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Interleukin-12 receptor β2 from grass carp: Molecular characterization and its involvement in *Aeromonas hydrophila*-induced intestinal inflammation

Xue-Hong Song, Jian Tang, Tian-Tian Gao, Xu-Fang Xu, Hui-Xing Yang, Kang Wu, Cai-Gen Yang, Zhong-Qin Cheng, Bing-Yao Sun*

School of Biology and Basic Medical Sciences, Soochow University, 199 Ren'ai Road, Suzhou, Jiangsu, 215123, China

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ABSTRACT

Interleukin-12 receptor β2 (IL-12Rβ2) is a signaling subunit of heterodimeric receptors for IL-12 and IL-35. It plays important regulatory functions in the development of Th1 cells and in the expression of inflammatory cytokines in mammals and other higher vertebrates. However, little is known about IL-12Rβ2 in teleost fish. In this work, we have cloned and characterized IL-12Rβ2 from grass carp (*Ctenopharyngodon idella*). The full-length cDNA of grass carp IL-12Rβ2 is 2875 bp, which encodes a mature protein with 741 amino acids. This mature protein contains three fibronectin type III domains, a transmembrane helix, and CXW and WSXWS-like motifs that are characteristic of the type I cytokine receptor family. Phylogenetic analysis revealed that cyprinid fish IL-12Rβ2 formed a single branch, clearly separated from those of other vertebrates. We expressed and purified a recombinant grass carp IL-12Rβ2 protein containing major antigenic regions, which was used to raise a polyclonal antibody. The specificity of the antibody was assessed by Western blotting analysis of whole cell lysates from *Escherichia coli* cells expressing the recombinant IL-12Rβ2, grass carp intestinal intraepithelial lymphocytes, and cultured *C. idella* kidney cells. To explore the potential regulatory role of IL-12Rβ2 in inflammation, we generated an intestinal inflammation model by anal intubation of fish with *Aeromonas hydrophila*. Immunohistochemical staining of the inflamed intestines revealed that IL-12Rβ2 expression is consistent with inflammatory cell recruitment during intestinal inflammation. Real-time quantitative PCR revealed that IL-12Rβ2 is widely expressed in normal tissues and is up-regulated in most tissues after infecting with *A. hydrophila*. We found that IL-12Rβ2, IL-12p35, and interferon-γ were expressed in similar patterns in the intestines during inflammation. Taken together, our results suggest that IL-12Rβ2 is involved in the regulation of intestinal inflammation.

1. Introduction

Interleukin-12 receptor (IL-12R) is a heterodimeric protein [1]. IL-12R is composed of two β-type cytokine receptor subunits (β1 and β2 subunits) that are designated as IL-12Rβ1 and IL-12Rβ2 [2,3]. Both IL-12R subunits are necessary for effective binding to IL-12, a heterodimeric cytokine consisting of the p40 and p35 subunits. It has been demonstrated that IL-12Rβ1 binds IL-12 mainly through the p40 subunit, while IL-12Rβ2 interacts with IL-12 through a heterodimer specific region in which both the p40 and p35 subunits are involved [4,5].

IL-12Rβ1 is shared by the IL-12 and IL-23 receptors while IL-12Rβ2 is shared by the IL-12 receptor and three different IL-35 receptors [6]. Previous studies have indicated that the expression of IL-12Rβ1 and IL-12Rβ2 in activated T and NK cells is regulated through different mechanisms [7]. Numerous studies using IL-12Rβ1-or IL-12Rβ2-deficient mice revealed that these two IL-12R subunits are required by different cell lineages and play differential roles in IL-12 signaling [5,8,9].

Using Stat4-and IL-12Rβ2-deficient mice, previous studies demonstrated that IL-12Rβ1 is primarily responsible for binding IL-12, whereas IL-12Rβ2 is responsible for the activation of intracellular signal transduction pathways and essential for IL-12 to execute its biological functions [8,10]. IL-12Rβ2 is differentially expressed in two rat strains with different Th1/Th2 bias, which determines the cytokine profile of an immune response [11]. Interestingly, IL-12Rβ2 was identified as a key mediator of its own signaling pathway independently of IL-12. In addition, although IL-12R signaling is not necessary for the development of T-bet$^+$ regulatory T cells, impaired expression of IL-12Rβ2 may lead to abortive Th1 cell differentiation from T-bet$^+$ regulatory T cells [12]. Furthermore, IL-12Rβ2 binds to gp130 to form an unconventional receptor complex that mediates the anti-inflammatory effects of IL-35

* Corresponding author.
E-mail address: sunbingyao@suda.edu.cn (B.-Y. Sun).

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Table 1
Nucleotide primers used in this study.

Primer	Sequence (5′ to 3′)	Application
12R5-1	TCCAGGAGCAGTGAATGTCTTGA	5′-RACE
12R5-2	CAACTGTTTGCTCTTCCCTGTGC	
12R3-1	GGACTGTGACACTGTGGAACCCTT	3′-RACE
12R3-2	GAGGATTCCCAGCCATTCACAC	
23R3-3	AGTTTTCGTGTTCGCTGCCACT	
IL-12Rβ2-F	TTTTGGTACCATGGTCGACACCTGCAACGTTACC	Recombinant construct
IL-12Rβ2-R	TTTTCTCGAGTTCACCAGAAGAACCGTCGTCTTCTTC	
12rcDNA-F	GCAGAGTAAACCGCTGAAGAAC	PCR amplification of the internal cDNA sequences
12rcDNA-R	CTAACCACTTCTCCGTACCTTCA	
q12RI-F	GCCGATGATTCCAACTATTTACAC	Quantitative real-time PCR (for IL-12Rβ2 gene)
q12RII-F	AAGAGAAGGCTGGACGTTTACTC	
q12R-R	TGACCTGTGTTGAAGACAGACTCT	
qp35F	TGGAAAAGGAGGGGAAGATG	Quantitative real-time PCR (for IL-12p35 gene)
qp35R	AGACGGACGCTGTGTGAGTGTA	
qIFNγF	AATTCCAAGGCTTCCTGCACT	Quantitative real-time PCR (for IFN-γ gene)
qIFNγR	TTCCCCTCAGCTTTTTCACCTC	
β-actinF	CCTTCTTGGGTATGGAGTCTTG	Quantitative real-time PCR (for reference gene β-actin)
β-actinR	AGAGTATTTACGCTCAGGTGGG	

All primers were commercially synthesized by GENEWIZ (Suzhou, China). The primers used in reverse transcription for generating cDNA templates, and the universal primers used for 3′- and/or 5′-RACE are not shown in this table. The underlined are restriction sites for *Kpn* I and *Xho* I, respectively.

