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Molecular characterization of grass carp interleukin-6 receptor and the agonistic activity of its soluble form in head kidney leucocytes

Xinyan Wang*, Yafei Guo, Chao Wen, Mengyuan Lv, Ning Gan, Hong Zhou, Anying Zhang, Kun Yang

Center for Informational Biology, School of Life Science and Technology, University of Electronic Science and Technology of China, Chengdu, 610054, PR China

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ABSTRACT

Interleukin (IL)-6 receptor (IL-6R) can specifically bind to IL-6 and the complex subsequently recruits a transmembrane signal transducer, gp130, to trigger the intracellular signal transduction. IL-6R exists in two forms, a transmembrane IL-6R and a soluble IL-6R (sIL-6R), leading to different signal transduction mechanisms as classic signaling and trans-signaling, respectively. There is now a general consensus that these two modes of signal transduction can mediate anti-inflammatory and pro-inflammatory activities of IL-6. The study on Il-6r is limited although Il-6 has been well studied in teleost. In the present study, a cDNA encoding grass carp Il-6r (gcIl-6r) was isolated. An *in-silico* analysis showed that gcIl-6r shared the same functional domains and conserved gene synteny at its loci with mouse homologue, and its amino acid sequence was conserved in fish species. A tissue distribution assay demonstrated that g*cil6r* mRNA was expressed with high levels in immune tissues including spleen and head kidney, and its expression was induced by LPS and Poly I:C in grass carp head kidney leucocytes (HKLs). An *in vitro* binding assay showed that recombinant soluble gcIl-6r (rgcsIl-6r) could specifically bind to recombinant gcIl-6 (rgcIl-6) protein. Moreover, rgcIl-6 stimulated *suppressor of cytokine signaling 3* (*socs3*)'s mRNA expression in grass carp HKLs and it combined with rgcsIl-6r increased *socs3* mRNA expression in CIK cells with gp130 but without Il-6r expression. In HKLs, rgcIl-6 stimulated the mRNA levels of both pro-inflammatory (*tnfa* and *il1b*) and anti-inflammatory (*il10*) cytokines, and rgcsIl-6r could augment these stimulatory effects of gcIl-6. Taken these data together, gcsIl-6r can mediate the immuno-regulatory functions of gcIl-6 and has an agonistic property in these actions of Il-6 in grass carp.

1. Introduction

Interleukin (IL)-6 is a glycosylated protein of 21–28 kDa and belongs to IL-6 cytokine family which has nine family members (IL-6, IL-11, CNTF, LIF, OSM, CLC, CT-1, NNT-1 and IL-27) [1]. It can be produced by almost all stromal cells and immune cells to play pleiotropic functions in inflammatory, metabolic, regenerative and neural processes [2]. The biological functions of IL-6 are mediated by IL-6 receptor (IL-6R) complex which comprises an IL-6-binding chain, IL-6R and a dimer of signal-transducing chain, gp130. IL-6R is the specific receptor for IL-6 and mainly expressed in immune cells and hepatocytes [1,3]. Compared with IL-6R, gp130 is a common receptor shared by all members of the IL-6 cytokine family [4] and expressed on all cells of the body [5]. IL-6 can bind to transmembrane IL-6R with nanomolar affinity [3] but this binding does not trigger signal transduction. The complex of IL-6 and IL-6R recruits two molecules of gp130, subsequently leading to the initiation of Jak (Janus kinase)/STAT, PI3K and MAPK transduction pathways (classic signaling) [6]. It is noteworthy that IL-6R has been shown to exist in two forms, transmembrane IL-6R and soluble IL-6R (sIL-6R), and the latter possesses no transmembrane and cytoplasmic regions. Interestingly, sIL-6R can be found in blood and bind to IL-6, leading to form an IL-6-sIL6R-membrane gp130 complex which is able to activate intracellular signaling cascade (trans-signaling). It permits IL-6 responsiveness in cell types that lack the IL-6R but have gp130 [7]. These different modes of signaling confer the capability of IL-6 to act as both a pro-inflammatory and an anti-inflammatory cytokine. It has been accepted that IL-6 trans-signaling largely mediates pro-inflammation, whereas the classic signaling pathway promotes anti-inflammatory processes [1]. In both classical and trans-signaling, dimerization of gp130 leads to auto-activation of Jak which subsequently activates STAT3 by phosphorylation to drive the biological response. Additionally, activated STAT3 also stimulates suppressor of cytokine signaling-3 (SOCS3) expression which in turn can terminate the Jak/STAT signaling cascade. This action of SOCS3

* Corresponding author.
E-mail address: wangxyhku@uestc.edu.cn (X. Wang).

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seems to be a primary mechanism by which IL-6 signaling is controlled [8].

Interleukin-6 (*il6*) gene has been identified in many fishes including zebrafish [9], yellow croaker [10], flounder [11], Japanese puffer [12], orange-spotted grouper [13], seabream [14] and rainbow trout [15]. Its constitutive or inducible expression is investigated in different fish species and its promoter activity has been analyzed in seabream [16] and rainbow trout [17]. It is noteworthy that the bioactivities of Interleukin-6 (Il-6) are examined in rainbow trout, showing that Il-6 is a differentiation factor for B cells [18] and can enhance macrophage growth [19]. In the same model, Il-6 has been shown to induce the expression of antimicrobial peptide but down-regulate the expression of *interleukin-1b* (*il1b*) and *tumor necrosis factor-a* (*tnfa*) in microphages [19]. In another fish, orange-spotted grouper, Il-6 is able to stimulate Th2 cell differentiation pathway and antibody production [13]. However, compared to the study on fish Il-6, functional characterization of its receptor is currently lacking although the gene sequences of Il-6 receptor have been identified in some fishes [9,20,21]. In the present study, the full-length cDNA sequence of grass carp (*Ctenopharyngodon idella*) *Il-6 receptor* (g*cil6r*) was cloned, and its constitutive and inducible expression was examined in different tissues and head kidney leucocytes (HKLs), respectively. To study the function of grass carp Il-6r (gcIl-6r), recombinant proteins of grass carp Il-6 (gcIl-6) and soluble gcIl-6r (gcsIl-6r) were prepared. Consequently, the specific binding between recombinant gcIl-6 and gcsIl-6r was demonstrated, prompting us to study the roles of gcsIl-6r in mediating the actions of gcIl-6 in grass carp cells. Accordingly, the roles of gcsIl-6r in the regulation of gcIl-6 on the mRNA expressions of *suppressor of cytokine signaling 3* (*socs3*) in *Ctenopharyngodon idella* kidney (CIK) cells and HKLs, and some inflammation-related cytokines in HKLs were examined.

2. Materials and methods

2.1. Animals

Healthy grass carp of about 1 kg each were purchased from Tongwei Aquatic Science and Technology Company (Chengdu, China). They were kept in pathogen free conditions before sacrificed for later experiments. All animal experiments were performed in accordance with the Regulation for Animal Experimentation of Sichuan province, China and were allowed by the ethics committee of the University of Electronic Science and Technology of China.

2.2. RNA isolation and cDNA synthesis

Grass carp tissues (~70 mg/ml) or cells (~2×10^6 cells/ml) were dissolved in TriPure Isolation Reagent (Roche, Basel, Switzerland) and total RNA was isolated following the manufacturer's instruction. About two micrograms of total RNA was converted into cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) and oligo $(dT)_{18}$ as the primer. The cDNA templates were used in molecular cloning and RT-qPCR.

