated that *Lc*GCSFb may be involved in antibacterial immunity via promoting the inflammatory response and the phagocytic activity of leukocytes.

Notes

The authors declare no competing financial interest.

Acknowledgements

The work was supported by grants from the National Key R&D Program of China (2018YFD0900503), National Natural Science

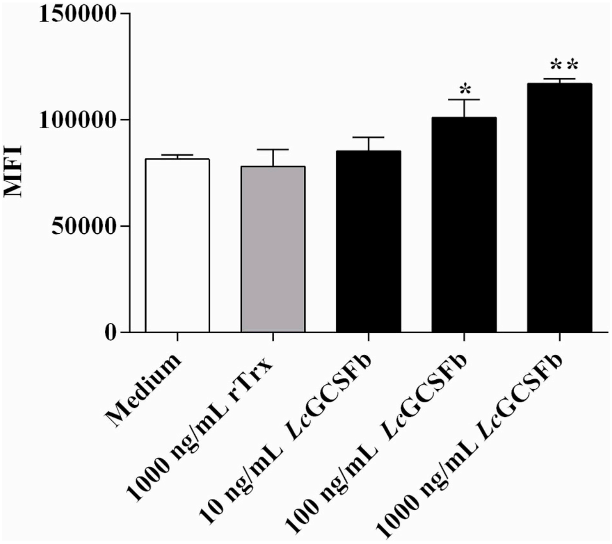

Fig. 8. Effect of *Lc*GCSFb on phagocytosis of PKL.
PKL grown in 24-well plates (1 × 10^6 cells/well) were incubated with a final concentration of 10, 100, 1000 ng/mL r*Lc*GCSFb for 24 h. The rTrx at a final concentration of 1000 ng/mL was used as a control. PKL were incubated with 1 μL red-fluorescent microspheres in all cases for 1 h. The cells were then examined using FACSCaliber flow cytometer and data were analyzed using FlowJo software. Deviation bars represent the standard error of the mean (SEM) of three independent experiments. Statistically significant differences were indicated with asterisks where $*$ stands for $p < 0.05$ and $**$ for $p < 0.01$.

Foundation of China (U1605211, 31772874, and 31602194), China Agriculture Research System (CARS-47), and Xiamen Ocean Economic Innovation and Development Demonstration Project (16PZP001SF16).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2019.04.048.

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