Fish and Shellfish Immunology 92 (2019) 315–321

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Functional characterization of three fish-specific interleukin-23 isoforms as regulators of Th17 signature cytokine expression in grass carp head kidney leukocytes

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ARTICLE INFO

Keywords:
Il-23
Isoforms
Grass carp
Functional identification
Il-17a/f1
Il-23r
Th17-like response

ABSTRACT

Mammalian Interleukin (IL)-23 is a heterodimeric cytokine with an IL-23-specific P19 subunit and a P40 subunit shared with IL-12, and plays a key role in the regulation of cell differentiation as well as inflammation. We previously demonstrated the existence of three soluble fish Interleukin (Il)-23 isoforms consist of a single P19 and one of three P40 isoforms (P40a/b/c) in grass carp. In the present study, three recombinant grass carp Il-23 (rgcIl-23) isoforms were prepared by linking gcP19 and gcP40a/b/c in a prokaryotic expression system, and then their functional properties were verified in grass carp head kidney leukocytes (HKLs). All three rgcIl-23 isoforms showed the bioactivities to divergently upregulate the mRNA expression of Th17 signature cytokines (*il17a/f1*, *il21*, *il22* and *il26*) as well as Il-23 receptor (*il23r*) in HKLs. Moreover, they also promoted gcIl-17a/f1 secretion in a dose-dependent manner, strengthening their roles in Th17-like response. Furthermore, induction of *il17a/f1* and *il23r* transcription by rgcIl-23 was blocked by a STAT3 inhibitor in grass carp HKLs, suggesting the involvement of STAT3 signaling in these inductions. Taken together, we for the first time identified the bioactivities of fish Il-23 isoforms and particularly revealed the existence of Il-23/Il-17a/f1 axis in fish, thereby advancing our understanding of Th17-like responses in fish immunity.

1. Introduction

Interleukin (IL)-23 belongs to IL-12 family which consists of IL-12, IL-23, IL-27 and IL-35. These cytokines have heterodimeric structures formed by an α-chain (including P35, P28 and P19) and a β-chain (including P40 and Epstein-Barr virus-induced gene 3, namely EBI3) [1]. IL-23 is formed by an IL-23-specific P19 subunit and a P40 subunit which is shared with IL-12 [2]. In response to various immunostimulants, IL-23 is produced by macrophages, dendritic cells, keratinocytes, and other antigen-presenting cells as well as T and NK lymphocytes [3–5]. Once IL-23 is secreted, it binds to a heterodimeric receptor which is comprised of two chains. One chain specifically binds to P40, namely IL-12Rβ1 while the other specifically binds to P19, namely IL-23R. IL-23 signaling depends on the activation of tyrosine kinase (TYK2) via IL-12Rβ1 and Janus associated kinase 2 (JAK2) via IL-23R, which predominantly result in STAT3 phosphorylation and to a lesser extent in STAT1, STAT4, and STAT5 phosphorylation [3,6]. In terms of its biological activity, mammalian IL-23 is well known to induce and maintain differentiation of Th17 cells characterized by prominent production of IL-17A and IL-17F [2–4]. Besides, activated Th17 cells also produce IL-6, IL-22, and IL-26 [3,4]. Increasing evidence suggests that IL-23 as well as other Th17-related cytokines have been implicated in chronic autoimmune diseases [7–9], as well as host defense against intracellular pathogens [10,11].

Unlike mammals, fish-specific whole genome duplication (WGD) events may result in multiple gene generation in teleost [12]. For *p40* gene, three distinct *p40* paralogues, namely *p40a*, *p40b* and *p40c*, are found in common carp, zebrafish and pufferfish [13]. Additionally, two *p19* paralogues, *p19a* and *p19b*, have been identified and characterized in Atlantic salmon and rainbow trout [14]. In our previous study [12], we have uncovered that grass carp (*Ctenopharyngodon idella*) possessed a single *p19* gene and three *p40* paralogues, and particularly proved the existence of three Il-23 isoforms as soluble heterodimeric cytokines in grass carp. However, the functional information of these Il-23 isoforms remains obscure.