[13,14].

Fish homologues of the mammalian IL-12p35 and IL-12p40 genes have been identified in several teleost species, such as amberjack (*Seriola dumerili*), Atlantic salmon (*Salmo salar*), common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), rainbow trout (*Oncorhynchus mykiss*), orange-spotted grouper (*Epinephelus coioides*), pufferfish (*Fugu rubripes*), rock bream (*Oplegnathus fasciatus*), and sea bass (*Dicentrarchus labrax*) [15–19]. Differential expression of the p35 and p40 subunits of IL-12 was also observed in pathogen-infected fish [17]. The role of the IL-12/IL-12R signaling axis in fish immune responses against pathogenic microorganisms is largely unknown, mainly because of the scarce information on fish IL-12R genes. Up to now, fish IL-12R subunits, IL-12Rβ1 and IL-12Rβ2, have not been characterized at the molecular level.

In the present study, we report the cloning and characterization of an IL-12Rβ2 gene from grass carp. We generated a recombinant grass carp IL-12Rβ2 protein and a polyclonal antibody against this protein. Immunohistochemical analysis of the posterior intestine with this antibody demonstrated the involvement of IL-12Rβ2 in intestinal inflammation induced by *Aeromonas hydrophila*.

2. Materials and methods

2.1. Animals

Grass carps (2 years old, mean body weight 62.3 ± 3.4 g) were provided by Wujiang Aquaculture Co., Ltd., Jiangsu, China. The fish were reared in a recirculating system under conditions described previously [20].

Six week old female Balb/c mice were purchased from the Laboratory Animal Center of Soochow University, Suzhou, Jiangsu, China. Mice were fed with pathogen-free diet and water and kept under pathogen-free conditions. All animal experiments were approved by the Animal Ethics Committee of Soochow University.

2.2. Induction of intestinal inflammation in grass carp

A strain of *A. hydrophila* (strain preservation number CCTCC M2013089) was used to induce intestinal inflammation in grass carp. Fish anesthetized with MS-222 were infected with an *A. hydrophila* suspension at 1×10^7 cfu/mL by anal intubation as described previously [20]. Each fish received 150 μL of the bacterial suspension. Control fish were mock-infected with an equal volume of physiological saline solution (PSS). After infection, control or infected fish were raised separately at 28 ± 1 °C. By scoring swimming movement, ingestion rate, anal inflammation, body surface bleeding, abdominal dropsy, and intestinal mucosal lesions, the disease activity index (DAI) was calculated at d 1, 3, 7, 14, and 21 after bacterial infection as described previously [20].

2.3. Tissue sampling, total RNA extraction, and cDNA synthesis

One d before infection with *A. hydrophila*, fish feeding was stopped to reduce fecal contamination of the intestinal mucus. Fish were killed under anesthesia and tissues were excised. One d after infection, muscle, thymus, heart, intestine, trunk kidney, spleen, head kidney, gill, skin, tail fin, liver, and blood were collected from healthy fish and from infected or mock-infected fish. Tissues were extracted from three fish. The intestines were also collected at d 1, 3, 7, 14, and 21 after infection by anal intubation. Tissues were used for total RNA extraction and subjected to quantitative real-time PCR (qPCR) analysis. For immunohistochemical analysis, the posterior intestine was extracted from fish at d 1, 3, 7, 14, and 21 after infection. After washing thoroughly with cold PSS, tissues were fixed in 10% formaldehyde.

Total RNA was extracted using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RNA was quantified by measuring absorption at 260 and 280 nm with a spectrophotometer (NanoDrop ND-1000, Wilmington, DE, USA). The integrity of total RNA was checked with agarose gel electrophoresis. One microliter of total RNA was used for the synthesis of first strand cDNA using the SMARTScribe Reverse Transcriptase kit and Oligo$(dT)_{18}$ primers (TaKaRa, Dalian, China). The resulting cDNA was then used for qPCR.

2.4. Molecular cloning and sequence analysis of grass carp IL-12Rβ2

To clone the full-length cDNA encoding grass carp interleukin-12 receptor β2 (designated as gcIL-12Rβ2), we performed a rapid amplification of cDNA ends (RACE) using the SMARTer® RACE 5′/3′Kit (TaKaRa Bio USA, Mountain View, CA, USA). First strand cDNA templates for 5′- and 3′-RACE were prepared according to the user's manual using 1 μg of total RNA isolated from intestines of *A. hydrophila*-infected fish. Two rounds of 5′- RACE and three rounds of 3′-RACE were carried out using primers designed from a 992-bp sequence of unigene 25229 (Table 1) [21]. The PCR products were gel-purified, cloned into the pMD19-T vector (TaKaRa, Dalian, China), and sequenced by Genewiz (Suzhou, Jiangsu, China). Subsequently, a pair of primers (12rcDNA-F

Fig. 1. Nucleotide sequence and predicted amino acid sequence of grass carp IL-12Rβ2. The start and stop codons are in bold. The predicted 22-amino acid signal peptide is boxed. The sequence of fibronectin type III (FN3) domains is indicated by italic letters in bold. The transmembrane helix region is shown in bold and shaded in gray. In the extracellular domain, the conserved CXW and WSXWS-like motifs defining the type I cytokine receptor family are boxed in gray. The conserved cysteine residue upstream of the CXW domain is shaded. The potential antigenic regions are wavy-underlined.

