Fish and Shellfish Immunology 90 (2019) 91–101

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Molecular characterization and biological functioning of interleukin-8 in Siberian sturgeon (*Acipenser baeri*)

Xiaowen Wang[a,b], Guoqing Ma[a,b], Rong Zhang[a,b], Lili Liu[a,b], Jianya Zhu[a,b], Hua Zhu[a,b,*]

[a] Beijing Fisheries Research Institute& Beijing Key Laboratory of Fishery Biotechnology, Beijing, 100068, China
[b] National Freshwater Fisheries Engineering Technology Research Center, Beijing, 100068, China

ARTICLE INFO

Keywords:
Interleukin-8 (IL-8)
Siberian sturgeon (*Acipenser baeri*)
Inflammatory response
Immunoregulation

ABSTRACT

Interleukin-8, otherwise known as CXCL8, is a CXC chemokine that plays a pivotal regulatory role in immune and inflammation responses of animals. Here, we identified an interleukin-8 homologue from Siberian sturgeon (*Acipenser baeri*), named AbIL-8, which belongs to the lineage 1 group of teleost fish IL-8s. The cDNA of *Abil-8* is 1130 bp in length, containing a 5′- untranslated region (UTR) of 50 bp, a 3′- UTR of 783 bp, and an open reading frame (ORF) of 297 bp that encodes a protein consisting of 98 amino acids. The deduced AbIL-8 contained five cysteines, four of which are highly conserved, and an ELR motif typical of known mammalian CXC chemokines was also found preceding the CXC motif. Our phylogenetic analysis showed that AbIL-8 clustered with the CXCL8_L1 sequences from other teleosts, being clearly distinct from those of either birds or mammals. *Abil*-8 mRNA was constitutively expressed in all tested tissues and significantly up-regulated in the liver and spleen tissues by the bacteria *Aernomas hydrophila.* The *in vitro* experiment using primary spleen cells stimulated with heat-killed *Aernomas hydrophila* or lipopolysaccharide (LPS) revealed a similar expression pattern to that found *in vivo*, whereas stimulation on spleen cells with β-glucan or polyI:C elicited negligible changes in levels of *Abil*-8 mRNA. Purified recombinant AbIL-8 not only exhibited chemotactic activity for lymphocytes and monocytes in peripheral blood leukocytes (PBLs) and, to a lesser extent, spleen cells, but also stimulated the proliferation of spleen cells at 10 ng/mLor above. Furthermore, intraperitoneal injection of rAbIL-8 also up-regulated the expression of immuno-related genes (*IL-6*, *IgM* and *MHCIIβ*) at 24 h. Collectively, these results enhance our understanding of how IL-8 functions in the regulation of the immune responses in sturgeon.

1. Introduction

Chemokines are a key family of cytokines with chemotactic activity that are known to regulate various steps in the immune responses by migrating immune cells [1]. According to the arrangement of the first two cysteines, chemokines are usually classified into five subfamilies: CXC, CC, CX3C, CX, and XC [2]. CXCL8, also known as interleukin-8 (IL-8), was first identified as a chemokine in mammals and belongs to the CXC subfamily [3]. In mammals, the CXC chemokines are further divided into two main subgroups: ELR$^+$CXC and ELR$^-$CXC, depending on whether or not the Glu-Leu-Arg (ELR) motif is preceding the CXC domain. In general, mammal CXC chemokines with an ELR motif recruit polymorphonuclear leukocytes, whereas CXC chemokines lacking this ELR motif always direct the migration of lymphocytes and monocytes [4]. Functionally, IL-8 is involved in various pathological processes, not only via its effective chemotaxis for several immune cell types but also by inducing respiratory burst, exocytosis, degranulation of storage proteins, and production of lipid mediators [5,6].

In teleost fish species, the IL-8 genes have been increasingly identified in variants that phylogenetically resemble the CXCL8 of mammals. Recent studies suggest these teleost fish CXCL8 chemokines can be divided into three divergent lineages: CXCL8_L1, CXCL8_L2, and CXCL8_L3 [1,7,8]. CXCL8_L1 is considered a fish-specific CXCL8 chemokine [8] that has been found in several fish species, such as grass carp (*Ctenopharyngodon idellus*) [9], ayu (*Plecoglossus altivelis*) [10], zebrafish (*Danio rerio*) [11], Atlantic cod (*Gadus morhua*) [12], rainbow trout (*Oncorhynchus mykiss*) [12,13], common carp (*Cyprinus carpio*) [14], half-smooth tongue sole (*Cynoglossus semilaevis*) [15], and Japanese flounder (*Paralichthys olivaceus*) [16]. By contrast, the third lineage (CXCL8_L3) seems restricted to fewer fish species, including large

Abbreviations: UTR, untranslated region; ORF, open reading frame; LPS, lipopolysaccharide; PBLs, peripheral blood leukocytes; qRT-PCR, Quantitative real time PCR; rAbIL8, recombbinant AbIL8; *A.h*, *Aeromonashydrophila*; PKM, head kidney macrophages; SD, standard deviation; ELR, Clu-Leu-Arg

* Corresponding author. Beijing Fisheries Research Institute& Beijing Key Laboratory of Fishery Biotechnology, Beijing, 100068, China.
E-mail address: zhuhua@bjfishery.com (H. Zhu).

https://doi.org/10.1016/j.fsi.2019.04.010
Received 8 January 2019; Received in revised form 14 March 2019; Accepted 5 April 2019
Available online 09 April 2019

yellow croaker (*Larimichthys crocea*) [17], and rainbow trout (*Oncorhynchus mykiss*) [8], likewise, lineage 2 (CXCL8_L2) has only been found in the cyprinids, (including zebrafish, common carp, and grass carp) [7,18]. In common carp, recombinant CXCa_L1 and CXCL8_L2 induced a moderate increase in their *IL-1β* expression, and *in vivo* could induce leukocytes' recruitment [18]. Recombinant CXCL8_L1 from half-smooth tongue sole was shown to have chemotactic properties for peripheral blood leukocytes (PBLs) and head kidney lymphocytes and capable of stimulating the proliferation of head kidney lymphocytes in a dose-dependent manner [15]. In yellow croaker, recombinant LycCXCL8_L3 chemotactically attracted lymphocytes and eosinophils in peripheral blood leukocytes (PBLs), and enhanced the respiratory burst activity of primary head kidney macrophages (PKM) as well [17]. In sum, research to date indicates that the three CXCL8 lineages mentioned above appear to be functional homologs of the mammalian CXCL8.

Sturgeon is an ancient and endangered yet lucrative commercial aquaculture species that belongs to the chondrostean fish group. Its highly-prized caviar, fast growth, and tasty meat make sturgeon an attractive fish species for farming in China [19]. However, intensive feeding in confined waters can cause many disease outbreaks that result in substantial economic losses to sturgeon farmers. There is no doubt that inflammatory cytokines become activated upon recognition of pathogen [20], with chemokines in particular of pivotal importance in recruiting leukocytes to the site of inflammation. Surprisingly, however, to date chemokines remain poorly characterized in sturgeon species.

To fill this knowledge gap, in this study we identified CXCL 8 (interleukin-8, IL-8) in Siberian sturgeon (*Acipenser baeri*), a chondrostean fish. Then, we analysed its tissue distribution and expression modulation in response to stimulators both *in vitro* and *in vivo*. Going further, we investigated its potential chemotactic activity and immunoregulatory effect using the recombinant AbIL-8. Cloning and functional analysis of AbIL-8 may allow us to better understand the involvement and mechanisms of IL-8 in regulating the immune response in sturgeons and perhaps other chondrostean fish.

2. Materials and methods

2.1. Fish and sampling

Acipenser baeri juveniles (83.6 ± 9.03 g, n = 100) were obtained from Beijing Shidu base for sturgeon breeding, and cultivated in the recirculating-water tanks for 2 weeks before carrying out the experiment. During both the adaption and experimental phases of this research, water temperature was kept at 24 ± 1 °C and all fish were fed with commercial standard diets (Beijing HANYE science and technology Co. Ltd, China) on a 2% body weight basis. Fish were anaesthetized with 100 mg/L of tricaine methane sulfonate (MS-222, Hangzhou Animal Medicine Factory, China) prior to any tissue collection. All experimental procedures conformed to the standards of the China Council on Animal Care.

2.2. Cloning of full-length Abil-8 cDNA

Total RNA was extracted from the mixture of spleens from three Siberian sturgeons using RNAiso plus (Takara, Cat. No. 9108, Japan) according to the manufacturer's protocol, and then reverse-transcribed into first strand cDNA (Takara, Cat. No. RR047A, Japan) in a total volume of 20 μL. To isolate the *il-8* cDNA from this sturgeon species, two primers were designed (IL-8F0 and IL-8R0; Table 1) based on the known partial cDNA sequence of *Acipenser ruthenus* obtained from information of its transcriptome. Polymerase chain reaction (PCR) amplification was conducted as follows: 5 min denaturing step at 95 °C, 35 cycles of 15 s at 95 °C, 20 s at 55 °C, and 45 s at 72 °C, followed by an additional 7 min at 72 °C for the extension. The PCR product was purified, ligated into the pMD19-T Vector (Takara, Cat. No. 6013, Japan) and transformed into *E. coli* DH5α competent cells (Takara, Cat. No. 9057, Japan). Finally, positive clones were selected for sequencing by the TSINGKE Biological Technology Company (Beijing, China).

Table 1
List of primers and their applications.