In the present study, we prepared three recombinant proteins of

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https://doi.org/10.1016/j.fsi.2019.06.028
Received 25 March 2019; Received in revised form 12 June 2019; Accepted 12 June 2019
Available online 13 June 2019
1050-4648/

gcIl-23 (rgcIl-23) isoforms by using a prokaryotic expression system followed with protein refolding method. In grass carp head kidney leukocytes (HKLs), these three rgcIl-23 isoforms showed bioactivities to promote the mRNA expression of grass carp *il17a/f1*, *il21*, *il22*, *il26* and *il23r* to different extents, suggesting the conserved functions of gcIl-23 as seen in mammals. This notion was further supported by the findings that three isoforms stimulated the secretion of Il-17a/f1 in the same model. Moreover, we found that STAT3 pathway was involved in gcIl-23-mediated the transcription of *il17a/f1* and *il23r* in grass carp HKLs. Taken together, our results for the first time define the functional role of teleost Il-23.

2. Materials and methods

2.1. Animals

Healthy grass carp, weighing about 1.0 kg, were purchased from Chengdu Tongwei Aquatic Science and Technology Company (Chengdu, China) and kept in a running water system and fed to satiation daily with commercial carp pellets. The fish were acclimated to laboratory environment for at least two weeks prior to use. Grass carp head kidney was collected using the procedure of Secombes [15] with some modifications. Briefly, the fish was anaesthetized and exsanguinated in 0.05% MS222 (Sigma-Aldrich, MO, USA). Subsequently, the peritoneal cavity of the fish was opened by an incision and the swim bladder and other organs were removed aseptically. Finally, the exposed head kidney was dissected out and put into Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) supplemented with 1% Antibiotic-Antimycotic Gibco™ (Thermo Fisher Scientific, MA, USA) and heparin (10 units/mL, Sangon Biotech, Shanghai, China). All animal experiments complied with the Regulation of 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. Construction of expression plasmids

A peptide with 15 amino acids of three repeats of GGGGS was used to link P40 and P19 to produce three gcIl-23 isoforms (P40-linker-P19). Briefly, the cDNA sequences encoding mature gcP19 was amplified by PCR using the primers of Not I–P19-Linker-F and Hind III-P19-R (Supplementary Table S1) with Phusion High-Fidelity DNA Polymerase (New England Biolabs, Beverly, MA) and then subcloned into PET30a (+) expression vector (Novagen, Darmstadt, Germany) after digested with Not I and Hind III (New England Biolabs). Next, the Not I-Not I fragments encoding the mature gcP40a, gcP40b or gcP40c was amplified by PCR using the primers with Not I restriction site (Supplementary Table S1) and subsequently inserted into PET30a(+) expression vector containing *gcp19* after the digestion with Not I. The integrity of the inserted DNA was verified by sequencing. Finally, three grass carp Il-23 expression plasmids [pET30a(+)/gcIl-23a, pET30a(+)/gcIl-23b and pET30a(+)/gcIl-23c] were prepared for transformation.