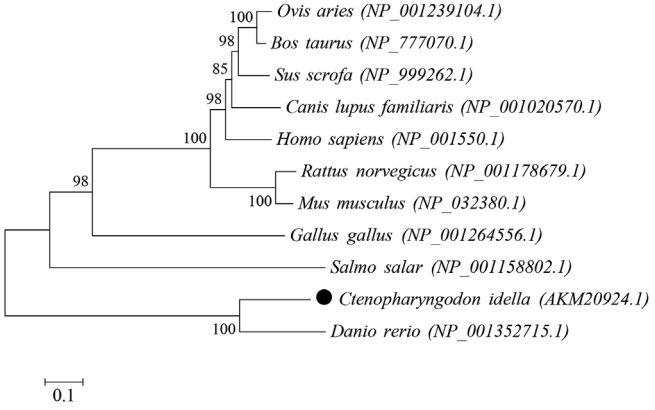

Fig. 2. Phylogenetic tree derived from neighbor-joining analysis of the IL-12Rβ2 proteins from representative species. Protein sequences were retrieved by standard protein BLAST from NCBI using grass carp IL-12Rβ2 sequence (AKM20924.1) as a query. The phylogenetic tree was constructed using the neighbor-joining method of amino acid sequences with identity to grass carp IL-12Rβ2 using MEGA (version 5.2).

and 12rcDNA-R, see Table 1) corresponding to the 5'-untranslated region (UTR) and to the sequence immediately proximal to 3'-UTR were designed and used to amplify the intervening DNA. The full-length cDNA of gcIL-12Rβ2 was assembled by aligning the internal cDNA sequences and the 5′- and 3′-RACE sequences using DNASTAR. Structural features, such as the signal peptide and conserved domains, were predicted using SMART software (http://smart.embl-heidelberg.de). The potential proteolytic cleavage sites of gcIL-12Rβ2 were predicted using PeptideCutter (https://web.expasy.org/peptide_cutter/).

2.5. Phylogenetic analysis and sequence alignment

Standard protein BLAST from NCBI was performed using gcIL-12Rβ2 (GenBank accession no. AKM20924.1) sequence as a query. The amino acid sequences of IL-12Rβ2 in fish, chicken, and mammalian species were retrieved and used for sequence alignment using ClustalX (version 2.0). Phylogenetic analysis was conducted using the neighbor-joining method in MEGA software (version 5.2) using 100 replicates for bootstrapping. The aligned sequences were saved in FASTA format and used to identify conserved motifs using SMS software (http://www.bioinformatics.org/sms/).

2.6. Gene synthesis, recombinant expression of gcIL-12Rβ2, and fusion protein production

2.6.1. Antigenic-site mapping and gene synthesis

Prediction of B-cell epitopes within the amino acid sequence of gcIL-12Rβ2 was performed using the online BCEPRED program (http://

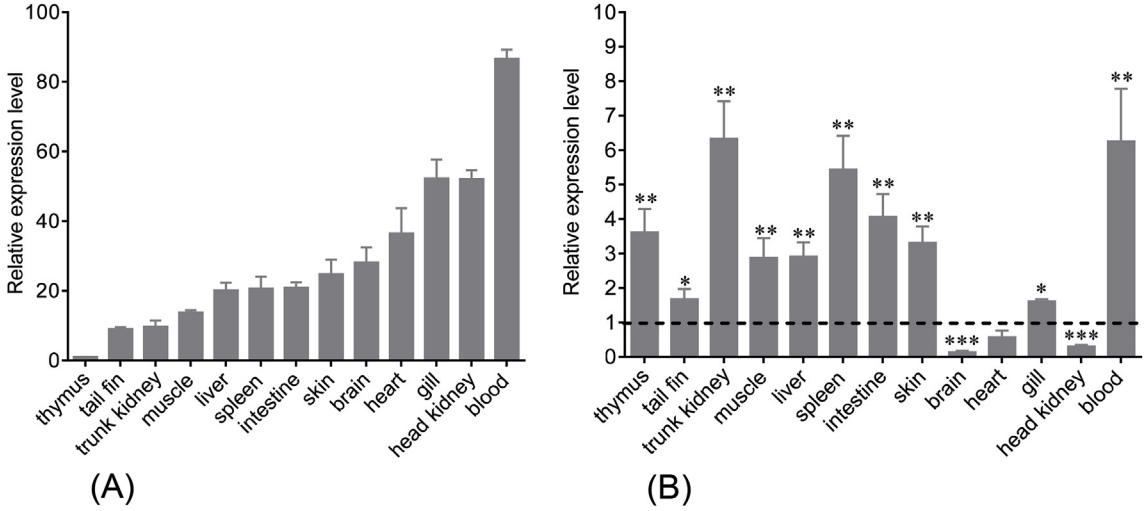

Fig. 3. Tissue distribution of IL-12Rβ2 and its expression pattern in response to bacterial infection. (A) The IL-12Rβ2 mRNA levels in normal grass carp tissues. IL-12Rβ2 expression in each tissue was normalized to β-actin and is shown as fold increase over the lowest expression level in the thymus. (B) The IL-12Rβ2 expression pattern in response to bacterial infection. The IL-12Rβ2 expression level at 1 d after anal intubation with *A. hydrophila* was normalized to the baseline levels of normal tissues, which were set to 1, as indicated by the dotted line.

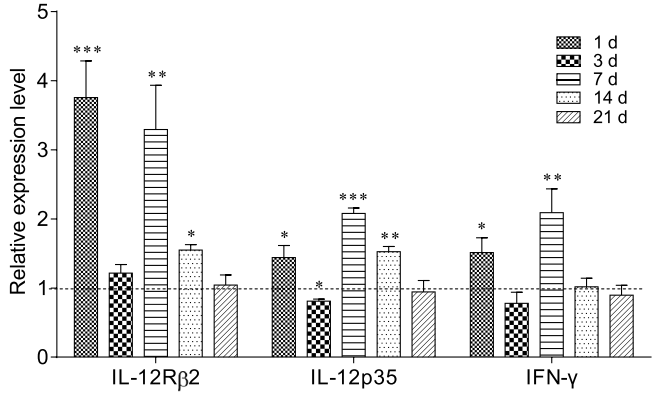

Fig. 4. Time course of expression of IL-12Rβ2, IL-12p35, and IFN-γ in inflamed intestines. Intestinal expression of IL-12Rβ2, IL-12p35, and IFN-γ at five time points after anal intubation with *A. hydrophila* or physiological saline solution (PSS) (control) was examined. Expression for these genes was normalized to β-actin, and normalized to their corresponding control levels, which were set to 1, as indicated by the dotted line.