Primers	Primer sequence 5′-3′	Purpose used
IL-8F0	TGCACTATCTGAAGGGATGTCTTTG	cDNA fragment of AbIL-8
IL-8R0	ACTATCTTGACCCAGCGGGCAGTTG	cDNA fragment of AbIL-8
GSPII	TCTTTTTTAATGCACTGACAACGGAG	5′RACE
NP5	ATTCTGGATCTGCCTGGGATGG	Nested 5′RACE PCR
GSPI	CAAGGATGCTGAAGTCATTGCCA	3′RACE
NP3	GCCCGCTGGGTCAAGATAGTTA	Nested 3′RACE PCR
18SF	TGCCCTATCAACTTTCGATGG	qRT-PCR
18SR	CTGCCTTCCTTGGATGTGGT	qRT-PCR
qAbIL-8F	CATCCATCCCAGGCAGATC	qRT-PCR
qAbIL-8R	TTGACCCAGCGGGCAGTT	qRT-PCR
AbIL-8F	CCGGATATCATGTCTTTGAAAAGC	recombinant construct
AbIL-8R	CCGCTCGAGTCAGGCCTTGGAGCT	recombinant construct

Note: The restriction sites for *Eco*RV and *Xho*I are underlined.

The 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) was carried out with the SMART™ RACE cDNA Amplification Kit (Clontech, Cat. No. 634858, Japan). Based on the partial nucleotide sequence of *Abil-8*, two specific primers (GSPII, NP5) were designed for the 5′-RACE reactions and two specific primers (GSPI, NP3) were designed for the 3′-RACE reactions, according to manufacturer's protocol. The sequences of all the above primers are listed in Table 1.

2.3. Bioinformatics analysis

The *Abil-8* cDNA sequence was analysed using the open reading frame (ORF) finder tool available at NCBI (https://www.ncbi.nlm.nih.gov/orffinder/), to deduce its translation initiation site (ATG) and amino acid sequence. Then, the basic information and structural features of the AbIL-8 protein were analysed using DNAstar and the online SMART tool (http://smart.embl-heidelberg.de/) [21]. Its signal peptide was predicted using SignalP v5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) [22] and a multiple amino acid sequence alignment was conducted in ClustalX software [23]. MEGA v6.06 software [24] was used to construct a phylogenetic tree using the neighbor-joining method with 1000 bootstrap trials [25]. Identity at amino acid level was calculated by the Matrix Global Alignment Tool (MatGAT) v2.0.3 [26].

2.4. Quantitative real time PCR (qRT-PCR) analysis of Abil-8 expression under different conditions

2.4.1. Tissue specific expression of Abil-8 in healthy Siberian sturgeon

Ten tissues (heart, eye, muscle, gut, gill, liver, skin, spleen, head kidney, and brain) were collected aseptically from three sturgeons and used for total RNA extraction. qRT-PCR was carried out using SYBR® Premix Ex Taq™ II (TaKaRa, Cat. No. RR820A, Japan) with the ABI 7500 Real-Time PCR System (Applied Biosystems, USA). The 18S ribosomal RNA, a housekeeping gene, served as the internal control to normalize the template amounts [27]. The primers used here were qAbIL-8F and qAbIL-8R for *Abil-8*, and 18SF and 18SR for *18S*. Thermal cycling included an initial denaturation step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 59.6 °C for 30s. To confirm the amplification of a single product per primer pair, a melt-curve analysis was implemented once the PCR reaction ended. In particular, the primers AbIL-8F/R and 18SF/R should display an amplification efficiency of 90%–110% when assessing the standard curve by qPCR. The expression levels of *Abil-8* were normalized by *18S* using the $2^{-\triangle\triangle CT}$ method, and expressed as the ratio of *Abil-8* expression levels in the muscle. All data were obtained from three independent PCR assays with three replicates in each assay.

2.4.2. Expression of Abil-8 in response to the Aernomas hydrophila challenge

The fish bacterial pathogen *Aernomas hydrophila* (*A.h*) NX830 (obtained from National Aquatic Pathogen Collection Center, China) was cultured in LB medium and re-suspended in PBS to a density of 1×10^8 CFU/mL. Sturgeons (84.4 ± 3.57 g, n = 60) were randomly divided into two groups of 30 fish each. After 2-week acclimation phase, fish in the challenged group were intraperitoneally injected with *A.h* at a dose of 2×10^7 CFU per fish in 200 μL of PBS, while those in the other group were injected with 1 × PBS at a dose of 200 μL per fish to serve as the control. The spleen, liver, and head kidney were sampled randomly from three fish in each group at 6 h, 12 h, 24 h, 72 h, and 7 days post-challenge, and used for the qRT-PCR analysis as described above. The fold-changes in gene expression levels were normalized by *18S*, and expressed as the ratio of the *Abil-8* expression levels in sturgeons injected with PBS at 6 h post-injection (defined by a value of 1).

2.5. Expression of Abil-8 in sturgeon spleen cells with in vitro stimulation

This *in vitro* stimulation experiment was performed as described by Zhang et al. [27] but with slight modification. After disinfecting their body surfaces, the spleen tissue was separately collected from four individual fish (87.3 ± 3.27 g) and washed twice with 1 × PBS containing 100 U/mL of penicillin and 100 U/mL of streptomycin (Solarbio, China). Using a 5-mL disposable syringe, spleen cells were gently squeezed through a 150-μm filter in RPMI1640 medium (Solarbio, China) without fetal bovine serum (FBS). The filtered spleen cells were washed three times with 1 × PBS at × 840 *g* at 4 °C for 10 min. After washing, cells were re-suspended in complete RPMI1640 medium with 5% FBS (Gibico, Cat. No. 10099141, USA). The cells were adjusted to 1×10^6 per mL and put into four 24-wells plates (Corning, Cat. No. 3524, USA) giving 5 wells of cells from the same fish at each plate. After 24 h incubation at 25 °C, the culture medium was removed and replaced with a complete RPMI1640 medium. LPS (100 μg/mL, Sigma, Cat. No. L3129, USA), poly I: C (50 μg/mL, Sigma, Cat. No. P1530G, USA), β-glucan (100 μg/mL, Sigma, Cat. No. G6513, USA), heat-killed *A.h*, and 1 × PBS were subsequently added into four wells (replicates from four individual fish with one well from one fish) at each plate, respectively. At 6 h, 12 h, 48 h, and 96 h after stimulation, the cells in each well were harvested individually in 1 mL RNAiso plus (Takara, Cat. No. 9108, Japan) reagent. Total RNA was then extracted and reversely transcribed into cDNA, the *Abil*-8 mRNA levels were determined by qRT-PCR.

2.6. Production and purification of rAbIL-8

The ORF fragment of AbIL-8 excluding its signal peptide was amplified with the forward primer (AbIL-8F) containing an *Eco*RV restriction enzyme site and the reverse primer (AbIL-8F) include an *Xho*I site. Both the gene fragment and the pET30a (+) expression vector (Merck millipore, Cat. No. 69909, USA) were digested by *Eco*RV and *Xho*I (Thermo, USA), purified DNA fragments were ligated, and the constructed recombinant plasmid pET30a (+)-AbIL-8 was then transformed into *E. coli* Rosetta gami2 complete cells (Merck millipore, Cat. No. 71350, USA). Expression of the fusion protein containing a 6 × His tag was induced by 1 mM of isopropyl β- D -thiogalactoside (IPTG) at 37 °C for 5 h and analysed by SDS-PAGE. The vector pET30a (+) was also transformed into *E. coli* Rosetta gami2 complete cells, to serve as the control. After IPTG induction, the cells were harvested by centrifugation, and re-suspended with 1 × PBS. After sonication on ice, the inclusion bodies were obtained by centrifugation of the bacterial lysate.

The inclusion bodies were washed twice with a lysis buffer and re-suspended in Buffer A (6 M Gua-HCl, 50 mM Trise-HCl, 10% glycerol, 50 mM NaCl, pH 8.0). After centrifugation at × 10 000 g for 10 min, the supernatant was filtered through a 0.45-μm filter membrane and the rAbIL-8 was purified by Co^{2+} affinity resin (Takara, Cat. No. 635503, Japan). The purified protein was refolded in a refolding buffer containing 20 mM of Tris- HCl, 150 mM of NaCl, and reduced urea (6 M, 4 M, 2 M) for 12 h at 4 °C. The resulting rAbIL-8 protein was finally dialyzed against PBS, and this dialyzed sample was centrifuged at × 10 000 g for 30 min at 4 °C to remove any precipitate, then concentrated to 1 mg/mL by protein concentrators (Merck millipore, Cat. No. UFC901024, USA). The recombinant Siberian sturgeon IL-1β protein (rAbIL-1β) was also expressed and purified according to the methods described above.

2.7. In vitro chemotaxis assay

2.7.1. Isolation of peripheral blood leukocytes (PBLs) and spleen cells

The PBLs were isolated following the method described by Jinsheng Sun et al. [15] with a slight modification. Briefly, the blood was sampled from the caudal vein sinus of Siberian sturgeon using a 2-mL sterile syringe and quickly transferred into tubes containing heparin sodium (10 U/mL, Solarbio, Cat. No. H8060, China). After diluted by RPMI1640 culture medium at a ratio of 1:1, blood was loaded onto the same volume of freshly prepared 65% Percoll (Solarbio, Cat. No. P8370, China) and separated via centrifugation at × 850 g for 25 min at 4 °C. After centrifugation, PBLs were obtained from the interface between plasma and Percoll, and washed twice with cell culture medium. Finally, PBLs were adjusted to a final concentration of 1×10^6 cells/mL in complete medium containing 5% of FBS, 100 U/mL of penicillin and 100 U/mL of streptomycin.

The spleen cells were prepared as described in section 2.5. The obtained spleen cells in the chemotaxis assay were adjusted to a concentration of 1×10^6 cells/mL in a complete medium.