2.3. Molecular cloning of gcil6r cDNA sequence

The mixture of the cDNAs from different tissues including gill, intestine, brain, thymus, skin, heart, head kidney and spleen was used as template to clone the full-length cDNA sequence of g*cil6r*. The primers of g*cil6r* partial F and g*cil6r* partial R (Supplementary Table 1) for cloning of g*cil6r* partial sequence were designed based on the conserved region of *il6r* homologues in zebrafish (GenBank accession no.: NM_001330258.2), golden-line barbell (GenBank accession no.: XM_016267780.1) and common carp (GenBank accession no.: XM_019119191). Subsequently, the 5′- and 3′-sequences of g*cil6r* were obtained by 5′- and 3′- RACE System for Rapid Amplification of cDNA Ends Rapid kits (Thermo Scientific, Waltham, MA, USA), respectively, using the primers listed in Supplementary Table 1. Finally, the full-length cDNA sequence of g*cil6r* was validated by PCR amplification using Phusion DNA polymerase (New England Biolabs, Beverly, MA, USA). Amino acid sequence alignment was carried out by DNAMAN software (Lynnon Biosoft, Pointe-Claire, Canada). Exon and intron organization of the genes was analyzed by comparing the mRNA sequences with corresponding genomic sequences. The genome sequence of mouse could be found in GenBank (https://www.ncbi.nlm.nih.gov/genome/52?genome_assembly_id=334509). Grass carp genome sequence could be found at the site of http://www.ncgr.ac.cn/grasscarp/ [22]. Functional domains were analyzed by online InterPro server (http://www.ebi.ac.uk/interpro/) and diagrams were constructed using Domain Graph (DOG) [23], version 2.0. Gene synteny at the *il6r* loci was analyzed using the data from NCBI database. The protein sequence was also analyzed by online ScanProsite server (https://prosite.expasy.org/).

2.4. RT-qPCR analysis of gcil6r transcripts expression

A Bio-Rad CFX96 Real-time detection system (Bio-Rad, Hercules, CA, USA) was used to examine the levels of g*cil6r* and *bactin* mRNA by RT-qPCR. The specific primers used in these experiments were listed in Supplementary Table 1. The qPCR mixture was prepared as follows: 10 μl of RealMasterMix (Tiangen, Beijing, China), 2 μl of the cDNA, 0.5 μl each of forward and reverse primer, and 7 μl of PCR water. The qPCR program was 94 °C for 2 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 20 s and 65 °C for 30 s. Direct comparison of g*cil6r* mRNA expression was made through $2^{-\Delta\Delta CT}$ method. In tissue distribution assay, the level of g*cil6r* mRNA in gill was used to calibrate its levels in other tissues. For the *in vitro* cell experiments, the levels of g*cil6r* mRNA were expressed as the ratio of that in control group.

2.5. Recombinant expression of gcIl-6 and gcsIl-6r

Recombinant gcIl-6 (rgcIl-6) was expressed by BL21 (DE3)/pET-30a (+) (Merck Millipore, Billerica, MA, USA) system and purified by metal ion affinity chromatography. Briefly, the DNA fragment encoding mature gcIl-6 (GenBank accession no.: KC535507.1) was subcloned into pET-30a (+) vector to generate expression plasmid pET-30a/gcIl-6. After the plasmid was amplified in *E. Coli* and extracted by using a TIANprep Rapid Mini Plasmid Kit (Tiangen), it was co-transformed with the expression vector pGro7 (Takara Bio, Beijing, China) encoding GroEL/ES chaperone proteins into *E. Coli* BL21 (DE3) strain. The positive clone was grown in Luria Broth (LB) medium (5 g of yeast extract, 10 g of peptone, and 5 g of NaCl per liter; 300 ml in 500-ml conical flasks) with kanamycin (100 mg/l, Sigma-Aldrich, St. Louis, MO, USA), L-arabinose (0.5 g/l, Sigma-Aldrich) and chloramphenicol (34 mg/l, Sangon Biotech, Shanghai, China) in an orbital shaker at 37 °C and at 180 rpm for 2 h. When the OD at 600 nm reached 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich) was added to a final concentration of 0.5 mM, and the temperature was lowered to 16 °C. After 20 h of induction, the cells were washed with 10 mM phosphate buffer (pH 7.4) twice and pelleted by centrifugation. The cells were resuspended in ice-cold binding buffer [20 mM imidazole (Sigma-Aldrich), 20 mM phosphate buffer, 0.5 M NaCl, pH7.4] supplemented with 1 mg/ml lysozyme (Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride (PMSF, Amresco, Solon, OH, USA). The sample was sonicated on ice, and the cell lysate was clarified by centrifugation at 10,000 × *g* for 30 min at 4 °C. The supernatant was collected and filtered by a 0.22 μm filter. The rgcIl-6 was purified on a HisTrap affinity column (GE Healthcare, Waukesha, WI, USA) and desalted on a Superdex G25 prep grade column (GE Healthcare). The rgcIl-6 concentration was determined by a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) and the purified protein was analyzed by SDS-PAGE and Western blotting. Finally, the purified protein was lyophilized and stored at −80 °C for later experiments.

For production of recombinant gcsIl-6r (rgcsIl-6r, 23–490 aa) protein, the plasmid pET-30a/rgcsIl-6r was transformed into *E. Coli* BL21 (DE3) (Merck Millipore) competent cells. A positive single colony was cultured in 50 ml of LB medium with shaking at 180 rpm and 37 °C. IPTG was added to a final concentration of 1 mM to induce rgcsIl-6r expression at 30 °C for 12 h when the OD_{600nm} of the culture reached 0.5. The bacteria cells were harvested by centrifugation (4000 g, 5 min) at 4 °C and washed by 10 mM ice-cold phosphate buffer (pH 7.4) twice. The cells were resuspended in 20 ml of 10 mM phosphate buffer (pH 7.4) supplemented with 1 mM PMSF and were sonicated on ice. The sediment was collected by centrifugation at 12,000 g for 20 min at 4 °C and resuspended in washing buffer (20 mM Tris-HCl, 10 mM EDTA and 1% Triton X-100, pH7.5). The inclusion body was washed by washing buffer via centrifugation (10,000 g, 10 min) at 4 °C for three times and dissolved in ice-cold denaturation buffer (30 mg inclusion body/ml; 8 M urea, 10% glycerol, 50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA and 10 mM DTT, pH8.0). The supernatant was collected after centrifugation at 10,000 g for 10 min at 4 °C. About 1 mg protein in the supernatant was added into 50 ml of renaturation buffer (100 mM Tris-HCl, 400 mM arginine, 2 mM EDTA, 5 mM oxidized glutathione and reduced glutathione each and 0.5 mM PMSF, pH8.0) slowly (5–6 drops/min) with magnetic stirring at 100 rpm and the solution was incubated at 4 °C overnight. After that, rgcsIl-6r was purified on a HisTrap affinity column (GE Healthcare) followed by ultrafiltration using an Amicon Ultra-15 Centrifugal Filter Unit (Amicon®Ultra 50 K device, Merck Millipore).