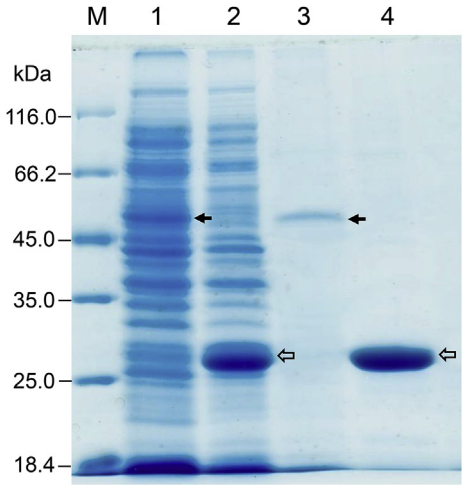

Fig. 5. SDS-PAGE analysis of the recombinant grass carp IL-12Rβ2. M, protein molecular weight marker (kDa); lane 1, crude lysate of IPTG-induced *E. coli* BL21 (DE3) transformed with pGEX5T-rgcIL-12Rβ2; lane 2, crude lysate of IPTG-induced *E. coli* BL21 (DE3) transformed with the empty pGEX5T vector; lane 3, purified recombinant grass carp IL-12Rβ2 (rgcIL-12Rβ2); lane 4, purified GST.

www.imtech.res.in/raghava/bcepred/). Three potential antigenic regions, corresponding to amino acids 99–205, 333–381, and 715–763, respectively, were identified according to physicochemical properties including hydrophilicity, flexibility, accessibility, turns, exposed surface, and polarity, which were set as the default thresholds. We constructed a synthetic gene, which consists of these three antigenic regions and two flexible peptide linkers (Gly-Gly-Gly-Ser and Ser-Ser-Gly-Gly-Ser-Ser). After codon optimization using DNAWorks (https://hpcwebapps.cit.nih.gov/dnaworks/), the gene was synthesized by Genewiz and subcloned into the Kpn I and Xho I sites of pGEX5T. The sequence of the resulting recombinant plasmid, pGEX5T-rgcIL-12Rβ2, was verified by colony PCR and Sanger sequencing and a recombinant grass carp IL-12Rβ2 (rgcIL-12Rβ2) fusion protein with glutathione S-transferase (GST) was expressed in *E. coli*.

2.6.2. Recombinant expression and fusion protein production

To generate rgcIL-12Rβ2 protein, the pGEX5T-rgcIL-12Rβ2 plasmid was transformed into competent *Escherichia coli* strain BL21 (DE3) using standard methods. Cells were grown in Luria–Bertani (LB) medium containing ampicillin (100 μg/mL) for 3 h with shaking at 37 °C and then incubated overnight at 25 °C under induction with IPTG (0.05–0.5 mM). One milliliter of the bacterial culture was centrifuged to obtain the cell pellet, which was washed and lysed in 0.5 mL phosphate buffered saline (PBS) by sonication. The lysate was centrifuged at 12,000 × *g* for 10 min to obtain the supernatant, which was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The cultures producing the target protein were inoculated into 10 mL LB medium, cultured overnight, and then transferred into 1 L of fresh LB medium containing ampicillin for culture at 25 °C, followed by IPTG induction. Cells were harvested by centrifugation and lysed by ultrasonic disruption. Lysates were centrifuged at 12,000 × *g* for 10 min to collect the supernatant, which was loaded onto a nickel (Ni)-affinity column (Ni-NTA, Genscript, Nanjing, China) to purify the rgcIL-12Rβ2 protein. This fusion protein was eluted from the column

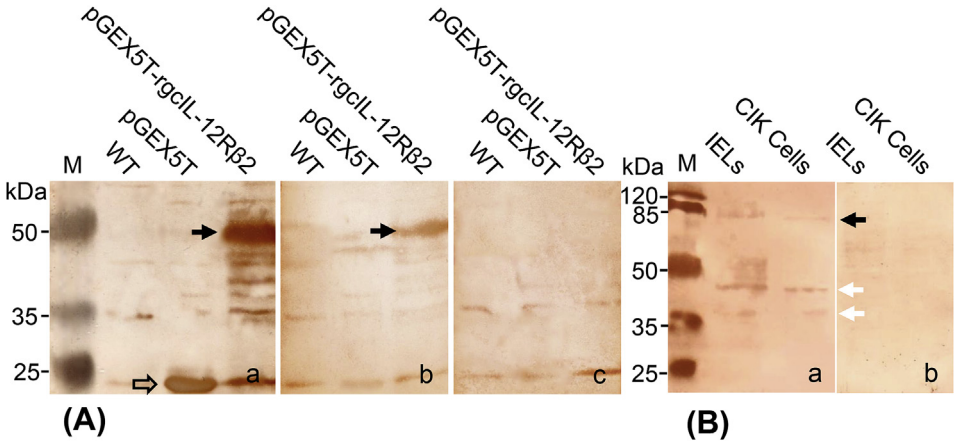

Fig. 6. Specificity of anti-rgcIL-12Rβ2 polyclonal antibody. (A) Western blotting analysis of recombinant grass carp IL-12Rβ2 expressed in *E. coil* BL21 (DE3). Nitrocellulose membranes were incubated with rgcIL-12Rβ2 pAb (a), rgcIL-12Rβ2 pAb pre-incubated with purified GST (b), or rgcIL-12Rβ2 pAb pre-incubated with purified rgcIL-12Rβ2 (c). M, protein molecular weight marker (kDa); WT, crude lysate of wild-type *E. coli* BL21 (DE3) without plasmid transformation; pGEX5T, crude lysates of *E. coli* BL21 (DE3) transformed with the empty pGEX5T vector; pEGX5T-rgcIL12Rβ2, crude lysate of *E. coli* BL21 (DE3) transformed with pGEX5T-rgcIL-12Rβ2. The rgcIL-12Rβ2 and GST protein bands are indicated by a solid arrow and a hollow arrow, respectively. (B) Western blotting analysis of gcIL-12Rβ2 in intestinal intraepithelial lymphocytes (IELs) and *C. idella* kidney (CIK) cells. Nitrocellulose membranes were incubated with rgcIL-1b pAb (a) or with rgcIL-12Rβ2 pAb pre-incubated with purified rgcIL-12Rβ2 (b). The band indicated by a black arrow corresponds to the predicted molecular weight of 84.0 kDa of mature IL-12Rβ2 and the bands indicated by white arrows are possibly different isoforms of IL-12Rβ2.