2.7.2. Transwell migration assay

In vitro chemotaxis assay was performed using a 24-well Costar Transwell apparatus (Corning, Cat. No. 3422, USA) following Chang-Qing Chu et al. [10] with some minor modifications. Purified rAbIL-8 was diluted in complete RPMI1640 medium to 100, 500, 1000, and 5000 ng/mL. Then, 600 μL of each dilution was added to the lower chamber of the transwell unit, meanwhile purified recombinant AbIL-1β dilution (5 μg/mL) was set as the negative control, and adding 5 ng/mL fMLP (Sigma, Cat. No. F3506-5 MG, USA) was set as the positive control. Polycarbonate filters with a pore diameter of 8-μm were then placed onto the lower wells, and 100 μL target cells (1×10^6 cells/mL) were added in the upper chamber. The plates were incubated at 25 °C for 45 min. At the end of reaction, the cells on the filter were fixed by methyl alcohol for 3 min, and washed with 1 × PBS. After wiping off the non-migrated cells, the migrated cells were stained by DAPI and counted in three fields (× 400) under a fluorescence microscope (Olympus IX71, Japan). Thus, the average number of cells per field was determined. Each migration assay was performed in triplicate.

2.7.3. Morphological identification of attracted leukocyte in PBLs

To determine the cell types upon which rAbIL-8 might exert chemoattractant effect, the dilution of 1000 ng/mL purified rAbIL-8 was used to perform the chemotaxis assay for Siberian sturgeon PBLs as described above; At the end of reaction, cells migrated to the reverse side of polycarbonate filters in the upper chamber were digested by trypsin (Gibico, Cat. No. 25200056, USA) and collected for morphological identification using rapid Wright-Giemsa staining (Solarbio, cat. No. G1020, China).

2.8. Effects of rAbIL-8 on spleen cell proliferation

The MTT assay was performed to determine the proliferation of spleen cells according to established methods [15,28], albeit with slight modifications. A suspension of 150 μL of spleen cells (1×10^6 cells/mL) was added to each well of a 96-well plate, and incubated with different concentrations of rAbIL-8 (i.e., 10 ng/mL, 100 ng/mL, or 500 ng/mL) for 72 h; treatment with just 1 × PBS was regarded as the control. Three

replicates were set in each treatment. After the cells were cultured for 72 h, the spleen cells received 20 μL of 5 mg/mL MTT {3- (4,5- dimethyl-2-thiazolyl)-2,5- diphenyl- 2H- tetrazolium bromide} (Solarbio, Cat, No. M8180, China) per well; they were then further incubated for 4 h to form the crystals. Next, the supernatant was discarded and 150 μL of DMSO was added. Each plate was then read at 490 nm using a microplate reader (BioTek Eon, USA). Each experiment was repeated three times.

2.9. Expression analysis of immuno-related genes by qRT-PCR

To investigate the transcription of immuno-related genes after injection of rAbIL-8, Siberian sturgeons (83.9 ± 4.37 g, n = 24) were randomly divided into two groups with 12 fish in each group. Purified rAbIL-8 was dissolved in PBS (pH 7.4) to a concentration of 0.2 mg/mL, and injected into the abdominal cavities at a volume of 200 μL per fish. BSA (200 μL 2 mg/mL; Solarbio, Cat. No. A8010, China) was also administered in the same way, serving as the negative control. At 6 h and 24 h post-injection, the spleen and the head kidney were randomly sampled from three sturgeons. qRT-PCR was applied to evaluate the transcription of several immuno-related genes, including *IL-6*, *IgM*, and *MHCIIβ*. The fold-changes in each gene expression levels were normalized by *18S*, and expressed as the ratio of the gene expression levels in sturgeons injected with BSA at 6 h post-injection (defined by a value of 1).

2.10. Statistical analysis

All data were analysed with SPSS v19.0 and expressed as mean + standard deviation (SD), with a threshold of $P < 0.05$ considered statistically significant. The normal distribution and homogeneity of variance among treatment groups' data were first tested, then One-way ANOVA analysis was carried out and followed by Tukey's method for pairwise comparisons of means [29], whereas Independent-Samples T test was used to determine the statistical significance of differences observed between the experimental and control groups. When a non-parametric method was used, Kruskal-Wallis analysis was applied and when there is a significant difference, Mann-Whitney U test was adopted in post hoc test. If $P < 0.05$, a statistically significant level was considered [29].

3. Results

3.1. Molecular cloning and sequence analysis of Abil-8

The whole nucleic acid sequence of Siberian sturgeon *il-8* cDNA (GenBank Acc. No. MK140599.1) was assembled from the results of 5′ and 3′ RACE, and the ORF was further verified with successful PCR. The full-length cDNA of *Abil-8* is 1130 bp in length, with a 50 bp 5′-untranslated region (UTR), a 297 bp open reading frame (ORF), and a 783 bp 3′-UTR containing a polyadenylation signal (AATAAA) (Fig. 1A). The ORF was predicted to encode a protein of 98 amino acids (aa), with a 22 aa signal peptide and a 76 aa mature peptide. The deduced AbIL-8 protein contains five cysteines, four of which are highly conserved cysteines (C^{34}, C^{36}, C^{61} and C^{77}) with a glutamine residue situated between the two cysteines (C^{34} and C^{36}), thus clearly forming a CXC motif; and extra one (C^{13}) located at the signal peptide (Fig. 1A).

The phylogenetic tree based on the amino acid sequences of other known CXCL8s showed that CXCL8 in selected species formed five obvious clades (Fig. 2). It was clear that Siberian sturgeon IL-8 fell into a major clade of fish CXCL8_L1 sequences, which is distinct from the clusters of the mammalian and avian CXCL8, fish CXCL8_L2 and CXCL8_L3 sequences (Fig. 2). In CXCL8-L1 cluster, AbIL-8 had a closer phylogenetic relationship with *Stegastes partitus* CXCL8-L1 (XP_008276674.1) and *Larimichthys crocea* CXCL8_L1 (AKM12660.1) (Fig. 2). Homology comparison revealed that AbIL-8 exhibited a relatively high identity of 32.1–50.0% with known members of the CXCL8_L1 subgroup, but a low identity with CXCL8_L2 and CXCL8_L3 sequences from other species (respectively, 27.5–29.2% and 29.6%; Table 1B). Similar to the mammalian and avian IL-8s, AbIL-8 has a highly conserved Glu-Leu-Arg (ELR) motif that is essential for angiogenesis but one most other teleost fish lack. Nevertheless, it is evident the CXC motif is highly conserved in almost all animals (Fig. 1B).

3.2. Tissue distribution of Abil-8 mRNA in healthy sturgeon

The *Abil*-8 mRNA was constitutively expressed in all tissues examined (Fig. 3). The highest mRNA levels were detected in the liver and head kidney, followed by the gill and spleen, whereas lower levels of expressions were found in the brain, skin, and muscle tissues of sturgeon.

3.3. Inducible expression of Abil-8 in vivo following bacterial infection

After live *A.h* infection, the expression of *Abil*-8 mRNA in the spleen ($P = 0.01$) and liver ($P = 0.016$) increased significantly at 6 h (Fig. 4A, C). Showing a different pattern, *Abil*-8 mRNA levels in the head kidney slightly increased at 12 h and thereafter decreased (Fig. 4B). The levels of *Abil*-8 mRNA reached their maximum value at 72 h in spleen ($P = 0.019$) and peaked at 12 h in liver ($P = 0.027$). Generally, *Abil-8* maintained a higher expression level in liver, whereas in the spleen and head kidney *Abil*-8 mRNA levels were gradually down-regulated at 7 days post-*A.h* injection.

3.4. Inducible expression of Abil-8 in sturgeon spleen cells by in vitro immune stimulation

To investigate the effects of stimulants—namely LPS, inactivated *A.h*, poly I: C, and β-glucan—on *Abil-8* expression in primary immune cells, spleen cells were applied. The level of *Abil*-8 mRNA in cells treated with inactivated *A.h* increased, significantly exceeding that of control cells at 6 h and 96 h post-treatment ($P < 0.01$) (Fig. 5A). Likewise, LPS dramatically induced the transcripts of *Abil-8* at 48 h and 96 h, but only the latter time point was statistically significant ($P < 0.05$). In contrast to LPS and inactivated *A.h*, both β-glucan and poly I: C significantly down-regulated the expression of *Abil-8* in spleen cells ($P < 0.05$; Fig. 5B); notably, at all tested time-points, the *Abil*-8 mRNA levels of treated cells were lower than that of control cells.

3.5. Production and purification of recombinant AbIL-8

To examine its potential activity, rAbIL-8 was produced as a His-tagged recombinant protein in *E. coli* Rosetta gami2 complete cells previously transformed with pET-30a (+)-AbIL-8. This recombinant AbIL-8 was over-expressed in response to the IPTG treatment according to the SDS-PAGE analysis (Fig. 6A). The purified protein was analysed by Western blot using anti- 6 × His tag monoclonal antibody (Fig. 6B), and the molecular weight of the purified AbIL-8 was determined to be approximately 14.6 kDa with the His-tag, which corresponded to the expected molecular size.