2.6. Western blotting analysis

The protein samples were separated on 10% SDS-PAGE gels and electrophoretically transferred to PVDF membranes (Merck Millipore) using a Mini Trans-Blot cell system (Bio-Rad). The membranes were blocked in TBS/T buffer (25 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20, pH 7.4) containing 5% (w/v) nonfat dried milk for 2 h at room temperature and incubated with corresponding primary antibodies overnight at 4 °C. The samples were exposed to horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse secondary antibody (1:5000, ZSGB-BIO, Beijing, China) for 1 h at room temperature. Finally, the signals were detected using an ECL kit (Roche) according to the manufacturer's instruction.

2.7. Binding assay

Binding of gcIl-6 and gcsIl-6r was analyzed *in vitro* by quantitative ELISA. Different amounts (from 0 to 15 pmol/well) of rgcsIl-6r and bovine serum albumin (BSA, Ctrl, Sigma-Aldrich) were coated into 1 × 8 Stripwell high-binding plates (Costar, Cambridge, MA, USA) and incubated overnight at 4 °C. After the plate was washed by phosphate buffered saline containing 0.05% Tween 20 (PBST, pH7.4), it was blocked by 0.3% fetal bovine serum and 5% milk in phosphate buffered saline (PBS, pH7.4) at 25 °C for 2 h. Four pmol/well of rgcIl-6 was added into the pre-coated wells and the plate was incubated at 25 °C for 2 h. The wells were washed for three times with PBST, then incubated with 1:2500 diluted rabbit horseradish peroxidase-conjugated polyclonal anti-gcIl-6 antibody (Abmart, Shanghai, China) at 25 °C for 1 h. After washing, positive signals were developed with the TMB color reaction and the reaction was stopped by adding 50 μl of 2 M H_2SO_4. Finally, the OD at 450 nm was measured by an iMark Microplate Absorbance Reader (Bio-Rad).

2.8. Isolation and culture of grass carp HKLs

Grass carp HKLs were prepared by density gradient centrifugation as our previous study [24]. Healthy grass carp was scarified, and its head kidney was collected. After washed twice with D-Hanks solution (Sigma-Aldrich), the head kidney was squeezed gently to release the cells into the RPMI-1640 medium (Thermo Scientific). The tissue debris was removed by filtration through a 200-gauge stainless steel mesh. The cell suspension was layered on Ficoll-Hypaque (1.083 kg/l, TBDscience, Tianjin, China) and centrifuged for 30 min at 400 × *g* at 25 °C. After that, the cells at the interface were harvested, washed twice and resuspended in RPMI-1640 medium supplemented with 10% FBS (Thermo Scientific). About 6×10^5 cells/well were seeded in 24-well plate (BD Biosciences, San Jose, CA) and incubated overnight at 27 °C under 5% CO_2 and saturated humidity. On the following day, drug treatment was initiated for the durations as indicated in individual experiments and then total RNA was extracted from the HKLs. The mRNA expression was measured by RT-qPCR. Before drug treatment, the concentration of rgcIl-6 and rgcsIl-6r was determined by a BCA Protein Assay Kit (Beyotime Biotechnology). Recombinant gcIl-6 (32 nM) and gcsIl-6r (48 nM) were separately inactivated for 20 min in a boiling water bath and used as controls.

2.9. Culture of CIK cells

CIK cells were obtained from China Center for Type Culture Collection (Wuhan, China) which were derived from grass carp kidney [25]. The cells were maintained in DMEM-F12 medium (Thermo Scientific) supplemented with 10% FBS and 1 × antibiotic-antimycotic (Thermo Scientific). About 1×10^5 cells/well were plated in a 24-well plate in 500 μl of DMEM-F12 medium with 10% FBS and 1 × antibiotic-antimycotic. After incubation overnight, rgcIl-6 (32 nM) and/or rgcsIl-6r (48 nM) were added to treat the cells. After that, the gene expression levels were evaluated by RT-qPCR as described in Section 2.10.

2.10. RT-qPCR analysis of socs3 and some inflammation-related cytokines' mRNA levels

The mRNA levels of *socs3*, *tnfa*, *il1b*, *interleukin 10* (*il10)* and *bactin* were detected by RT-qPCR using the specific primers for them (Supplementary Table 1) by a Bio-Rad CFX96 Real-time detection system (Bio-Rad). These experiments were carried out as described in Section 2.4. The annealing temperatures for detection of *socs3*, *tnfa*, *il1b*, *il10* and *bactin* mRNA were 59 °C, 58 °C, 59 °C, 58 °C and 60 °C, respectively. The mRNA level in control group was used to calibrate those in other groups.

3. Results

3.1. Molecular cloning and sequence analysis of gcil6r

The full-length cDNA sequence of *gcil6r* was 2223 bp (GenBank accession no.: MG188797.1) encoding a polypeptide with 594 amino acids (Fig. 1). The functional domains (Supplementary Fig. 1A) of gcIl-6r were highly conserved with those of mouse IL-6R although the amino acid identity of them was only 19% (Fig. 2). Syntenic analysis showed that the genes of *atp8b2* and *aqp10* neighboring *il6r* locus of grass carp were coincident with those of mouse (Supplementary Fig. 1B). Amino acid alignment showed that the signal peptides and transmembrane domains of Il-6rs were highly conserved in cyprinid fishes including grass carp, zebrafish, golden-line barbell and common carp. The Ig-like and fibronectin type-III (FNIIIs) domains were highly conserved in fish species and some amino acids (As an example: four cysteines in 1st FNIII domain, indicated by asterisks in Fig. 2) in them were also highly conserved in all vertebrates that we analyzed (Fig. 2). Unexpectedly, a WSxWS motif which was conserved in fugu, trout, mouse and human (Blue box, Fig. 2) was mutated in the cyprinid fishes.

3.2. Tissue distribution of gcil6r transcript in grass carp and its inducible expression in HKLs

Grass carp *il6r* mRNA was highly expressed in head kidney and

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1     acttttggttctctcacgaacgaacgagctccaggtcgcaatcttaccttgaagcaactgaaaatgttt
70    tttgtcaaacgaagagttaattcttgtcccagatctgtacaatgcttcaaatgagaacgtaacagtttt
139   aggttgtcgctgtaggctatgcgtttttcccttctaggctgtctaaaatattgatgtgaggaaaatgta
208   agatcatctcgttaaacttgctgaacgggttgacattatgacccgttttacccatatttcatattttgg
277   tggctgattatttttgatagtgaaaactataata