with elution buffer containing 500 mM imidazole. Pooled eluents were dialyzed against PBS at 4 °C to remove imidazole. An additional centrifugation step was performed to remove precipitates. The purity and size of eluted proteins were estimated using SDS-PAGE and Coomassie staining. The GST protein was produced and purified as a negative control using the same procedure using *E. coli* strain BL21 (DE3) transfected with the empty pGEX5T plasmid. Protein concentration was determined using a commercial bicinchoninic acid protein assay kit (Beyotime, Shanghai, China).

2.7. Polyclonal antibody production and western blotting analysis

The rgcIL-12Rβ2 protein purified from the nickel column was used as an immunogen to obtain polyclonal anti-rgcIL-12Rβ2 antibodies (rgcIL-12Rβ2 pAb). Balb/c mice were immunized according to the procedure described in our previous study [22]. One week after the final immunization, blood was collected by puncturing the caudal vein and centrifuged at 12,000 × *g* for 15 min. Serum was collected, aliquoted, and stored at −80 °C until further use.

The specificity of the rgcIL-12Rβ2 pAb was assessed with Western blotting analysis. Lysates from *E. coli* cells transformed with either pGEX5T or pGEX5T-rgcIL-12Rβ2 were resolved with 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated overnight at 4 °C in blocking solution (PBS containing 1% ovalbumin (w/v)). After washing twice with PBST (PBS with 0.05% Tween 20), membranes were incubated for 2 h at room temperature with rgcIL-12Rβ2 pAb (1:1000 dilution), or rgcIL-12Rβ2 pAb pretreated with either purified GST protein or Ni-NTA-purified rgcIL-12Rβ2 protein. Thereafter, the primary antibodies were detected by incubating the membrane with goat anti-mouse IgG secondary antibody (1:300 dilution). Antibody binding bands were visualized using conventional diaminobenzidine staining.

To verify the antibody specificity in grass carp cells, Western blotting analysis was also performed using intestinal intraepithelial lymphocytes (IELs) and cultured *C. idella* kidney (CIK) cells. IELs were isolated as described previously [23]. Briefly, one d after anal intubation with *A. hydrophila*, the fish were anesthetized and the posterior intestine was excised, washed with PBS to remove fecal material, and then cut into 0.5 cm fragments. The intestinal fragments were immersed in calcium- and magnesium-free PBS containing 2 mM dithiothreitol, 5 mM EDTA and 2% fetal bovine serum and shaken at 37 °C for 30 min. The resulting slurry was filtered by passing through a nylon 100-mesh sieve. Cells were harvested by centrifugation at 290 × *g* for 5 min at 4 °C and then resuspended in 5 mL of 40% Percoll. Percoll suspensions were centrifuged at 800 × *g* for 15 min at 4 °C. The precipitates were washed with PBS and IELs were obtained after centrifugation at 290 × *g* for 5 min at 4 °C.

IELs (~5×10^7 cfu) were suspended in 100 μL PBS, mixed with an equal volume of 2 × SDS loading buffer, and then boiled for 10 min. The cell lysates were centrifuged at 12,000 × *g* for 10 min at 4 °C. Lysates of CIK cells were prepared following the same procedure as for IELs. After centrifugation, cell lysates were separated using SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to immunoblotting as described above.

2.8. Immunohistochemical analysis

Paraffin sections (8 μm thick) of formaldehyde-fixed intestinal tissues were prepared for histological analysis using standard methods. After removing paraffin using Clear-Rite (Rite Scientific, Pittsburgh, PA, USA), the sections were hydrated with gradient ethanol, and then immersed in distilled deionized water. Heat-induced epitope retrieval was performed by incubating tissue sections in a sodium citrate buffer (pH 8.0) at 100 °C for 10 min. Then, tissue sections were blocked with 5% non-fat milk and oxidized with 3% H_2O_2. Tissue sections were further incubated with mouse anti-rgcIL-12Rβ2 pAb (1:1000 dilution) overnight at 4 °C. After incubation with HRP-labelled goat anti-mouse secondary antibody for 50 min at room temperature, the sections were stained with DAB and then counterstained with Harris hematoxylin. Tissue sections were examined under a microscope.

2.9. qPCR analysis

Gene expression analysis was performed using qPCR on tissues obtained from healthy fish, from fish infected with *A. hydrophila*, or from mock-infected fish. qPCR was also performed using intestinal tissues to analyze the expression of IL-12Rβ2, IL-12p35, and interferon (IFN)-γ following a time course of intestinal inflammation. The primers for the amplification of the gene of interest and of the reference gene β-actin are listed in Table 1. The threshold cycle value for each sample was obtained by three independent amplifications. The gene expression level in each sample was calculated using the threshold cycle value as described previously [20]. Significant differences in gene expression were evaluated by one way analysis of variance using SPSS13.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was indicated in figure legends by asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