3.6. Chemotactic activity of purified rAbIL-8

Our *in vitro* transwell migration assay showed that stimulation with all the tested concentrations (100, 500, 1000, 5000 ng/mL) of rAbIL-8 induced migration of PBLs (Fig. 7A) and also of spleen cells at all concentrations expect 100 ng/mL (Fig. 7B). By contrast, the control protein rAbIL-1β had no chemotactic activity towards PBLs and spleen cells; hence, this ruled out the possibility that the chemotactic effect observed with rAbIL-8 had resulted from a contaminated endoxin present in the purified protein. Moreover, rAbIL-8 induced the migration of these cells in a concentration-dependent manner at the concentration

A

```
1    GTGAAAAGCT TCAGCTTCAT TTTCCAGAAA CGAACAAGCT CACGAAAATC ATGGACTCCA
                                                        M  D  S
61   AAACTACAGT GACCGTGGTC ATTTTCTGCC TGGCCGTTGT TGCACTATCT GAAGGGATGT
     K  T  T  V  T  V  V  I  F  C  L  A  V  V  A  L  S  E  G  M
121  CTTTGAAAAG CATTGGACTG GAGCTCCGTT GTCAGTGCAT TAAAAAAGAA TCCAGATTCA
     S  L  K  S  I  G  L  E  L  R  C  Q  C  I  K  K  E  S  R  F
181  TCCATCCCAG GCAGATCCAG AATGTAGAGC TCTTCCCCAG TGGACCGCAC TGCAAGGATG
     I  H  P  R  Q  I  Q  N  V  E  L  F  P  S  G  P  H  C  K  D
241  CTGAAGTCAT TGCCACTTTG AAATCTGGTG ATCAGATTTG CTTGGAACCA ACTGCCCGCT
     A  E  V  I  A  T  L  K  S  G  D  Q  I  C  L  E  P  T  A  R
301  GGGTCAAGAT AGTTATAAAG AAAATATTGG AAAGCTCCAA GGCCTGAATC CTGCCATGGA
     W  V  K  I  V  I  K  K  I  L  E  S  S  K  A  *
361  AATTTAGAGG AACAAACCTA AAGAGAAACT GAAGTTAATC AAGGGTTGAT CTTTTTAACC
421  ACTTGTTTAT TTATTAGTGT GTTTATTTAT TTGCCAATTT AGTTAACAAA TTGTATTTAA
481  TATGTTATTA GACTGAACAG TTGTCATTCG ACATATAGGG TATGGAAGGA TTGTATCAGA
541  CCACTCAGGA AAGGATTTAA ACTACAAACT CTGTTTGCTT AATACAGTGT TTTTCAAGTT
601  TTTGTTAAAT TCTGTTAATT GTCTTGCAGT ATCACTCAGT GGGTTTTTAA TTTTTTTTAT
661  AAAAAATTGA AAACGTGAAC ATGATATGTA AATTGTACCT TTTTTTAAAA AAAAACTGGA
721  AATTGGTCAA AAACAGATGA AGGAAGGGTT TTTAAACCCT TATTTTATAT TGTGCATTCC
781  TTAAACCAAT GGCCGGGGAT GGCAAACTGG CCTATTTCTT TTGGTTGGTT GGCCAACTTG
841  ATTATTTTTG AAACTATACA TATAATGGAT TAATTCATTG AATTTAATCT GGATATTTTT
901  GAAACCAATT GGAATAATTA AACTGGTAAA GTTATTGGTT AAGTGTGGCA GTATGAATGG
961  GGTTAGTTTT GCCATGTTTT TTTTTAATAA TTTTTTTTTT TTTACAAAGG GGGGAAACCC
1021 AATTTTTAAA AATTCGGGGG CCCCCCCTCT TTTTTAGGGC CCCAAAACAA ATTGTTAAAA
1081 AAAAAAATTT TATTTTTTGA TTATCAAAAA AAACAAATAA AAAAAAAAAA
```

B

```
                                                                                identity(%)
sturgeon  ---MNSKTTVTVVIFCLAVVAISEGMSLKSIGLELRCQCIKKESRFIHP-RQIQNVELFPSGPHCKDAEV  66
   Gadus  MKMTSGKIPIGSLLVLLVLLTITEGRSLRGLGMELRGRCIQTESRQIG--RHIGMVEIIPANSHCEETEI  68   39.2
    Homo  ---MTSKLAVALLAAFLISAALCEGAVLPRSAKELRCQCIKTYSKPFHP-KFIKELRVIESGPHCANTEI  66   39.4
  Gallus  ---MNGKLG-AVLALLLVSAAMSQGRTLVKMGNELRCQCISTHSKFIHP-KSIQDVKLTPSGPHCKNVEI  65   47.1
Oryctolagus  ---MNSKLAVALLATFLLSLTLCEAAVLTRIGTELRCQCIKTHSTPFHP-KFIKELRVIESGPHCANSEI  66   38.6
Dicentrarchus  --MMSSKVFATSIVVLLAFLAISEGMSLRSLGVELHGRCIQTESKPIG--RHIGKVELIPANSHCEETEI  66   50.0
   Pagrus  --MMSSRVFVISIVGLLAFLAISEAMSLRSLGVELHGRCIQTESKPIG--RHIEKVELIPANSHCEETEI  66   47.5
Oncorhynchus  ---MSIRMSASLVVVLLALLTITEGMSLRGMGADLRGRCIETESRRIG--KLIKKVEMFPPSSHCRDTEI  65   46.5
Xiphophorus  ---MSL---STVVVAILVFLTIHEGSVVGNQVVNLRGRCITKERKPIG--RRIVALEVHPVSSHCAEIQI  62   32.1
  Stegastes  ---MS----TIATVALLVFLIIHEGISVGDQGGPLRGHCIKKEKRKIG--RFIGEVEVRPGSSHCPEIEI  61   39.4
  Oryzias-L3  -------MKLYSLLLLGTLVVLIDGIPPINRDYNTRCQCLKVESRIIPP-DSLKSIKLVTEGPHCPETEV  62   39.6
Cyprinus-L2  -----MKLTVSAFMLLICTAALSTTEGRPKSQQLSGRCPRMHSEPAIPANKVLSLRVIPAGPICKNENI  65   27.5
   Danio-L2  ----MMKLSVSAFMLLICTTALLCANEGEALPPPQRGCQCIKTHSKPPIPKRQVLGLKVTPAGSHCRNEEI  66   29.2

sturgeon  IATLK-SGDQICLEPTARWVKIVIKKILESSKA----------  98
   Gadus  IATLKRTGQEVCLDADAPWVKNVIERMISSRRH----------  101
    Homo  IVKLS-DGRELCLDPKENWVQRVVEKFLKRAENS----------  99
  Gallus  IATLK-DGREVCLDPTAPWVQLIVKALMAKAQLNSDAPL----------  103
Oryctolagus  IVKLV-DGRELCLDPKEKWVQKVVQIFLKRAEQQES----------  101
Dicentrarchus  IATLKKTGQEVCLDPEAPWVKKVIQKILSNRRR----------  99
   Pagrus  IATLKRTGQEVCLDPEAPWVKKVIQKIMSNSRRR----------  100
Oncorhynchus  IATLSKSGQEICLDVSAPWVKKVIEKMLANNK----------  97
Xiphophorus  IATLKMEKRKVCLDPKAPWVQKLLKNRRARQKVQEQTPKA----------  102
  Stegastes  IATLKKDGKRICLDPNAPWVKEVLANRPAVQTT----------  94
  Oryzias-L3  IAGLV-TGEKVCLNPRSAWVKKLVQFVLERQLNHQKAPAKNQA----------  104
Cyprinus-L2  IATMK-KG-QVCLDPTKDWVISLNEEIKKRNLKSQP----------  99
   Danio-L2  IATLK-KG-QICLNPTETWVISLKEKFAASATKLAATAAPAQTTTTFSTIMTTN  118
```

Fig. 1. Sequence analysis of AbIL-8 gene. (A) The cDNA sequence of *Abil-8* and its deduced amino acid sequence. The initiation codon and stop condon are boxed. The five cysteine residues are shaded in gray. The characteristic signature of the CXC chemokines (CQC) is shaded. The typical polyadenylation signal (AATAAA) is indicated by the double underscore. (B) Multiple sequence alignment of the deduced IL-8 in Siberian sturgeon (*Acipenser baeri*) and other selected vertebrates. The consensus residues are shaded. Accession numbers of genes are given in Supplementary Table 1.

ranging from 100 to 1000 ng/mL. However, above the concentration of 1000 ng/mL, the migrated cells declined to a certain degree. Lastly, the chemotactic effect of rAbIL-8 was more pronounced on the PBLs than spleen cells.

To check which type of leukocytes is attracted towards rAbIL-8, the migrated cells were digested by trypsin and collected for Wright-Giemsa staining at the end of transwell assay. The stained smears revealed that lymphocytes and monocytes were the main cell types attracted in PBLs (Fig. 7C)

3.7. Effect of rAbIL-8 on spleen cell proliferation

In the MTT assay, when rAbIL-8 was added to the spleen cell cultures, it was found to stimulate the proliferation of these cells, with significant enhancements observed under all protein concentrations tested (P-values < 0.05) (Fig. 8). Notably, 100 ng/mL of rAbIL-8 appeared to induce the maximal response.