312   ATGTGGACCCGATCTACATTCATGTTTCTCTTCGGTGTATTCTCTGCGATTAAAGTTCAATCTGCCCAA
1      M  W  T  R  S  T  F  M  F  L  F  G  V  F  S  A  I  K  V  Q  S  A  Q
381   GAAGATGAGCTGTGTCCTCGACAAGAGCCCCTTCCTGGAGTTTTGGTCTTGAAGTTAGGAAGCAATGTT
24     E  D  E  L  C  P  R  Q  E  P  L  P  G  V  L  V  L  K  L  G  S  N  V
450   GTGCTTGGCTGCAGAGGCCATGTCACTGTGGACGACGTGCTGTTGGCTTCGACCAGTGTCATGAATAAA
47     V  L  G  C  R  G  H  V  T  V  D  D  V  L  L  A  S  T  S  V  M  N  K
519   AAATACAGAAACAAGCAGAGAGAAGACATCACTGTCAGCTGGACATCCAAAAGAGTTTATGCTAATGCT
70     K  Y  R  N  K  Q  R  E  D  I  T  V  S  W  T  S  K  R  V  Y  A  N  A
588   ACACAAATAACCAGTATGAAGGGAGATGCTACAATTGGAACATACCAAAAGACTGATTCTGGTATGTAT
93     T  Q  I  T  S  M  K  G  D  A  T  I  G  T  Y  Q  K  T  D  S  G  M  Y
657   ACCAAAACTAAAGGGGACACGACGGTCACTATGAAACCCCCTGTAGCCATAAGAAAGGAACAAAGCACT
116    T  K  T  K  G  D  T  T  V  T  M  K  P  P  V  A  I  R  K  E  Q  S  T
726   TCTAGACGAGTTTTGCGTGCTGTCAGTCAAACTGCTGGACATGAAGAAGAGGCCTTCGGTATCACCATG
139    S  R  R  V  L  R  A  V  S  Q  T  A  G  H  E  E  E  A  F  G  I  T  M
795   GGCACAGATCCTGATCTAGATGATTATGAGGACTATGATTATGAAGAGGAAGGCTCCAGGGTTACACGA
162    G  T  D  P  D  L  D  D  Y  E  D  Y  D  Y  E  E  E  G  S  R  V  T  R
864   GGCATCAAAAAGCGAACACGCTGGACTCTCAATGGACGACAAGTGCATGTTGGTGTGGAAAGGGGTGGG
185    G  I  K  K  R  T  R  W  T  L  N  G  R  Q  V  H  V  G  V  E  R  G  G
933   ATTTTAAGACGTCCGCATCTCAGCTTGGCAGATGCTGGGAATTACAGCTGCTACAGAGGAGAGAGACTC
208    I  L  R  R  P  H  L  S  L  A  D  A  G  N  Y  S  C  Y  R  G  E  R  L
1002  ATTTCCACATTCAAAATCAGCGTAGGGGCGCCTCCAGAGAGACCCACTGTCTTTTGCTACAGGAAGTTT
231    I  S  T  F  K  I  S  V  G  A  P  P  E  R  P  T  V  F  C  Y  R  K  F
1071  CACACTAGTAAGGTCCGCTGTGATTGGACGTCCAAAGATCCGGTAACCCCACGGCCGCTGTGTTACCTA
254    H  T  S  K  V  R  C  D  W  T  S  K  D  P  V  T  P  R  P  L  C  Y  L
1140  CTTCTTAATAGAGGATTGTTCGGTAACATCACTCATGTACCTTGTTCATTCTCCCGGTCCCGCTGTTGG
277    L  L  N  R  G  L  F  G  N  I  T  H  V  P  C  S  F  S  R  S  R  C  W
1209  TGTGCTTTCCCTGTGGAGGAGGGTGACAGAACTCTCCATGTGGCAAAAATGTGTGTGTCTAACACAGCA
300    C  A  F  P  V  E  E  G  D  R  T  L  H  V  A  K  M  C  V  S  N  T  A
1278  GGCAGTGCCACGAGCCCTGAACTCAGCTTCAGGCTACACGACATCATTAAGCCAGACCCCCCAACCAGA
323    G  S  A  T  S  P  E  L  S  F  R  L  H  D  I  I  K  P  D  P  P  T  R
1347  GTGGTGGTGAGAGCAGTCGAGGGCCAGAAGCATATCCTTAAAGTATCCTGGTCTTATCCCAGTTCCTGG
346    V  V  V  R  A  V  E  G  Q  K  H  I  L  K  V  S  W  S  Y  P  S  S  W
1416  AAGCATGGCTTCTATGTCCTGCATTTCCAGCTGAGATACCGACCACAACTCGCAGAGCAGTACCAGCCA
369    K  H  G  F  Y  V  L  H  F  Q  L  R  Y  R  P  Q  L  A  E  Q  Y  Q  P
1485  GTGTTGATAGGTGATAGGGCTGACAGACAACAGTCCTGGACAATTTATGATGCTCTGCCTAACACTCAG
392    V  L  I  G  D  R  A  D  R  Q  Q  S  W  T  I  Y  D  A  L  P  N  T  Q
1554  TATGAAGTGCAGCTTCGGGCCAAGGATGAGTTTGATGGCGTCTGGAGTGACTGGACTGATACTGTCTTG
415    Y  E  V  Q  L  R  A  K  D  E  F  D  G  V  W  S  D  W  T  D  T  V  L
1623  GCAGCCACTTGGTCAGATCTTGAGACGACTACTTCCTCTGAGAGCAATACCTTGGAACCCTTTGAGATG
438    A  A  T  W  S  D  L  E  T  T  T  S  S  E  S  N  T  L  E  P  F  E  M
1692  TTTCCTGAAGGTTCTGGAGGATCTGGAGAAGATCCTGGTGTAGGATCAGTAGTAATAGTTGGAGCTGAT
461    F  P  E  G  S  G  G  S  G  E  D  P  G  V  G  S  V  V  I  V  G  A  D
1761  GATATTGGACATGCTACTGCGTGGCTGTATGTGTCGTGTGTTTTTGGGCTGTTTTTTCTTGTAGCCCTC
484    D  I  G  H  A  T  A  W  L  Y  V  S  C  V  F  G  L  F  F  L  V  A  L
1830  ACCATGCTCACAGTCTTTTTCTTCAGGAATAAGCTGCATTTTATGTCTAGATTAGGAAAACAGACTTTG
507    T  M  L  T  V  F  F  F  R  N  K  L  H  F  M  S  R  L  G  K  Q  T  L
1899  CCCTTTACGTTGCTCCTTCATTCCTCTCGCCCTTTTCCCGCTCCTGCTGCCTCTGAACAGCTTCCAGGG
530    P  F  T  L  L  L  H  S  S  R  P  F  P  A  P  A  A  S  E  Q  L  P  G
1968  GAGGGAAAGTCACTGATGTCACCTCCCAAGCACAGCCTGCAACACTTCCTTCCTGTTGAGCAGGAGGGC
553    E  G  K  S  L  M  S  P  P  K  H  S  L  Q  H  F  L  P  V  E  Q  E  G
2037  ATTCATCTGCATAACATGGATTATTTCCTGTCTCCAGGGTCTGAGAGTGTGAGGGTCTGA
576    I  H  L  H  N  M  D  Y  F  L  S  P  G  S  E  S  V  R  V  -

2097  ctgagggacagacatgagtagatgaaacctcactgtagtagatacactgtgattgactaaaggtcacac
2166  actgtgtctttgttatggttcaataacagtattatgagtttgtaaaaaaaaaaaaaaa
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Fig. 1. The cDNA sequence and amino acid sequence of gcIl-6r. The coding sequence was presented in upper case whereas the sequences of untranslated regions were presented in lower case. Putative amino acid sequence was shown under the triplet codon.

spleen, followed by heart and was moderately expressed in thymus and skin. Lowest level of *gcil6r* mRNA was found in gill, intestine and brain (Fig. 3).