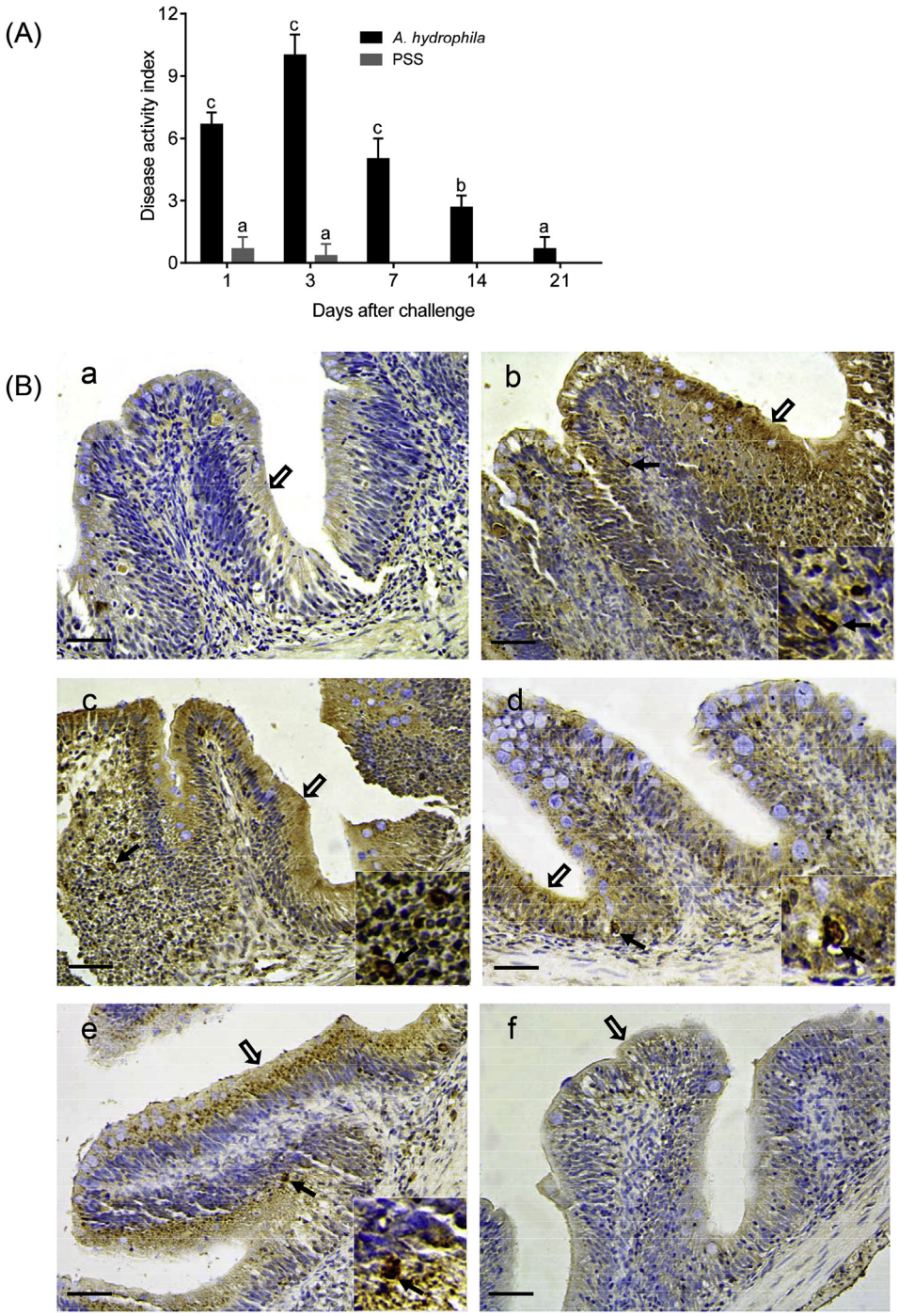

Fig. 7. Involvement of grass carp IL-12Rβ2 in *Aeromonas hydrophila*-induced intestinal inflammation. (A) Disease activity index (DAI) for fish that were infected with *A. hydrophila* or mock-infected with PSS. Data are mean ± SD (Standard deviation). Different lowercase letters represent differences that are statistically significant ($p < 0.05$). (B) Immunohistological analysis of grass carp posterior intestine during *A. hydrophila*-induced inflammation by immunostaining with rgcIL-12Rβ2 pAb. a, normal intestine in mock-infected fish (control); b–f, intestinal tissues at 1, 3, 7, 14, and 21 d after infection with *A. hydrophila*. In each panel, the hollow arrow indicates a positive reaction for IL-12Rβ2; the solid arrow indicates inflammatory cells positive for IL-12Rβ2. The arrow in each inset points to inflammatory cells. Scale bars in all panels are 50 μm.

3. Results

3.1. Cloning of cDNA encoding grass carp IL-12Rβ2

We obtained both 5′ and 3′ portions of the gcIL-12Rβ2 cDNA sequence by RACE amplification using gene-specific primers that were designed from a 992-bp unigene sequence of transcriptome data. Thereafter, conventional PCR was performed using a forward primer corresponding to the 5'-UTR and a reverse primer corresponding to a sequence immediately upstream of the stop codon. Sequences resulting from these amplification strategy were aligned and assembled into a complete cDNA encoding the entire reading frame of gcIL-12Rβ2 (GenBank accession no. KP412310.1).

The IL-12Rβ2 cDNA is 2875 bp long, containing a 306 bp 5'-UTR, a 2292 bp coding region, and a 277 bp 3'-UTR (Fig. 1). Comparison of cDNA with genomic sequences revealed that gcIL-12Rβ2 is composed of 15 exons and 14 introns (data not shown). Sequence analysis of the cloned cDNA revealed that gcIL-12Rβ2 consists of 763 amino acid residues, with a predicted molecular weight of 86.65 kDa. This protein includes a signal peptide of 22 amino acid residues. Domain prediction using SMART software showed that mature IL-12Rβ2 has three fibronectin type III domains, a transmembrane helix, and CXW and WSXWS-like motifs that are highly conserved in the type I cytokine receptor family (Fig. 1).

We performed a multiple alignment of the IL-12Rβ2 amino acid sequences against the sequences of human, bovine, mouse, chicken, and three teleost fish species. The CXW and WSXWS-like motifs and a cysteine residue upstream of the CXW motif are highly conserved among

vertebrates. However, other conserved structural features of mammalian IL-12Rβ2 proteins, such as the cytoplasmic box 1 and box 2 motifs, and three tyrosine residues are not present in fish IL-12Rβ2 proteins (Fig. S1).