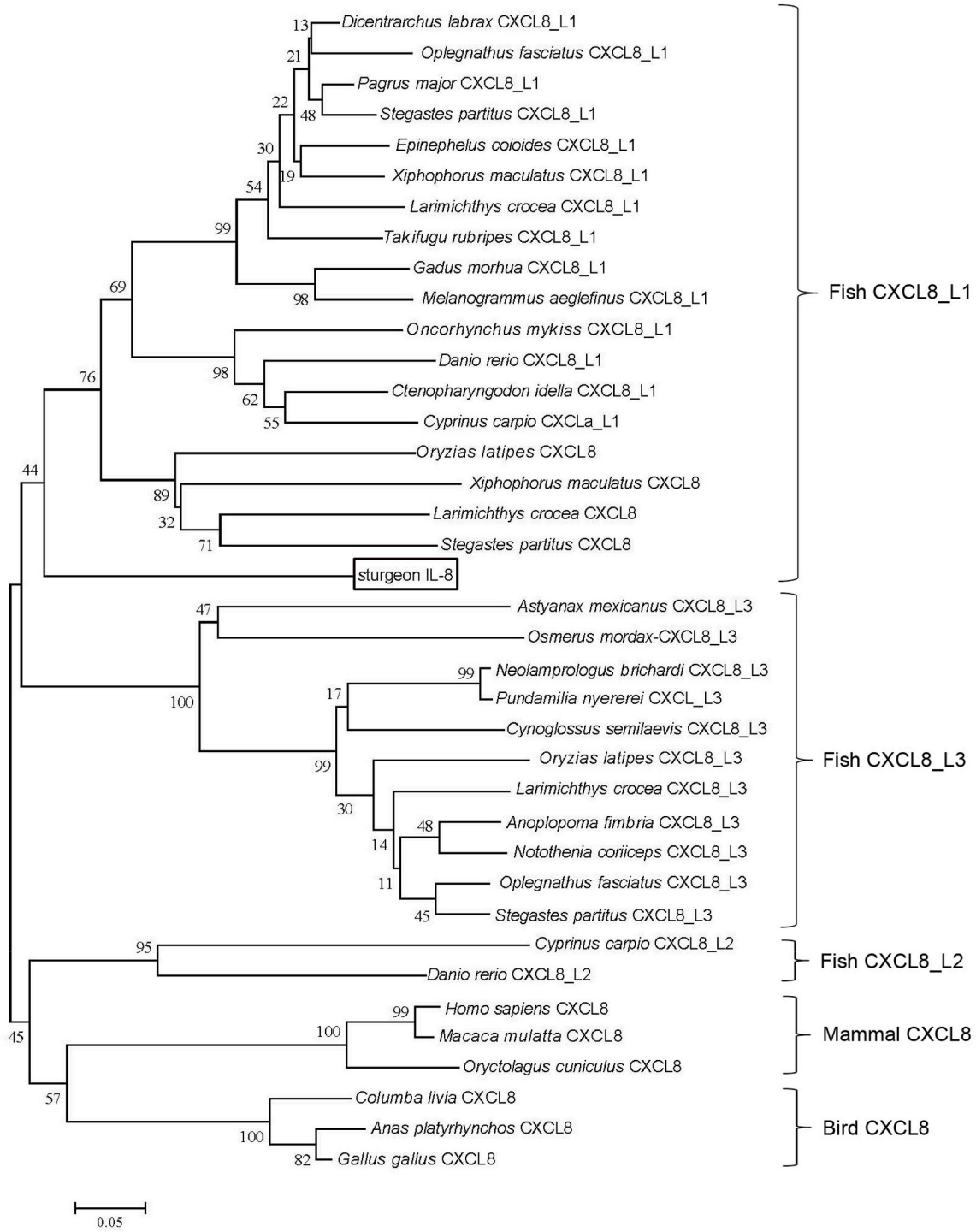


Fig. 2. Phylogenetic analysis of the IL-8 sequences. The tree was built in MEGA v6.06 to show the relationship between the deduced amino acid sequence of interleukin-8 from Siberian sturgeon (*Acipenser baeri*) and that of other species. The neighbor-joining method with bootstrap value of 1000 was applied. Accession numbers are given in Supplementary Table 1. Sturgeon IL-8 is boxed.

3.8. Effect of rAbIL-8 on the expression of immuno-related genes

Via qRT-PCR, the intraperitoneal injection of rAbIL-8 induced the expression of *IgM*, *MHCIIβ*, and *IL-6* in the spleen of Siberian sturgeon. All three genes were up-regulated at 24 h since the rAbIL-8 treatment, and significant changes were found in the levels of *IgM* and *IL-6* mRNA (P-values < 0.05; Fig. 9).

4. Discussion

Interleukin-8 (IL-8), a small cytokine, plays a key role in the inflammatory response of animals [30]. The IL-8 gene from Siberian sturgeon, named here *Abil-8*, was identified and characterized in this study, which was proved to belong to teleost CXCL8_L1 subgroup. This is the first report of the full-length cDNA sequence of *il-8* in sturgeon, a chondrostean fish. The deduced AbIL-8 protein, 98 aa in length, contained the characteristically four conserved cysteines (Fig. 1A) essential

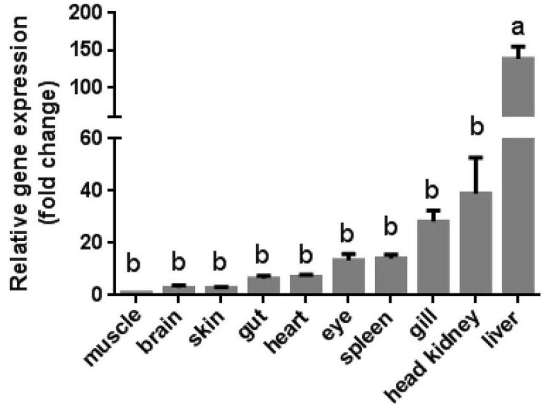


Fig. 3. The relative expression profile of *Abil*-8 mRNA was determined by qRT-PCR in 10 tissue types from three Siberian sturgeons. All data were obtained from three independent experiments with each performed in triplicate. The results were presented as mean + standard deviation (SD) (n = 3). Different lowercase letters between means indicate they are significantly different ($P < 0.05$).

for the tertiary structure and function of CXC chemokines [31]. Yet, AbIL-8 also possesses a fifth cysteine near its N-terminus, locating in the signal peptide region, which is considered less significant for CXCL8 functioning [32,33]. Unlike most teleost fish species, Siberian sturgeon IL-8 has a Glu-Leu-Arg (ELR) motif preceding the CXC domain (CQC), which is required for receptor binding and signaling in mammals [34–36].

Abil-8 was constitutively expressed in all tissues examined, with the highest level in liver, an important immune and metabolic organ, which was similar with the findings in large yellow croaker, half-smooth tongue sole, and grass carp, where CXCL8_L1 mRNA levels were also highest in the liver [9,15,37]. Greater expression levels were also detected in head kidney, an important immune organ, not unlike the levels of *il-8* expressed there in ayu [10], common carp [14], cobia [38], and rainbow trout [8]. In contrast, we found relatively low levels of *Abil*-8 mRNA in muscle, skin, and brain tissues, as also found for many other teleosts, such as tongue sole [15], grass carp [9], and rainbow trout [8]. High constitutive *Abil-8* expression in the liver, head kidney, spleen, and gill, indicated its primary function in fish immune responses and metabolism [36,39,40].

The participation of IL-8 is critical for activating inflammasomes, as well as for inducing antimicrobial responses in host animals [41]. Induction of *il*-8 mRNA by bacterial infection in liver, spleen and head kidney organs was observed in cobia [15,38]. Administration of *Listonella anguillarum* also increased the *il-8* expression levels of head kidney tissue in half-smooth tongue sole [15]. Greater *il-8* expression in response to bacterial invasion is not unexpected; it has been reported in many other fish species, such as ayu and large yellow croaker [10,31,37], suggesting an important role in antiviral immune response of fish IL-8. In our study, the expression levels of *Abil-8* were significantly increased in spleen (2.4-fold) and liver (5.4-fold) by *A. hydrophila* (Fig. 4A, C), implying that *Abil-8* may be involved in host immune responses against bacterial infections. However, *Abil-8* expression levels in head kidney did not show obvious increasement, differing with reported responses for head kidney of half-smooth tongue sole and cobia [15,38], which suggested that *il-8* induction in head kidney may be diversely regulated in different fish species. Additionally, these findings were strengthened by the *in vitro* results we obtained, showing that *Abil-8* expression in spleen cells was sharply elevated post stimulation with inactivated *A.h* or LPS, maintaining a high level at late time-points. In stark contrast, *Abil*-8 mRNA levels were down-regulated in spleen cells following β-glucan or poly I: C treatments. A similar spike in *il-8* expression was reported in bovine alveolar macrophages following stimulation with heat-killed bacteria [42]. The

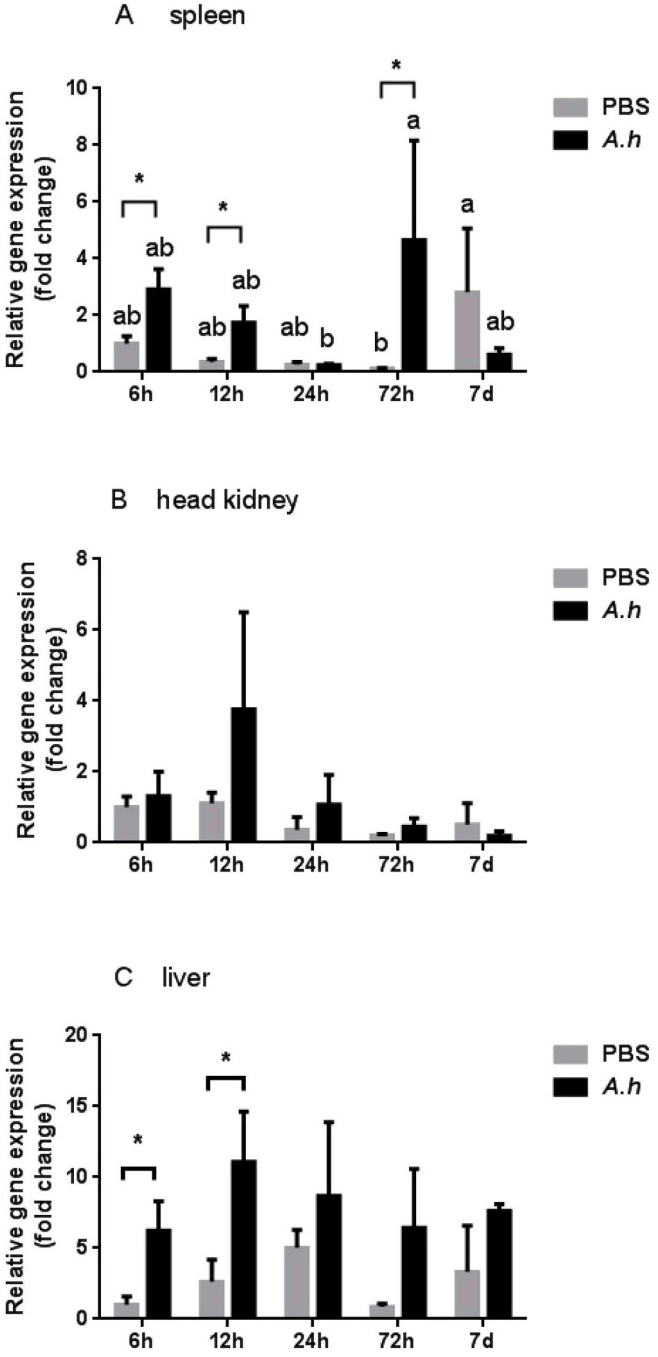


Fig. 4. Expression of *Abil-8* in spleen (A), head kidney (B) and liver (C) tissues of Siberian sturgeon (n = 3) within 7 days post-*A.h* injection. The relative expression method was applied in the calculation with *18S* as the reference gene. All data were obtained from three independent experiments with each performed in triplicate. Different lowercase letters indicate significantly different means ($P < 0.05$) across any time point. Error bars represent standard deviation.

induction of *il*-8 mRNA following LPS treatment was also observed in the PBLs of Japanese flounder [16] and in the head kidney macrophages of rainbow trout [43], Together, our results provide strong evidence for the participation of AbIL-8 in the anti-bacterial immune responses of sturgeon.