The tissue distribution study showed that *gcil6r* mRNA was expressed in head kidney with high level. Hence, we investigated inducible expression of *gcil6r* mRNA in HKLs. The results showed that both 50 μg/ml of polyinosinic: polycytidylic acid (Poly I:C) (Fig. 4A) and 30 μg/ml of lipopolysaccharide (LPS) (Fig. 4B) stimulated *gcil6r* mRNA expression in HKLs after 12, 24, 36 and 48 h treatment.

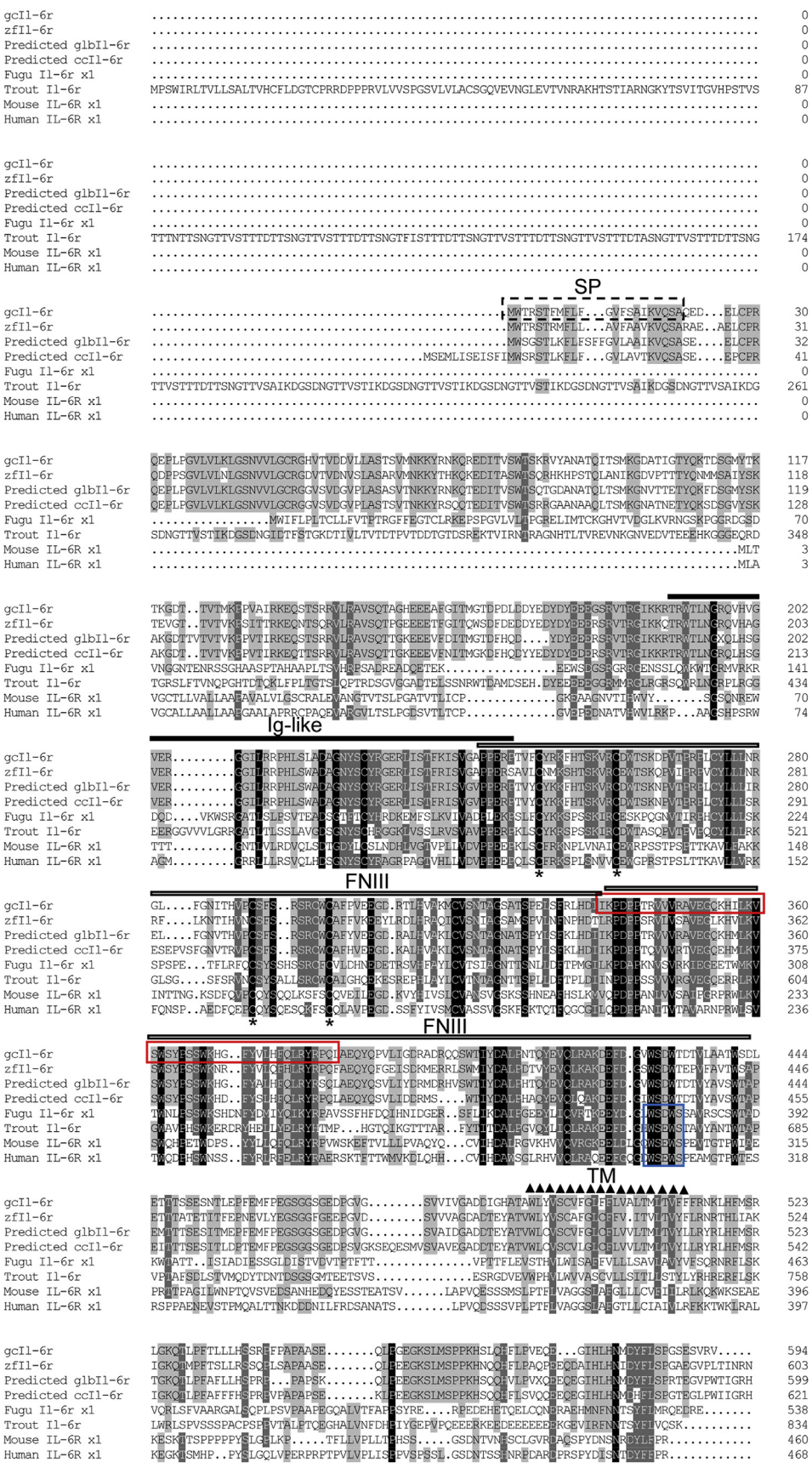

Fig. 2. Animo acid aligment of gcIl-6r with its homologues in other fishes, human and mouse. SP, signal peptide was indicated by dot box; Ig-like, immunoglobin-like domain was indicated by black box; FNIII, Fibronectin type-III domains were denoted by open black boxes; TM, Transmembrane domain was indicated by black triangles; Long hematopoietin receptor, soluble alpha chains family signature was shown in red line box. WSxWS motif was shown by blue line box. zf: zebrafish; glb: golden-line barbell; cc: common carp. GenBank accession no.: gcIl-6r: AVI05199.1; zfIl-6r: NP_001317187.2; Predicted glbIl-6r: XP_016123266.1; Predicted ccIl-6r: XP_018930773.1; Fugu Il-6r x1: BAN28552.1; Trout Il-6r: NP_001268335.1; Mouse. Il-6r x1: NP_034689.2; Human Il-6r x1: NP_000556.1.

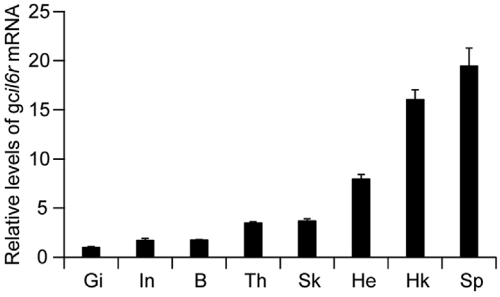

Fig. 3. Tissue distribution of g*cil6r* mRNA. Expression of g*cil6r* mRNA in the tissues was detected by RT-qPCR and *bactin* was amplified to serve as internal control. The level of g*cil6r* mRNA in gill was used to calibrate its levels in other tissues. Four grass carp were used in the tissue distribution assay and data were shown as means ± SEM of g*cil6r* mRNA levels. Gi: gill; In: intestine; B:brain, Th: thymus; Sk: skin; He: heart; Hk: head kidney; Sp: spleen.

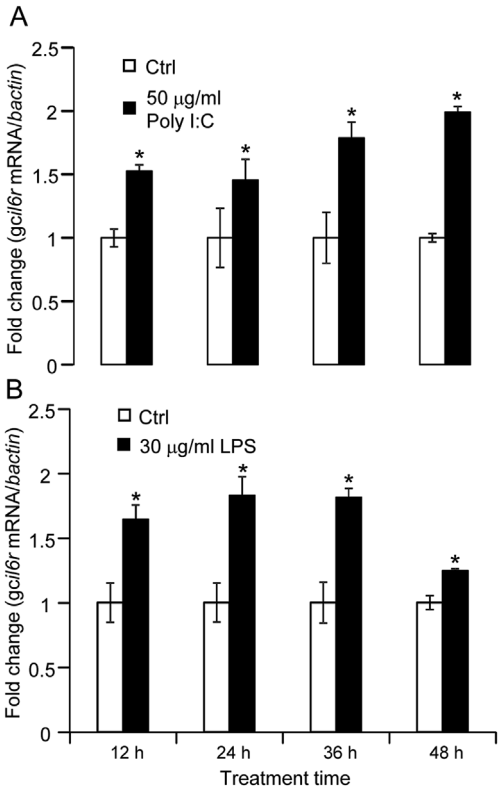

Fig. 4. Effects of Poly I:C (A) and LPS (B) on g*cil6r* mRNA expression in grass carp HKLs. The mRNA levels were determined by RT-qPCR and normalized to that of *bactin*. The fold changes were calculated by comparing the average expression level of each treatment group with that of the time-match control group. Bars indicate means ± SEM of gene expression levels in four individual samples (N = 4). One-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) tests were used to analyze the data. The * indicated statistical significance at $P < 0.05$ *vs* time-match control.