2.3. Expression and purification of rgcIl-23a, rgcIl-23b and rgcIl-23c

The expression plasmids for three grass carp Il-23 isoforms were transformed into *Escherichia coli* (*E. coli*) BL21 (DE3) competent cells (Thermo Fisher Scientific). A single colony of *E. coli* BL21 (DE3) harboring the pET30a(+)/gcIl-23a, pET30a(+)/gcIl-23b or pET30a (+)/gcIl-23c was cultured in 5 mL LB medium with 30 μg/mL kanamycin (Sangon Biotech, Shanghai, China) and grown overnight at 37 °C. This culture was subsequently added to 100 mL fresh LB medium with 30 μg/mL kanamycin and shaken at 180 rpm at 37 °C for about 2 h until the $OD_{600\ nm}$ reached 0.6–0.8, and then IPTG (Merck, Darmstadt, Germany) was added with a final concentration of 1 mM. Next, the culture was shaken at 180 rpm at 37 °C for additional 4 h. The bacteria were collected by centrifugation at 4000*g* for 10 min at 4 °C, washed twice with 10 mL phosphate buffer (PB, 20 mM, pH 8.0) and resuspended in 10 mL ice-cold PB (20 mM, pH 8.0) supplemented with 0.1 mg/mL lysozyme and 1 mM phenylmethanesulfonyl fluoride (PMSF, VWR, PA, USA). The cells were sonicated on ice, and the inclusion body was collected by centrifugation at 10,000 g for 30 min at 4 °C. The recombinant proteins were purified under denaturing conditions and refolded in a buffer containing arginine. In brief, inclusion bodies were washed twice with 10 mL washing solutions [200 mM Tris-HCl and 100 mM EDTA (Sigma-Aldrich), pH 7.5] containing 10% Triton X-100, 1% Triton X-114 (Sigma-Aldrich) or 3 M urea (Sigma-Aldrich), separately. Pre-purified inclusion bodies were solubilized with 20 mL of denaturing buffer [20 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole (Sigma-Aldrich) and 6 M guanidine hydrochloride (VWR), pH 8.0], passed through a 0.45 μm filter, and then loaded onto a HisTrap HP column (GE Healthcare, Waukesha, WI). After that, the bound proteins were eluted with elution buffer (500 mM imidazole, 20 mM PB, 500 mM NaCl and 6 M guanidine hydrochloride, pH 8.0) under denaturing conditions. About 1 mg of purified rgcIl-23 were refolded in 50 mL refolding buffer [100 mM Tris-HCl, 2 mM EDTA, 400 mM arginine (Sangon Biotech), 5 mM reduced glutathione (Biofroxx, Einhausen, German), 0.5 mM oxidation glutathione (Biofroxx) and 0.5 mM PMSF, pH 8.0], and finally the refolding buffer was replaced with PB (20 mM, pH 8.0) by passing through a Superdex 75 column (GE Healthcare). Endotoxin (LPS) was removed by Endotoxin Removal Agarose Resin (Yeasen, Shanghai, China). Affinity and gel filtration chromatography were run on ÄKTA Explorer 100 system (GE Healthcare). The protein concentration was determined by Bradford protein assay. Protein samples were applied to 8% SDS-PAGE and protein bands were analyzed by optic densitometry using Quantity One software (Bio-Rad, CA, USA).

2.4. Western blotting (WB) assay

The proteins were separated on 8% SDS-PAGE gel and electrophoretically transferred to a PVDF membrane (Millipore, Billerica, MA). The membrane was blocked by TBST buffer (25 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 7.4) containing 5% defatted dry milk for 2 h at room temperature and then incubated with *anti*-His Tag mAb (ZSGB-BIO, Beijing, China) at 1:1000 dilution with gentle shaking overnight at 4 °C. The membranes were exposed to horseradish peroxidase-conjugated goat *anti*-mouse secondary antibody (1:5000, ZSGB-BIO) for 1 h at room temperature. Finally, positive signals were detected using an ECL kit (Millipore) according to the instruction of the manufacture. To confirm the specificity of gcIl-17a/f1 pAb, total protein from grass carp HKLs lysates, and recombinant gcIl-17a/f1 (rgcIl-17a/f1) protein prepared in our previous study [16] were analyzed by WB in which the membrane was incubated with *anti*-gcIl-17a/f1 Ab pre-absorbed with excessive rgcIl-17a/f1.

2.5. Isolation of grass carp HKLs and primary cell culture

Grass carp HKLs were prepared by discontinuous density gradient centrifugation. In brief, head kidney was collected from freshly killed grass carp, washed twice and gently pressed in RPMI-1640 medium (Thermo Fisher Scientific, MA, USA). The cell suspension was filtrated through a 200-gauge stainless steel mesh and centrifuged at 500 g for 25 min on a discontinuous density gradient (Histopaque 1.083 kg/L, Sigma-Aldrich). After that, leukocytes enriched at the interface were collected and washed with PBS for two times. The cells were resuspended in RPMI-1640 medium supplemented with 10% FBS (Thermo Fisher Scientific), seeded with the density of 2×10^6 cells/well in 24-well plates (Corning, NY, USA) and incubated at 28 °C under 5% CO_2 and saturated humidity. The cells were treated with rgcIl-23 (rgcIl-23a, rgcIl-23b or rgcIl-23c) or STAT3 inhibitor (S3I-201, 30 μM, EMD Millipore, MA, USA) for the durations indicated in individual experiments, and the treatment was performed 2 h later after seeding.