Rapid migration of immune cells to the site of tissue injury or bacterial invasion is thought to be a critical step in the inflammation response. The chemotactic activity of recombinant fish CXCL8 has been confirmed in a number of fish species, including large yellow croaker

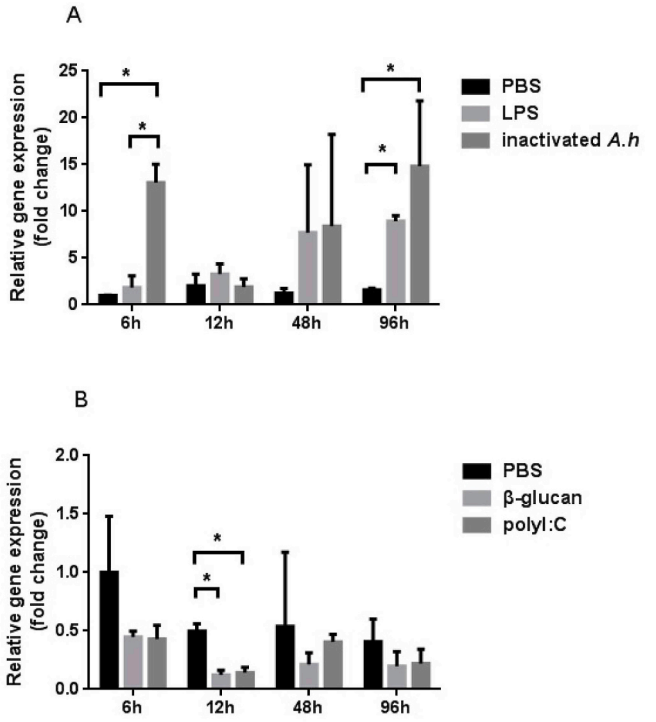


Fig. 5. Transcript levels of *Abil-8* in primary spleen cells of Siberian sturgeon (*Acipenser baeri*) (n = 4) upon stimulation with either LPS (100 μg/mL), inactivated *A.h*, β-glucan (100 μg/mL), or poly I: C (50 μg/mL). The relative expression method was applied using the *18S* as the reference gene. The results were presented as mean + standard deviation (SD) (n = 4). The x-axis represents the stimulation time (h). Asterisk (*) represents a statistical significance between two groups within one time point. Error bars represent standard deviation.

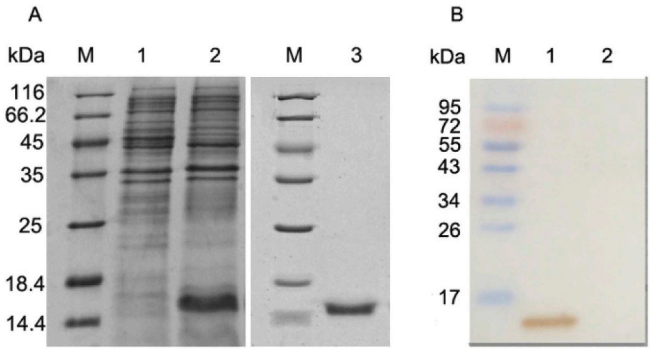
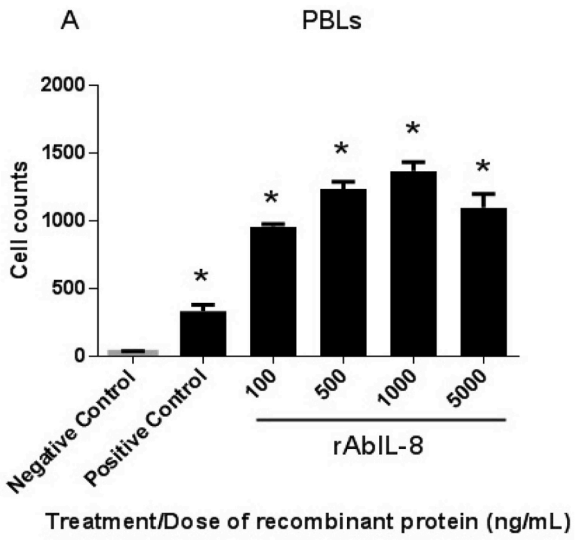
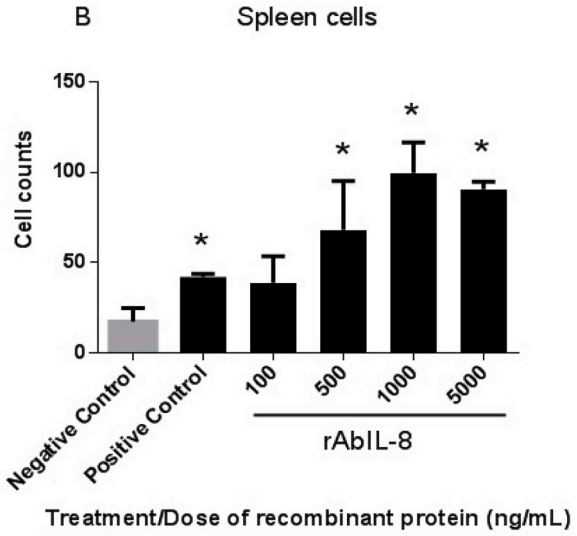
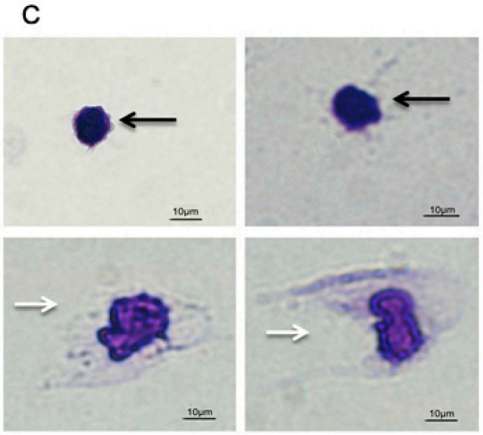


Fig. 6. Prokaryocyte expression and Western blot analysis of AbIL-8. (A) SDS-PAGE analysis of recombinant AbIL-8. Lane M: protein marker; 1: lysate of *E. coli* cells containing the empty pET30 (+) vector; 2: lysate of *E. coli* cells containing the recombinant construct pET30 (+)-AbIL-8; 3: the purified AbIL-8 protein. (B) Western blot analysis of the recombinant AbIL-8. Lane 1: purified recombinant protein; 2: control protein (BSA).

[17,37], grass carp [9], half-smooth tongue sole [15], mandarin fish (*Siniperca chuatsi*) [44] and ayu [10]. In this study, recombinant AbIL-8 exhibited obvious chemotactic activity for sturgeon PBLs and spleen cells *in vitro*; however, reduced chemotaxis occurred at higher concentration of 5 μg/mL (Fig. 7A, B), probably due to receptor desensitization, which has been noted for chemokines [1,4]. The chemotactic







(caption on next page)

Fig. 7. Effects of various concentrations of rAbIL-8 on the chemotaxis for Siberian sturgeon PBLs (A) and spleen cells (B). Incubation with rAbIL-1β (5000 ng/mL) and fMLP (5 ng/mL) served as the negative and positive controls, respectively. Error bars represent standard deviation. Statistically significant differences between each experimental group and its negative control are indicated with asterisks ($P < 0.05$). (C) Wright-Giemsa staining cell image (× 1000). The attracted Siberian sturgeon PBLs were stained by Wright-Giemsa at the end of transwell assay. Lymphocytes were pointed by black arrow and monocytes by white arrow.

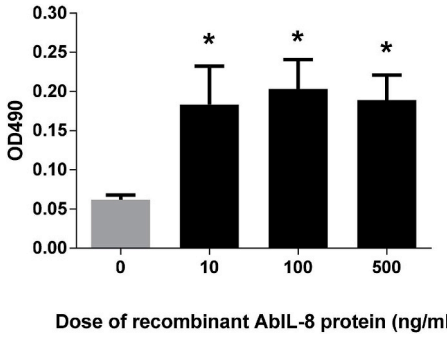


Fig. 8. Effects of AbIL-8 on proliferation of Siberian sturgeon spleen cells (n = 3). *In vitro* MTT assay was performed. Error bars represent the standard deviation. Statistically significant differences between each experimental group and its negative control are indicated with asterisks ($P < 0.05$).