3.3. Recombinant expression and purification of rgcIl-6 and rgcsIl-6r proteins

To examine the function of gcIl-6r, rgcIl-6 and rgcsIl-6r proteins were obtained by prokaryotic expression system. After IPTG induction, 6 × His-tagged rgcIl-6 protein was successfully produced in the supernatant of the crude protein extraction of *E. coli* cells (Fig. 5A, left panel, lane a). After purification by metal affinity chromatography and desalination by gel filtration chromatography, the purified rgcIl-6 was obtained and was visualized as a single band around 31 kDa on the gel of

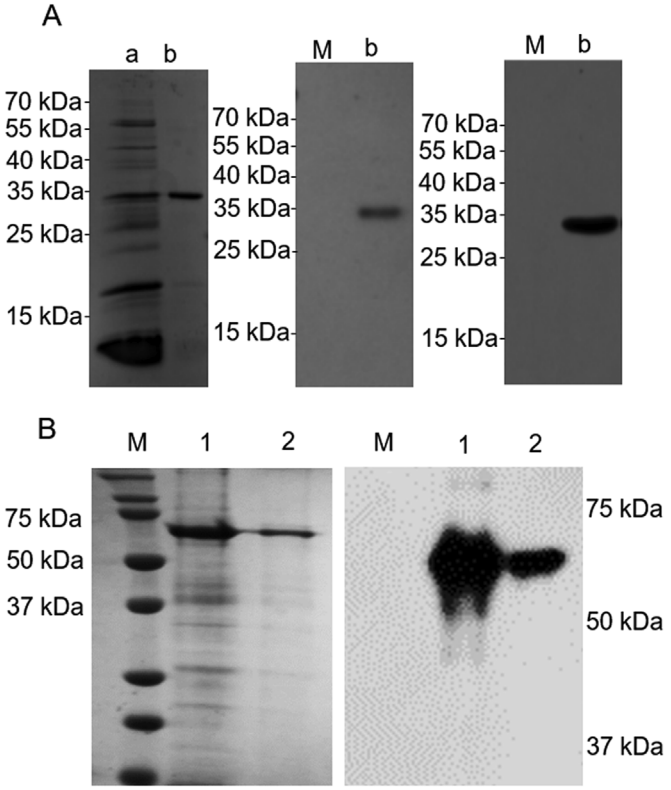

Fig. 5. Recombinant expression of gcIl-6 (A) and gcsIl-6r (B). A, left panel: SDS-PAGE analysis of purified rgcIl-6. A, middle panel: Western blotting analysis of rgcIl-6 using the antibody for gcIl-6. A, right panel: Western blotting analysis of rgcIl-6 using the antibody for His tag. Lane a: protein sample before purification. Lane b: purified rgcIl-6. B, left panel: SDS-PAGE analysis of purified rgcsIl-6r. B, right panel: Western blotting analysis of rgcsIl-6r using the antibody for gcIl-6r. M: molecular weight marker, lane 1: inclusion body sample, lane 2: purified rgcsIl-6.

SDS-PAGE (Fig. 5A, left panel, lane b). The rgcIl-6 was verified by Western blotting using a gcIl-6 antibody (Fig. 5A, middle panel) and a His-tag antibody (Fig. 5A, right panel). In both experiments, only one band with the same size as the band of SDS-PAGE was observed.

The same expression system was used to produce rgcsIl-6r. However, most of the recombinant protein was found in the inclusion bodies of the crude protein extraction of *E. coli* cells. The proteins of inclusion body were analyzed by SDS-PAGE and a strong band about 59 kDa was found (Fig. 5B, left panel, lane 1). The protein was denatured and refolded, and subsequently purified by metal ion affinity chromatography and desalted by centrifugal ultrafiltration. The purified rgcsIl-6r was analyzed by SDS-PAGE (Fig. 5B, left panel, lane 2) and Western blotting using an antibody for gcIl-6r (Fig. 5B, right panel, lane 2). Only one band was observed in both experiments. About 13% of rgcsIl-6r protein in inclusion body was obtained after refolding and purification.

3.4. Direct binding of gcIl-6 and gcsIl-6r

Given that ectodomain of transmembrane receptors was responsible for ligand binding and the extracellular domain of IL-6R (sIL-6R) has biological functions *in vivo* [26], the direct binding of gcIl-6 and gcsIl-6r was examined by quantitative ELISA. The results showed that rgcsIl-6r could bind to rgcIl-6 in a dose-dependent manner (Fig. 6). In parallel, the background binding of BSA controls was negligible.

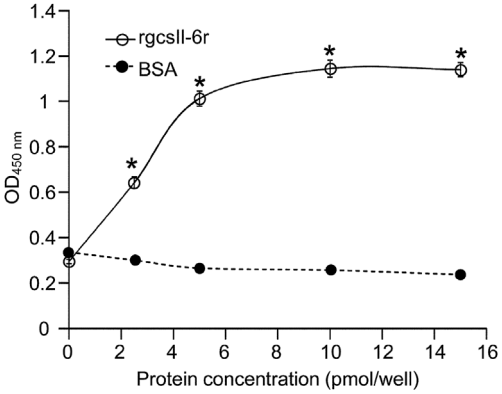

Fig. 6. Direct binding assay between gcIl-6 and gcsIl-6r. A 96-well plate was coated with increasing amounts (from 0 to 15 pmol/well) of gcsIl-6r or BSA (Negative control) at 4 °C overnight. After washing and blocking, rgcIl-6 (4 pmol/well) was added into the pre-coated wells and probed with a HRP-conjugated anti-gcIl-6 antibody. Data presented were expressed as means ± SEM (N = 4) of the OD values. One-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) tests were used to analyze the data. The asterisk (*) denotes a significant difference at $P < 0.05$ (vs. negative BSA control).

3.5. Biological activities of gcsIl-6r

Since gcsIl-6r protein was detected in the supernatant of HKLs culture after treated by 0.1 mM phorbol-12-myristate-13-acetate (PMA) for 3 h (Supplementary Fig. 2), biological activity of gcsIl-6r was examined in HKLs and CIK cells. The mRNA expression of *il6r* (Il-6-binding chain, detected by using the primers of *il6r* FLF & FLD, Supplementary table 1) and *gp130* (signal-transducing chain, detected by the primers of *gp130* F& D, Supplementary Table 1) was found in HKLs while *gp130* mRNA expression was only detected in CIK cells by using RT-PCR (Data not shown). In HKLs, rgcIl-6 dose-dependently increased *socs3* mRNA expression after 3 h-treatment (Fig. 7A). Thirty-two nanomolar of rgcIl-6 enhanced *socs3* mRNA expression at 3 and 6 h and it declined to basal level at 12 and 24 h (Fig. 7B). Accordingly, the dose of 32 nM of rgcIl-6 and treatment time of 3 h were used in the later experiments. In this condition, rgcIl-6 significantly stimulated *socs3* mRNA expression while heat-inactivated rgcIl-6 had no effect on the expression of this gene. Interestingly, 48 nM of rgcsIl-6r was able to enhance *socs3* mRNA expression and it also could slightly augment the rgcIl-6-stimulated *socs3* mRNA expression although the response was not significant (Fig. 7C). Compared with those results in HKLs, neither 32 nM rgcIl-6 nor 48 nM rgcsIl-6r affected *socs3* mRNA expression in CIK cells. However, combined treatment of them could increase *socs3* mRNA expression more than five folds (Fig. 7D).