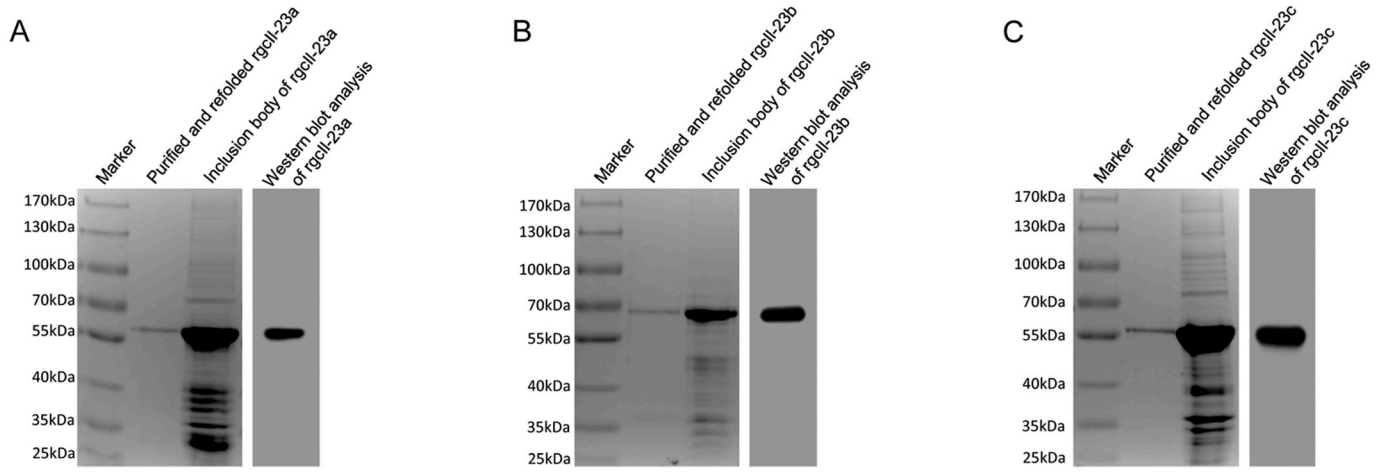

Fig. 1. SDS-PAGE and WB analysis of rgcIl-23a (A), rgcIl-23b (B) and rgcIl-23c (C). Refolded rgcIl-23a, rgcIl-23b, rgcIl-23c and their inclusion body proteins were analyzed by SDS-PAGE on 8% separation gels under reducing conditions. Proteins were stained with Coomassie blue R250. In WB assays, *anti*-His Tag mAb (1:1000) was used to detect the purified and refolded rgcIl-23a, rgcIl-23b and rgcIl-23c.

2.6. Gene expression analysis by real-time quantitative PCR (RT-qPCR)

Total RNA was extracted with TriPure Isolation Reagent (Roche, Basel, Switzerland), and subjected to reverse transcription using M-MLV Reverse Transcriptase (Promega, WI, USA) with Oligo $(dT)_{18}$ as the primer. The gene-specific intron-spanning primers for RT-qPCR were listed in Supplemental Table 1. RT-qPCR was performed on the qTOWER^3 G Thermocycler (Analytic Jena, Jena, Germany). To estimate the amplification efficiency, the standard curve for each target gene was generated by 10-fold serial dilutions (from 10^{-1} to 10^{-6} fmol/μL) of a plasmid containing the target gene sequences as the PCR template. In these experiments, *bactin* was amplified as the normalization control.