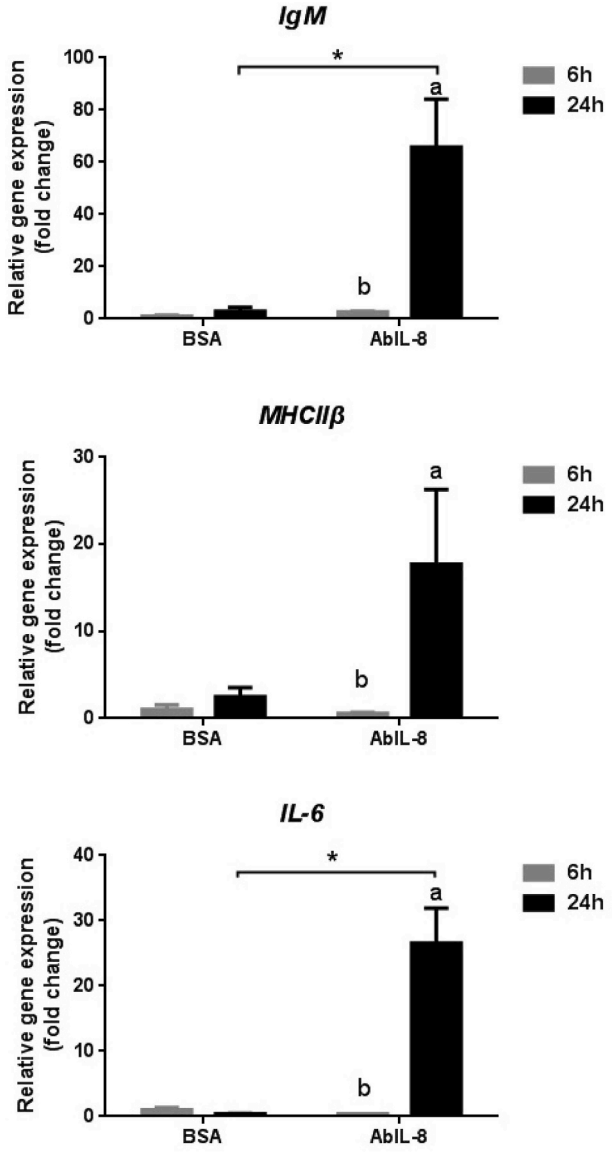


Fig. 9. Effects of recombinant AbIL-8 on the expression of immuno-related genes in the spleen of Siberian sturgeon (n = 3). The relative expression method was applied with *18S* used as the reference gene. Error bars represent the standard deviation. Statistically significant differences between each experimental group and its negative control at one time-point are indicated with asterisks ($P < 0.05$). Different lower letters indicate significant differences between paired means ($P < 0.05$) in one group at different time-points.

effect of rAbIL-8 on PBLs was more potent than that on spleen cells. It is possible that the spleen contained various cell types, but the leukocytes were not purified for transwell assay, and thus leading to a weak chemotactic activity. Several lines of evidence suggest that mammalian ELR^+ CXC chemokines usually recruit neutrophils and other polymorphonuclear leukocytes, while the ELR^- CXC chemokines specifically attract lymphocytes and monocytes [4]. However, in this study, rAbIL-8 with an ELR motif mainly attracted the lymphocytes and monocytes in PBLs (Fig. 7 C), consistent with what is observed in turbot [45] or half-smooth tongue sole [15] whose CXCL8s are lacking an ELR motif, suggesting that ELR motif is not specific for attracting neutrophils in fish. Besides, prior reports confirmed that an IL-8 lacking ELR motif could recruit not only neutrophils but also monocytes and lymphocytes in many teleost species. In the context of these previous observations, our results indicate that, fish IL-8 may interact with receptors via a mechanism different from that of mammal IL-8. Further, we found that rAbIL-8 stimulated the proliferation of spleen cells when the concentration reached or was above 10 ng/mL. A similar trend was reported in half-smooth tongue sole, where recombined IL-8 could stimulate the proliferation of PBLs and head kidney lymphocytes in a dose-dependent manner [15]. In turbot, rCXCL8 also induced the proliferation of PBLs, head kidney lymphocytes and macrophages [46]. These findings suggest that fish IL-8 probably play a direct role in immune response through modulation the migration and proliferation of immune cells.

Accumulating data show that IL-8 could induce the expression of several immuno-related genes in many fish species. For instance, recombinant IL-8 in half-smooth tongue sole [15], carp [18], and trout [45] all increased the expression of *IL-1β* in immune tissues. In Japanese flounder, *IL*-6 mRNA was significantly up-regulated at the rCXCL8_L1a injection-site; however, CXCL8_L1b, another type of CXCL8, induced gene expression of *IgM* [16]. In this study, rAbIL-8 not only significantly increased *IgM* and *IL-6* expression in the spleen ($P < 0.05$), but also slightly elevated the *MHCIIβ* mRNA levels ($P > 0.05$). IL-6 is recognized as an immunoregulatory cytokine that promotes the proliferation of B cells [47], and IgM is the predominant class of antibody present in teleost fish, together indicative of their adaptive immune system [48,49]. The major histocompatibility complex class II (MHCII) is critically involved in the presentation of antigen to the T lymphocytes receptors of mammals and birds [50]. Being able to induce *IL-6*, *IgM*, and *MHCIIβ* expression further suggested that AbIL-8 directly participates in the pro-inflammatory response and promoting adaptive immunity in sturgeon.

In conclusion, we identified *Abil-8* in Siberian sturgeon which is homologous to the teleost fish CXCL8_L1 subgroup, filling up the gap between jawless fish and teleosts. Expression of *Abil-8* was enhanced in response to *A.h* challenge and LPS treatment. Recombinant AbIL-8 not only exhibited chemotactic activity for lymphocytes and monocytes from PBLs and spleen cells, but stimulated the proliferation of spleen cells. Moreover, injection of rAbIL-8 increased the expression of *IL-6*, *IgM* and *MHCIIβ* in spleen. Collectively, these results indicate that AbIL-8 is closely involved in the inflammation and immunoregulation of Siberian sturgeon.

Author contributions

XW, HZ: conceived and designed the experiments. XW, GM, RZ, JZ and LL performed the experiments. XW: analysed the data and interpreted the results. XW, HZ: contributed reagents/materials/analysis tools. XW and HZ: wrote and revised the paper.

Declarations of interest

None.

Conflicts of interest

No conflict of interest existed.

Acknowledgements

This work was supported by the youth science Foundation of Beijing academy of agriculture and forestry (QNJJ201615); Beijing Municipal Excellent Talents Foundation (2017000020060G122); Natural Science Foundation of China (31602137); the Foundation of Beijing Municipal Science and Technology Project (Z161100004516003); and Beijing Sturgeon and Trout Innovation Team (BAIC08-2019).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.010.