3.2. Phylogenetic analysis of the IL-12Rβ2 proteins

A neighbor-joining tree was constructed using amino acid sequences of the IL-12Rβ2 proteins from fish, birds, and mammals (Fig. 2). It was seen that the IL-12Rβ2 sequences from grass carp and zebrafish were clustered into a single branch and clearly separated from other species. The mammalian IL-12Rβ2 sequences formed a large cluster, which was merged by the chicken sequence. It was noted that the Atlantic salmon IL-12Rβ2 did not cluster with other fish sequences and had substantial distance from others as the bootstrap values were extremely low.

3.3. Expression pattern of IL-12Rβ2 in normal grass carp tissues

IL-12Rβ2 expression in normal tissues was examined using qPCR analysis. IL-12Rβ2 expression level was normalized with internal control β-actin (Fig. 3A). The gcIL-12Rβ2 transcripts were detected in all tissues examined. The highest expression level was detected in blood, followed by in head kidney, gill, heart, brain, skin, intestine, spleen, liver, muscle, trunk kidney, and tail fin while the lowest expression level was observed in thymus.

3.4. Effects of A. hydrophila infection on IL-12Rβ2 expression in different tissues

To determine whether the expression pattern of IL-12Rβ2 is influenced by *A. hydrophila* infection, the expression of IL-12Rβ2 was examined with qPCR analysis 1 d after anal intubation with *A. hydrophila*. Fig. 3B shows that IL-12Rβ2 was significantly up-regulated 1 d after bacterial infection in most tissues while it was significantly down-regulated in brain and in head kidney.

3.5. Time course analysis of expression of IL-12Rβ2, IL-12p35, and IFN-γ in the intestine during inflammation

To explore the potential regulatory role of IL-12Rβ2 during inflammation, we used an experimental grass carp model of intestinal inflammation induced by anal intubation with *A. hydrophila*. The expression of IL-12Rβ2, IL-12p35, and IFN-γ in inflamed intestines or in control intestines from mock-infected fish was assessed with qPCR. IL-12Rβ2, IL-12p35, and IFN-γ had similar expression patterns during inflammation (Fig. 4). Relative to control intestines, the expression of these genes was increased 1 d after bacterial infection. Three d after infection, the expression of these genes was decreased. IL-12Rβ2 and IFN-γ expression was decreased to control levels, but IL-12p35 expression was decreased to a level that was lower than that of the control fish. At 7 d after infection IL-12Rβ2, IL-12p35, and IFN-γ expression was increased again. At this time point, the maximum expression of IL-12p35 and IFN-γ was reached. Thereafter, gene expression declined progressively. By d 21, IL-12Rβ2, IL-12p35, and IFN-γ expression returned to the control levels.

3.6. Generation of specific anti-rgcIL-12Rβ2 polyclonal antibody

The synthetic gene encoding three different antigenic regions of gcIL-12Rβ2 was cloned into pGEX5T and the GST fused rgcIL-12Rβ2 protein was expressed. After purification using nickel column affinity chromatography, SDS-PAGE revealed a major band of ~50 kDa (Fig. 5, lanes 1 and 3), which corresponds to the theoretical size of 51.2 kDa of the rgcIL-12Rβ2 protein. Thus, we used this fusion protein to immunize mice and generated an anti-rgcIL-12Rβ2 polyclonal antibody. Similarly, we also expressed and purified the GST protein alone, which appeared as a single band of approximately 26 kDa on SDS-PAGE (Fig. 5, lanes 2, and 4). The anti-rgcIL-12Rβ2 polyclonal antibodies were tested with Western blotting analysis of cell lysates from *E. coli* cells (Fig. 6A). Two bands that corresponded to the rgcIL-12Rβ2 or GST proteins appeared on the nitrocellulose membrane after incubation with the polyclonal antibody (Fig. 6A–a). By contrast, the main band corresponding to GST-rgcIL-12Rβ2 was detected when the membrane was incubated with the polyclonal antibody pre-incubated with purified GST (Fig. 6A-b). No specific bands for either rgcIL-12Rβ2 or GST were observed when the antibody was pre-incubated with purified GST-rgcIL-12Rβ2 (Fig. 6A–c). These results indicate that the anti-rgcIL-12Rβ2 polyclonal antibody specifically recognized rgcIL-12Rβ2 produced in prokaryotic cells. To verify antibody specificity in grass carp cells, Western blotting analysis was also carried out using lysates from IELs and CIK cells (Fig. 6B), which revealed the presence of three bands. The high molecular weight band (indicated by a black arrow in Fig. 6B–a) was 84.0 kDa, which corresponds to the mature IL-12Rβ2. The other two bands (white arrows) were presumed to be unknown isoforms of IL-12Rβ2. These bands were not observed when nitrocellulose membranes were incubated with antiserum pre-incubated with the purified rgcIL-12Rβ2 protein (Fig. 6B–b). This observation reveals that the antibody specifically recognizes native IL-12Rβ2 in grass carp cells.

3.7. Involvement of IL-12Rβ2 in A. hydrophila-induced intestinal inflammation

One d after anal intubation with *A. hydrophila*, fish started to swim slowly, lost appetite, and their anuses became red and swollen. At this time, DAI was significantly higher than that of mock-infected fish ($p < 0.05$) (Fig. 7A). Histopathological examination revealed the presence of damage to the local intestinal mucosa and adhesions in the severely inflamed posterior intestines. The intestinal epithelial cells were disintegrated and inflammatory cells infiltrating into intestinal tissues were observed (Fig. 7B–a, b). By d 3 after infection, although some of the damaged intestinal tissues had been repaired, mild inflammation was developed on the body surface and a small amount of ascites was observed, which resulted in increased DAI. Afterwards, these signs of inflammation were gradually relieved and almost disappeared completely by d 21 after infection. The possible involvement of IL-12Rβ2 during intestinal inflammation was examined with immunohistological analysis using the rgcIL-12Rβ2 pAb. Only very weak positive staining was found in control intestines from mock-infected fish (Fig. 7B–a). The strongest staining was detected early at d 1 after bacterial infection, and the IL-12Rβ2 protein was distributed in intestinal epithelial cells and in inflammatory cells (Fig. 7B–b). The IL-12Rβ2 protein levels declined steadily over time (Fig. 7B–c, d), but a slight increase of IL-12Rβ2 level in the cytoplasm of intestinal epithelial cells was observed on d 14 after infection (Fig. 7B–e). By d 21, the level of IL-12Rβ2 had nearly returned to control levels and there were almost no inflammatory cells in the intestinal tissues (Fig. 7B–f).