2.7. Competitive-inhibition enzyme linked immunosorbent assay (ELISA)

Competitive-inhibition ELISA was carried out to measure the concentration of gcIl-17a/f1 in the supernatant of grass carp HKLs culture. The gcIl-17a/f1 polyclonal antibodies (*anti*-gcIl-17a/f1 pAb) were custom products of Abmart (Shanghai, China). In this experiment, 96-well polystyrene plates (Sigma-Aldrich) were coated with 200 ng/well of rgcIl-17a/f1 at 4 °C for 16 h and blocked with 5% nonfat milk plus 0.3% BSA in PBS for 3 h at 37 °C. At the same time, 50 μL of culture medium or the titrated rgcIl-17a/f1 and 50 μL of *anti*-gcIl-17a/f1 pAb (1:100) were mixed and incubated at 37 °C for 2 h. After that, the plates were washed with PBST (0.05% Tween-20 in PBS) for three times, 100 μL of medium-antibody mixture was added to each well and further incubated at 4 °C for 16 h. The plate was washed with PBST for five times, and then 100 μL of horseradish peroxidase-conjugated goat *anti*-rabbit secondary antibody (1:1000, ZSGB-BIO) was added into each well. After incubation at 37 °C for 2 h, the plate was washed with PBST for five times and 100 μL of substrate buffer (1 mg/mL, 3, 3′, 5, 5′-Tetramethylbenzidine, TMD, Tiangen, Beijing, China) was added into the wells and incubated about 20 min at 37 °C. The reaction was stopped by 2 M H_2SO_4 and the optical density at 450 nm was measured with Bio-Rad iMark Microplate Reader (Bio-Rad). Control groups were pre-coated with BSA followed by the same procedures as described above. Absorbance reading was measured and the concentrations of samples were extrapolated from a standard curve for gcIl-17a/f1 inhibition.

2.8. Statistical analysis

The statistical analysis of gene expression was performed using the one-way ANOVA followed by LSD multiple group comparisons with SPSS Statistics 19.0 software (SPSS Inc., IL, USA). For comparison between two groups, Student's *t*-test was used. Data were expressed as mean ± SEM (N = 3). Differences were considered statistically significant at $P < 0.05$ and extreme significant at $P < 0.01$.

3. Results

3.1. Purification and refolding of rgcIl-23a, rgcIl-23b and rgcIl-23c

SDS-PAGE analysis showed the purified and refolded rgcIl-23a (Fig. 1A left), rgcIl-23b (Fig. 1B left) or rgcIl-23c (Fig. 1C left) exhibited a single band of about 60 kDa corresponding to their predicted size. WB assays by using *anti*-His Tag mAb also showed a single band of approximate 60 kDa for rgcIl-23a (Fig. 1A right), rgcIl-23b (Fig. 1B right) or rgcIl-23c (Fig. 1C right).

3.2. Effects of three gcIl-23 isoforms on the expression of Th17-related cytokines

To investigate biological activity of three rgcIl-23 isoforms, grass carp HKLs were incubated with increasing concentrations (30–1000 ng/mL) of rgcIl-23a, rgcIl-23b or rgcIl-23c for 12 h, and then the transcript levels of Th17-related cytokines were examined by RT-qPCR. Results showed that all of them potently enhanced the mRNA expression of *il17a/f1* in a dose-dependent manner (Fig. 2A–C). In this context, rgcIl-23a (Fig. 2A) and rgcIl-23b (Fig. 2B) at 1000 ng/mL induced a 50-fold and 38-fold increase of *il17a/f1* transcript levels, respectively, while rgcIl-23c at the same dose stimulated *il17a/f1* expression by about 6-fold (Fig. 2C). In parallel, rgcIl-23a induced an about 40-fold increase of *il26* transcript levels (Fig. 2D), but both rgcIl-23b and rgcIl-23c up-regulated *il26* mRNA expression lesser than 10 folds (Fig. 2E–F). In addition, *il21* transcription was significantly elicited by rgcIl-23a, rgcIl-23b or rgcIl-23c at different doses (Fig. 2G–I). Moreover, rgcIl-23a increased the mRNA expression of *il22* in a dose-dependent manner (100–1000 ng/ml, Fig. 2J), and rgcIl-23b only at 1000 ng/ml could stimulate *il22* mRNA expression (Fig. 2K). However, rgcIl-23c had no effect on mRNA expression of *il22* (Fig. 2L).