References

[1] L.M. van der Aa, C. Magdalena, E. Tijhaar, P. Boudinot, B.M.L. Verburg-van Kemenade, CXCL8 chemokines in teleost fish: two lineages with distinct expression profiles during early phases of inflammation, PLoS One 5 (8) (2010) e12384.
[2] B. Gorgoglione, E. Zahran, N.G. Taylor, S.W. Feist, J. Zou, C.J. Secombes, Comparative study of CXC chemokines modulation in brown trout (*Salmo trutta*) following infection with a bacterial or viral pathogen, Mol. Immunol. 71 (2016) 64–77.
[3] C.G. Larsen, A.O. Anderson, J.J. Oppenheim, K. Matsushima, Production of interleukin-8 by human dermal fibroblasts and keratinocytes in response to interleukin-1 or tumour necrosis factor, Immunology 68 (1) (1989) 31–36.
[4] R.M. Strieter, P.J. Polverini, S.L. Kunkel, D.A. Arenberg, M.D. Burdick, J. Kasper, J. Dzuiba, J.V. Damme, A. Walz, D. Marriott, S.Y. Chan, S. Roczniak, A.B. Shanafelt, The functional role of the ELR motif in CXC chemokine-mediated angiogenesis, J. Biol. Chem. 270 (45) (1995) 27348–27357.
[5] P. Peveri, A. Walz, B. Dewald, M. Baggiolini, A novel neutrophil-activating factor produced by human mononuclear phagocytes, J. Exp. Med. 167 (5) (1988) 1547–1559.
[6] I. Lindley, H. Aschauer, J.M. Seifert, C. Lam, W. Brunowsky, E. Kownatzki, M. Thelen, P. Peveri, B. Dewald, V.V. Tscharner, Synthesis and expression in Escherichia coli of the gene encoding monocyte-derived neutrophil-activating factor: biological equivalence between natural and recombinant neutrophil-activating factor, Proc. Natl. Acad. Sci. U.S.A. 85 (23) (1988) 9199–9203.
[7] N.K. Abdelkhalek, A. Komiya, Y. Katounoki, T. Somamoto, M. Nakao, Molecular evidence for the existence of two distinct IL-8 lineages of teleost CXC-chemokines, Fish Shellfish Immunol. 27 (6) (2009) 763–767.
[8] J. Chen, Q. Xu, T. Wang, B. Collet, Y. Corripio-Miyar, S. Bird, P. Xie, P. Nie, C.J. Secombes, J. Zou, Phylogenetic analysis of vertebrate CXC chemokines reveals novel lineage specific groups in teleost fish, Dev. Comp. Immunol. 41 (2) (2013) 137–152.
[9] T.T. Wang, X.H. Song, G.M. Bao, L.X. Zhao, X. Yu, J. Zhao, Molecular characterization, expression analysis, and biological effects of interleukin-8 in grass carp *Ctenopharyngodon idellus*, Fish Shellfish Immunol. 35 (5) (2013) 1421–1432.
[10] C.Q. Chu, X.J. Lu, C.H. Li, J. Chen, Molecular characterization of a CXCL8-like protein from ayu and its effect on chemotaxis of neutrophils and monocytes/macrophages, Gene 548 (1) (2014) 48–55.
[11] S.H. Oehlers, M.V. Flores, C.J. Hall, R. O'Toole, S. Swift, K.E. Crosier, P.S. Crosier, Expression of zebrafish cxcl8 (interleukin-8) and its receptors during development and in response to immune stimulation, Dev. Comp. Immunol. 34 (3) (2010) 352–359.
[12] A. Rebl, H. Rebl, T. Korytář, T. Goldammer, H.M. Seyfert, The proximal promoter of a novel interleukin-8-encoding gene in rainbow trout (*Oncorhynchus mykiss*) is strongly induced by CEBPA, but not NF-κB p65, Dev. Comp. Immunol. 46 (2) (2014) 155–164.
[13] K.J. Laing, J.J. Zou, T. Wang, N. Bols, I. Hirono, T. Aoki, C.J. Secombes, Identification and analysis of an interleukin 8-like molecule in rainbow trout Oncorhynchus mykiss, Dev. Comp. Immunol. 26 (5) (2002) 433–444.
[14] M.O. Huising, E. Stolte, G. Flik, H.F. Savelkoul, B.M. Verburgvan Kemenade, CXC chemokines and leukocyte chemotaxis in common carp (*Cyprinus carpio L.*), Dev. Comp. Immunol. 27 (10) (2003) 875–888.
[15] J. Sun, L. Zhao, L. Sun, Interleukin-8 of Cynoglossus semilaevis is a chemoattractant with immunoregulatory property, Fish Shellfish Immunol. 30 (6) (2011) 1362–1367.
[16] B. Zhao, T. Katagiri, H. Kondo, I. Hirono, Comparative analysis of two types of CXCL8 from Japanese flounder (*Paralichthys olivaceus*), Dev. Comp. Immunol. 52 (1) (2015) 37–47.
[17] S. Zhou, Y. Mu, J. Ao, X. Chen, Molecular characterization and functional activity of CXCL8_L3 in large yellow croaker Larimichthys crocea, Fish Shellfish Immunol. 75 (2018) 124–131.
[18] L.M. Van Der Aa, M. Chadzinska, L.A. Golbach, C.M.S. Ribeiro, B.M.L. Verburg-van Kemenade, Pro-inflammatory functions of carp CXCL8-like and CXCb chemokines, Dev. Comp. Immunol. 36 (4) (2012) 741–750.
[19] Q. Wei, J. He, D. Yang, W. Zheng, L. Li, Status of sturgeon aquaculture and sturgeon trade in China: a review based on two recent nationwide surveys, J. Appl. Ichthyol. 20 (5) (2010) 321–332.
[20] J. Sigh, T. Lindenstrøm, K. Buchmann, Expression of pro-inflammatory cytokines in rainbow trout (*Oncorhynchus mykiss*) during an infection with Ichthyophthirius multifiliis, Fish Shellfish Immunol. 17 (1) (2004) 75–86.
[21] I. Letunic, T. Doerks, P. Bork, SMART: recent updates, new developments and status in 2015, Nucleic Acids Res. 43 (D1) (2015) D257–D260.
[22] T.N. Petersen, S. Brunak, G. von Heijne, H. Nielsen, SignalP 4.0: discriminating signal peptides from transmembrane regions, Nat. Methods 8 (2011) 785.
[23] J.D. Thompson, D.G. Higgins, T.J. Gibson, W. CLUSTAL, Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (22) (1994) 4673–4680.
[24] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Version 6.0, Mol. Biol. Evol. 30 (12) (2013) 2725–2729.
[25] N. Saitou, M. Nei, The neighbor-joining method - a new method for reconstructing phylogenetic trees, Version 6.0, Mol. Biol. Evol. 4 (4) (1987) 406–425.
[26] J.J. Campanella, L. Bitincka, J. Smalley, MatGAT, An application that generates similarity/identity matrices using protein or DNA sequences, BMC Bioinf. 4 (1) (2003) 29.
[27] H. Zhu, R. Song, X. Wang, H. Hu, Z. Zhang, Peritoneal bacterial infection repressed the expression of IL17D in Siberia sturgeon a chondrostean fish in the early immune response, Fish Shellfish Immunol. 64 (2017) 39–48.
[28] H. Weichert, I. Blechschmidt, S. Schröder, H. Ambrosius, The MTT-assay as a rapid test for cell proliferation and cell killing: application to human peripheral blood lymphocytes (PBL), Allerg Immunol 37 (3–4) (1991) 139–144.
[29] C. Dytham, Choosing and Using Statistics: A Biologist's Guide, Wiley-Blackwell Publishing, 1999.
[30] K.J. Laing, C.J. Secombes, Chemokines, Dev. Comp. Immunol. 28 (5) (2004) 443–460.
[31] C. Tian, Y. Chen, J. Ao, X. Chen, Molecular characterization and bioactivity of a CXCL13 chemokine in large yellow croaker *Pseudosciaena crocea*, Fish Shellfish Immunol. 28 (3) (2010) 445–452.
[32] I. Clarklewis, C. Schumacher, M. Baggiolini, B. Moser, Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs. Critical role of NH2-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities, J. Biol. Chem. 266 (34) (1991) 23128–23134.
[33] I. Clark-Lewis, K.S. Kim, K. Rajarathnam, J.H. Gong, B. Dewald, B. Moser, M. Baggiolini, B.D. Sykes, Structure-activity relationships of chemokines, J. Leukoc. Biol. 57 (5) (1995) 703–711.
[34] N. Gerber, H. Lowman, D.R. Artis, C. Eigenbrot, Receptor-binding conformation of the "ELR" motif of IL-8: X-ray structure of the L5C/H33C variant at 2.35 A resolution, Proteins 38 (4) (2015) 361–367.
[35] Y. Corripio-Miyar, S. Bird, K. Tsamopoulos, C.J. Secombes, Cloning and expression analysis of two pro-inflammatory cytokines, IL-1β and IL-8, in haddock (*Melanogrammus aeglefinus*), Mol. Immunol. 44 (6) (2007) 1361–1373.
[36] M. Seppola, A.N. Larsen, K. Steiro, B. Robertsen, I. Jensen, Characterisation and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (*Gadus morhua L.*), Mol. Immunol. 45 (4) (2008) 887–897.
[37] Y. Mu, K. Wang, J. Ao, X. Chen, Molecular characterization and biological effects of a CXCL8 homologue in large yellow croaker (*Larimichthys crocea*), Fish Shellfish Immunol. 44 (2) (2015) 462–470.
[38] T.T.T. Nguyen, H.T. Nguyen, P.-C. Wang, S.-C. Chen, Identification and expression analysis of two pro-inflammatory cytokines, TNF-α and IL-8, in cobia (*Rachycentron canadum L.*) in response to Streptococcus dysgalactiae infection, Fish Shellfish Immunol. 67 (2017) 159–171.
[39] C.Y. Feng, S.C. Johnson, T.S. Hori, M. Rise, J.R. Hall, A.K. Gamperl, S. Hubert, J. Kimball, S. Bowman, M.L. Rise, Identification and analysis of differentially expressed genes in immune tissues of Atlantic cod stimulated with formalin-killed, atypical *Aeromonas salmonicida*, Physiol. Genomics. 37 (3) (2009) 149–163.
[40] A.M. Philip, E.H. Jørgensen, A.G. Maule, M.M. Vijayan, Tissue-specific molecular immune response to lipopolysaccharide challenge in emaciated anadromous Arctic charr, Dev. Comp. Immunol. 45 (1) (2014) 133–140.
[41] I. Hirono, B.H. Nam, T. Kurobe, T. Aoki, Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder *Paralychthys olivaceus*, Journal of immunology (Baltimore, Md 165 (8) (2000) 4423–4427 1950.
[42] M.A. Morsey, Y. Popowych, J. Kowalski, G. Gerlach, D. Godson, M. Campos,

L.A. Babiuk, Molecular cloning and expression of bovine interleukin-8, Microb. Pathog. 20 (4) (1996) 203–212.
[43] S.V. Amaia, J.B. Lennington, T.J. Smith, Molecular cloning of an IL-8-like CXC chemokine and tissue factor in rainbow trout (*Oncorhynchus mykiss*) by use of suppression subtractive hybridization, Cytokine 17 (2) (2002) 66–70.
[44] G.L. Wang, M.C. Wang, X.W. Zhang, M.X. Chang, H.X. Xie, P. Nie, Molecular cloning, biological effect, and tissue distribution of interleukin-8 protein in Mandarin fish (*Siniperca chuatsi*) upon *Flavobacterium columnare* infection, Fish Shellfish Immunol. 66 (2017) 112.
[45] J. Montero, J. Coll, N. Sevilla, A. Cuesta, N.C. Bols, C. Tafalla, Interleukin 8 and CK-6 chemokines specifically attract rainbow trout (*Oncorhynchus mykiss*) RTS11 monocyte–macrophage cells and have variable effects on their immune functions, Dev. Comp. Immunol. 32 (11) (2008) 1374–1384.
[46] Y.-H. Hu, L. Chen, L. Sun, CXCL8 of *Scophthalmus maximus*: expression, biological activity and immunoregulatory effect, Dev. Comp. Immunol. 35 (10) (2011) 1032–1039.
[47] P. Ataie-Kachoie, M.H. Pourgholami, D.R. Richardson, D.L. Morris, Gene of the month: interleukin 6 (IL-6), J. Clin. Pathol. 67 (11) (2014) 932–937.
[48] J.M. Covello, S. Bird, R.N. Morrison, A.R. Bridle, S.C. Battaglene, C.J. Secombes, B.F. Nowak, Isolation of RAG-1 and IgM transcripts from the striped trumpeter (*Latris lineata*), and their expression as markers for development of the adaptive immune response, Fish Shellfish Immunol. 34 (3) (2013) 778–788.
[49] M.F. Flajnik, The last flag unfurled? A new immunoglobulin isotype in fish expressed in early development, Nat. Immunol. 6 (2005) 229–230.
[50] H.W. Davidson, P.A. Reid, A. Lanzavecchia, C. Watts, Processed antigen binds to newly synthesized mhc class II molecules in antigen-specific B lymphocytes, Cell 67 (1) (1991) 105–116.