4. Discussion

In the present study, we have cloned and characterized gcIL-12Rβ2 by generating a polyclonal antibody. This allows us to investigate how the biological effects of IL-12 and IL-35 are regulated by IL-12Rβ2 in fish, especially in cyprinid fish.

Previous studies have confirmed that mammalian IL-12Rβ2 subunits contain the functionally important motifs CXW and WSXWS, where X represents a non-conserved residue [24–27]. In the present study, we show that the CXW motif is also highly conserved in fish IL-12Rβ2 subunits. The non-conserved "X" residue was either threonine or serine, which are both hydrophilic neutral amino acids with similar side chains. The WSXWS motif was strictly conserved in IL-12Rβ2 proteins from mammals [3,28] and birds (*Gallus gallus*, GenBank NP_001264556.1). This motif was also present in IL-12Rβ2 sequences

from Atlantic salmon (GenBank NP_001158802.1). In IL-12Rβ2 sequences from cyprinid fish such as grass carp (this study) and zebrafish (GenBank NP_001352715.1), the MSWXS motif was present at the position corresponding to human WSXWS motif. Thus, this motif is not completely conserved in fish IL-12Rβ2 sequences. The box 1 and box 2 motifs, and the three conserved tyrosine residues within the cytoplasmic domain are structural features characteristic of IL-12Rβ2 [3,28,29]. However, these features are only found in IL-12Rβ2 of mammals and birds, but not in fish IL-12Rβ2 although a moderately conserved sequence (Ile/Val-Pro-Asp-Pro) is present within the box 1 motif. This difference among highly divergent sequences was also supported by phylogenetic analysis of IL-12Rβ2 protein sequences.

IL-12p35 expression can be detected in the intestine of several fish species, such as pufferfish [19] and grass carp [30]. The interaction of IL-12p35 with IL-12Rβ2 may directly contribute to IL-12 signaling pathway. IFN-γ is a known inducer of IL-12Rβ2 [31] and has been confirmed to be expressed in fish intestine [32,33]. In rainbow trout, intestinal IFN-γ expression was up-regulated after infection with *Aeromonas salmonicida* [33]. In this study, expression of IL-12p35 and IL-12Rβ2 in the intestine increased after infection with *A. hydrophila*, which suggests that T cells and natural killer cells are activated due to bacterial infection [3,14,34,35]. On these activated cells, IL-12Rβ2 expression could be further up-regulated by IL-12, resulting in increased IFN-γ production [34] in Th1 differentiation [36]. Subsequently, macrophages could be activated to acquire microbicidal effector functions to eradicate the invading pathogen [37–39]. With the progressive removal of pathogenic bacteria, inflammatory stimuli would be significantly reduced. In this study, the expression of IL-12p35, IL-12Rβ2, and IFN-γ is significantly declined 3 d after bacterial infection. Similar results for the expression of IL-12p35 in grass carp intestines after the fish were infected by *A. hydrophila* have been reported [30]. In addition, the expression levels of these three genes are increased 7 d after infection and then returned to normal levels. The intestinal inflammatory changes, as assessed by immunohistological stains, occurred after changes in expression of these pro-inflammatory cytokines. A possible explanation for this is that protein production occurs after mRNA synthesis [40], thus delaying protein production of pro-inflammatory cytokines and inflammatory cell recruitment [41]. Taken together, these results suggest that IL-12Rβ2 is involved not only in the early intestinal inflammatory process but also in the adaptive immune response.

We raised an antiserum against rgcIL-12Rβ2 in mice. The specificity of this antiserum was confirmed by Western blotting analysis of lysates from *E. coli* cells expressing rgcIL-12Rβ2 and grass carp IELs and CIK cells. Notably, in addition to the 84-kDa protein that corresponded to the predicted molecular weight of IL-12Rβ2, two other proteins were recognized by rgcIL-12Rβ2 pAb. The appearance of multiple protein bands on Western blotting could be explained by several possible mechanisms, such as post-translational modifications like glycosylation [42], proteolytic cleavage [43] and alternative splicing [44]. In our study, the appearance of two bands of lower molecular weight is unlikely to be the result of glycosylation since glycosylation usually increases molecular weight [45]. We also ruled out the possible involvement of proteolytic cleavage as the PeptideCutter failed to identify a proteolytic enzyme capable of cleaving the gcIL-12Rβ2 protein. On the other hand the presence of different IL-12Rβ2 isoforms has been reported in canine blood [24]. Such IL-12Rβ2 isoforms were also identified and predicted in cyprinid fish, including grass carp, *Sinocyclocheilus rhinocerous* and *S. anshuiensis*. All these isoforms are generated by a splicing event of exon skipping [24,46]. Alternative splicing is a strategy for the regulation of immune responses against pathogen infection in teleost fish [47]. Thus, we are inclined to ascribe the presence of multiple IL-12Rβ2 protein bands to alternative splicing.

To further ascertain the involvement of IL-12Rβ2 in *A. hydrophila*-induced intestinal inflammation, we performed immunohistochemical analysis on the inflamed posterior intestines using the rgcIL-12Rβ2 pAb. Immunohistochemical staining revealed that the intestinal epithelial cells and the inflammatory cells were positive for IL-12Rβ2. IL-12Rβ2 expression is consistent with recruitment of inflammatory cells during the time course of the inflammatory process. These findings lead us to propose that IL-12Rβ2 is involved in the regulation of the inflammatory response during intestinal inflammation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.016.

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