3.3. Effects of three gcIl-23 isoforms on gcIl-17a/f1 release in grass carp HKLs

To reinforce the functionality of gcIl-23 in regulating Th17-like response, grass carp HKLs were treated with increasing doses (300–1000 ng/mL) of rgcIl-23a, rgcIl-23b or rgcIl-23c for 36 h, and the

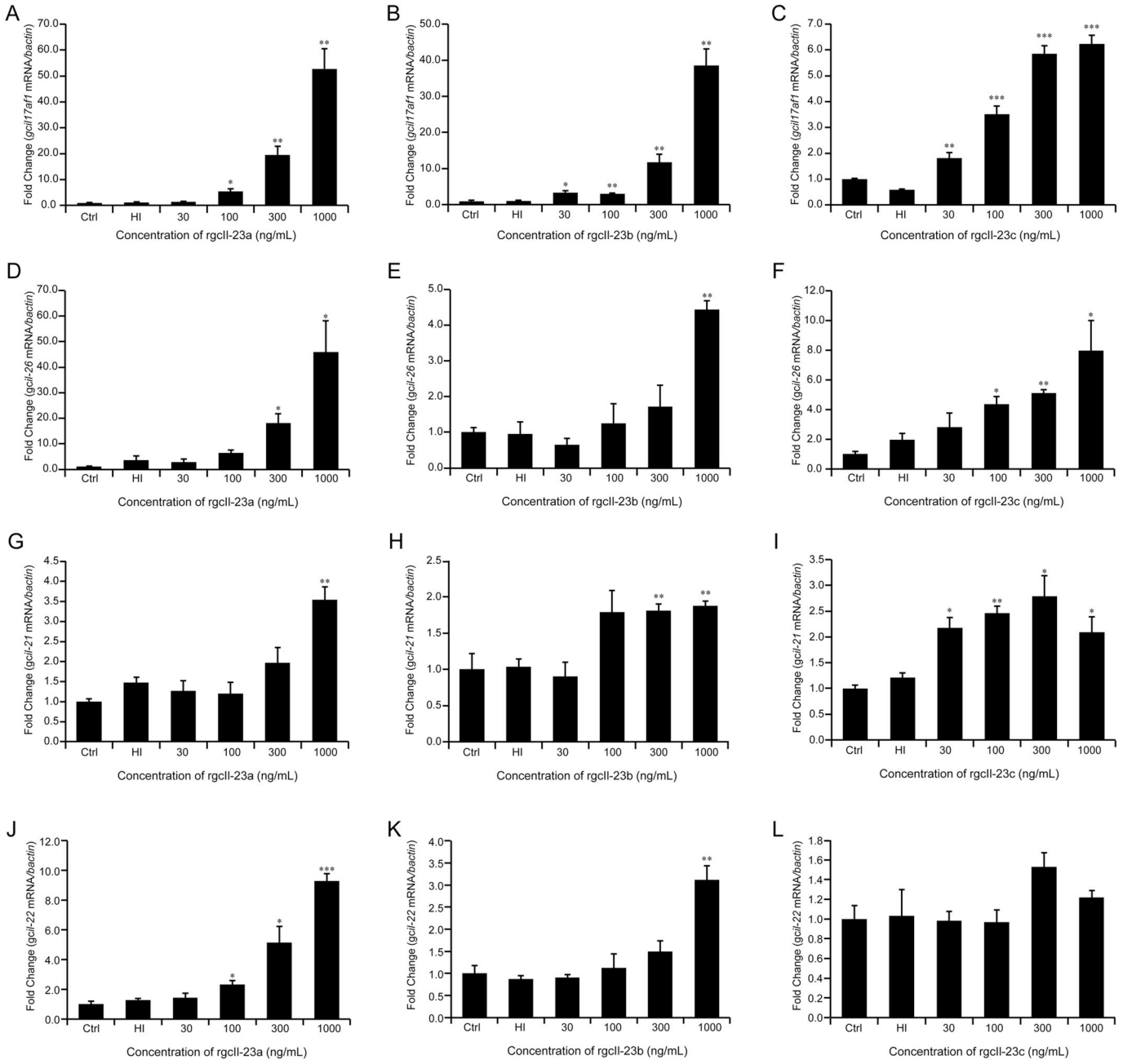

Fig. 2. Effects of rgcIl-23a, rgcIl-23b and rgcIl-23c on the mRNA expression of Th17 signature cytokines. Freshly prepared HKLs were seeded at the density of 2×10^6 cells/well in 24-well plates and were incubated with 30–1000 ng/mL of rgcIl-23a, rgcIl-23b, rgcIl-23c or their heat inactivated (HI) proteins (1000 ng/mL) for 12 h. The transcript levels of *bactin*, *il17a/f1* (A–C), *il26* (D–F), *il21* (G–I) and *il22* (J–L) were determined by RT-qPCR. The mRNA levels of each gene were normalized by *bactin* and expressed as fold changes of the mean value in the control group. Data are shown as mean ± SEM (N = 3). The "*" denotes significant differences ($P < 0.05$) relative to the HI group. "**" means $P < 0.01$, "***" means $P < 0.001$.

secretion of gcIl-17a/f1 in culture supernatant was detected by ELISA. In the experiment, the specificity of gcIl-17a/f1 pAb was validated, showing that the gcIl-17a/f1 pAb could specifically bind to rgcIl-17a/f1 (30 