3.6. Effects of gcIl-6, gcsIl-6r and their combined treatment on the mRNA expression of some inflammation-related cytokines in HKLs

Since gcsIl-6r could mediate the signal transduction of gcIl-6 as shown above, its roles in the regulation of gcIl-6 on some inflammation-related cytokines' mRNA expression were determined. In HKLs, rgcIl-6 stimulated pro-inflammatory cytokines' (*tnfa* and *il1b*) mRNA expression while heat-activated rgcIl-6 had no effect in this regard (Fig. 8A and B). Compared with its effects on the pro-inflammatory cytokines' expression, rgcIl-6 surprisingly stimulated anti-inflammatory cytokine (*il10*)'s mRNA expression at the same time (Fig. 8C). In these experiments, rgcsIl-6r augmented rgcIl-6's effects on the mRNA expression of these genes (Fig. 8A, B and C). It is noteworthy that rgcsIl-6r alone had a stimulatory effect on these genes' mRNA expression.

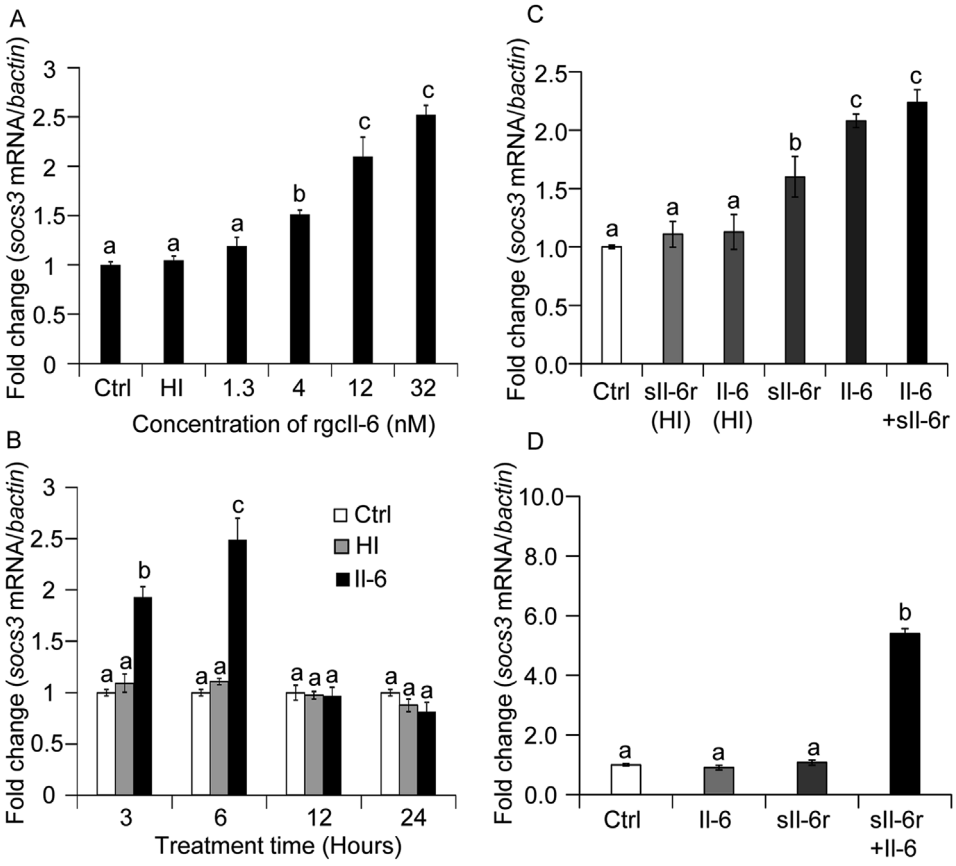

Fig. 7. Verification of the bioactivity of rgcIl-6 (A&B) and rgcsIl-6r in HKLs (C) and CIK cells (D). HKLs were treated with increasing doses of rgcIl-6 for 3 h or 32 nM of rgcIl-6 for different times (3, 6, 12 &24 h). Then, HKLs or CIK cells were treated with rgcIl-6 (32 nM), rgcsIl-6r (48 nM) or both of them for 3 h and the mRNA levels of *socs3* and *bactin* were detected by RT-qPCR. In the control groups, the same volume of culture medium was added at the same time. Statistical analysis was conducted by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Bars indicate means ± SEM of *socs3* mRNA levels in four individual samples. Different letters indicate the statistically significant differences ($p < 0.05$) *vs* control or time-match control. HI: heat inactivated.

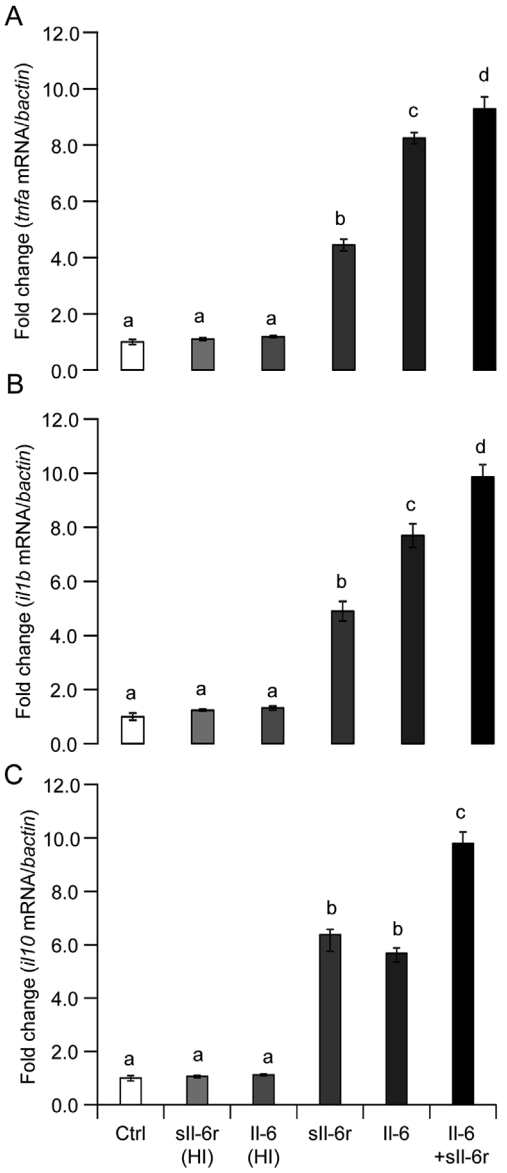

Fig. 8. Effects of rgcIl-6, rgcsIl-6r and their combined treatment on the mRNA expression of some inflammation-related factors in HKLs (A, B &C). The mRNA levels were detected by RT-qPCR. Statistical analysis was conducted by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Bars indicate means ± SEM of mRNA levels in four individual samples. Different letters indicate the statistically significant differences ($p < 0.05$) *vs* control. HI: heat inactivated.

4. Discussion

In teleost, *il6r* gene has been cloned in rainbow trout [21], fugu [20] and zebrafish [9]. However, the function of teleost Il-6r is poorly understood although its ability to mediate Il-6-activated Stat3 phosphorylation has been demonstrated [20]. In addition, the sequence for zebrafish *il6r* in GenBank (NM_001114318.1) has been removed because it is insufficient support for the transcript and the protein currently. Accordingly, it is indispensable to functionally define *il6r* gene in fish species. In line with this, grass carp *il6r* gene was cloned and its role in mediating gcIl-6's signaling was studied in the present study. Fish Il-6rs possess conserved functional domains (Fig. 2 [20,21]) and synteny of *il6r* loci (Supplementary Fig. 1 [20,21]) compared to their homologues in human and mouse. In particular, an Ig-like domain and two fibronectin type-III domains were found in gcIl-6r (Fig. 2 and Supplementary Fig. 1A). These domains were conserved in zebrafish, golden-line barbell, common carp, rainbow trout, fugu, mouse and human, indicating that the function of Il-6r and its interaction mode with the ligand are evolutionally conserved. The ectodomains in all fish Il-6r molecules are bigger than those in mouse and human because fish Il-6rs possess a longer sequence between signal peptide and Ig-like domain. It is noteworthy that a polymorphic domain before the Ig-like domain has been found in rainbow trout Il-6r which may affect the function of this receptor [21]. However, no similar sequence was found in Il-6r of other teleosts. The WSxWS motif proposed to facilitate cytokine binding [27] was conserved in trout [21] and fugu [20] but unexpectedly mutated to WSxWT in cyprinid fishes (Fig. 2). In human IL-6R, mutation of both serines (S) to alanines (A) in WSxWS motif significantly decreased IL-6-binding capability of sIL-6R, indicating that these two serines are critical for sIL-6R to mediate IL-6's functions [28]. Whether mutation of S to T among this motif in cyprinid fishes led to lower binding ability of Il-6r to its ligand is an interesting issue and warrants further study. The structure diversity of Il-6r proteins in fishes described above prompted us to investigate the functional roles of grass carp Il-6r in the present study.

The g*cil6r* mRNA was expressed with high levels in immune tissues including spleen and head kidney. This is consistent with the results in trout [21] and fugu [20] and in line with the function of IL-6 in immune response and inflammation. As an example, injection of recombinant Il-6r stimulates *igm* mRNA expression in head kidney of orange-spotted grouper [13]. In mammals, IL-6R is expressed only by hepatocytes, phagocytes and some lymphocytes [29]. Ubiquitous expression of *il6r* mRNA in the selected tissues of grass carp (Fig. 3) and trout [21] implies the functional diversity of fish Il-6 compared to its mammalian homologues. In grass carp HKLs, LPS and Poly I:C stimulated the mRNA expression of *il6r* in a time-dependent manner (Fig. 4). In trout, LPS can significantly enhance *il6r* mRNA expression in macrophage-like, epithelial and fibroid cell lines while Poly I:C can increase its expression only in an epithelial cell line [21]. LPS has the potential to stimulate cellular immunity and humoral immunity in fish species [30], and Poly I:C was considered a “viral mimic” in mammals [31] and has been extensively used as an immune stimulator in fish model. Thus, induction of *il6r* mRNA expression by LPS and Poly I:C in grass carp and trout indicates that Il-6r is involved in the immune responses during pathogen infection and subsequently affects the immune response induced by Il-6 in fish.

We demonstrated the specific binding of rgcsIl-6r and rgcIl-6 by using quantitative ELISA (Fig. 5). To our knowledge, this is the first report showing the direct binding between Il-6 and Il-6r in fish model. It provides evidence that the gene obtained is a genuine Il-6r in spite of its sequence variation compared to mammalian IL-6R. In mammals, SOCS3 is one of downstream target genes regulated by IL-6 [8]. In RTS-11 cells, a trout monocyte/macrophage-like cell line, a rapid increase of *socs3* mRNA expression stimulated by recombinant rainbow trout Il-6 was observed [19]. In grass carp HKLs, rgcIl-6 stimulated *socs3* mRNA expression (Fig. 7C), indicating that rgcIl-6 not only can bind to its receptor but also has bioactivities. Stimulation of *socs3* mRNA expression by Il-6 in trout and grass carp collectively suggests that this signal transduction pathway is conserved in fish species. In CIK cells, RT-PCR results showed that *gp130* but no *Il-6r* expression was detected (Data not shown), providing a promising cell model to investigate the bioactivities of gcsIl-6r. Not unexpectedly, rgcIl-6 alone had no effect on *socs3* mRNA expression in CIK cells (Fig. 7D). However, combined treatment of rgcIl-6 and rgcsIl-6r increased *socs3* mRNA levels to more than five folds (Fig. 7D). This together with our previous data of binding assay suggests that gcsIl-6r can bind to Il-6 and might activate intracellular signal transduction via transmembrane receptor, gp130. It is noteworthy that rgcsIl-6r alone stimulated *socs3* mRNA expression in HKLs (Fig. 7C) while heat-inactivated rgcsIl-6r had no effect in this regard. We speculate that this effect might be from the endogenous gcIl-6 secreted by the cells.

IL-6 can act as both an anti-inflammatory and a pro-inflammatory factor in mammals and its anti-inflammatory and pro-inflammatory activities are mediated by classic signaling and trans-signaling, respectively [1]. In grass carp HKLs, rgcIl-6, somewhat to our surprise, simultaneously stimulated the mRNA levels of both pro-inflammatory (*tnfa* and *il1b*) and anti-inflammatory (*il10*) cytokines after the same treatment (Fig. 8). In a macrophage-like rainbow trout cell line, recombinant Il-6 inhibited *tnfa1/2* mRNA after 4, 6 and 8 h-treatment and *ilb1* mRNA after 24 h-treatment while slightly stimulated *il10* mRNA after 4 h-treatment. The effects of recombinant Il-6 on *tnfa2* and *ilb1* mRNA are confirmed in primary microphages [19]. In large yellow croaker, overexpression of Il-6 or recombinant Il-6 can stimulate *tnfa* but not *il1b* mRNA expression in a kidney cell line, LCK cells [10]. Hence, effects of Il-6 on the expression of inflammation-related cytokines are largely dependent on the treatment time and cell type in fish species. However, combined treatment with rgcIl-6 and rgcsIl-6r further enhanced rgcIl-6-stimulated mRNA expression of *tnfa*, *il1b* and *il10* (Fig. 8), indicating that sIl-6r has agonistic activity in teleosts as in mammals [32]. This effect might be caused by activation of the HKLs which have gp130 but no membrane Il-6r expression by gcIl-6-gcsIl-6r complex.

In conclusion, grass carp *il6r* gene was conserved with its homologues in fish and mammals. It was expressed in immune tissues with high levels and its soluble form can specifically bind to Il-6 and mediate its bioactivities. In immune cells, gcsIl-6r possesses agonistic activities in the regulation of Il-6 on some cytokines' mRNA expression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.031.

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