kDa, Fig. S1A) and recognize a protein of 37 kDa from HKLs lysates (Fig. S1B). Moreover, no band was observed in the antigen pre-absorption experiment (Fig. S1C). ELISA assay showed that rgcIl-23a, rgcIl-23b or rgcIl-23c markedly stimulated the release of gcIl-17a/f1 with the peak response at 1000 ng/ml (Fig. 3). In parallel, 1000 ng/mL of heat-inactivated (HI) rgcIl-23a, rgcIl-23b or rgcIl-23c did not alter the release of gcIl-17a/f1 (Fig. 3).

3.4. Effects of three gcIl-23 isoforms on the expression of il23r in grass carp HKLs

Considering the involvement of IL-23R expression in mediating IL-23 signaling in mammals, we examined the effect of rgcIl-23 isoforms on the mRNA expression of *il23r* in grass carp HKLs. In this context, amino acid alignment showed that putative gcIl-23r (GenBank ID: MK676012) shared 71.53% and 78.47% identity to its homologs in zebrafish (GenBank ID: XM_021476745.1) and common carp Il-23r (GenBank ID: XM_019107190.1). Results of its expression showed that

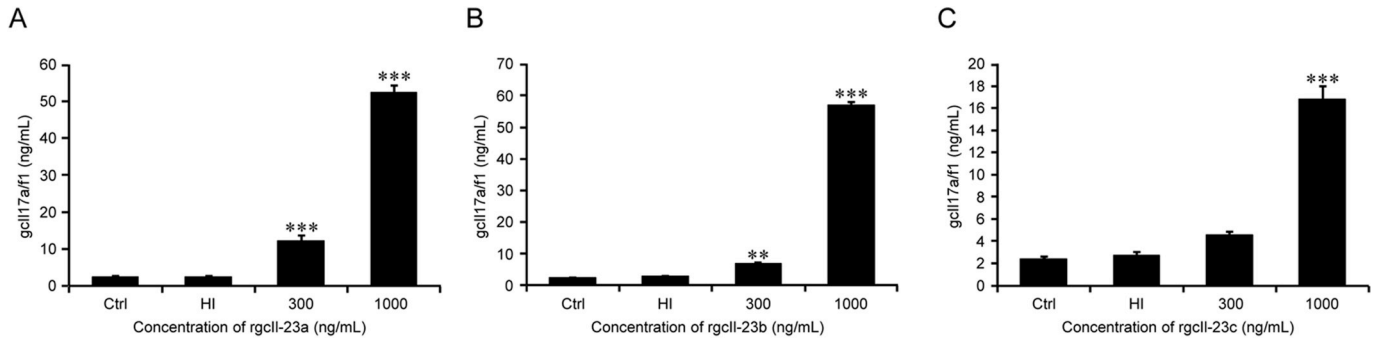

Fig. 3. Effect of rgcIl-23a, rgcIl-23b and rgcIl-23c on gcIl-17a/f1 release in grass carp HKLs. The HKLs were seeded at the density of 2×10^6 cells/well in 24-well plates and were treated with rgcIl-23a (1000 ng/mL) (A), rgcIl-23b (1000 ng/mL) (B), rgcIl-23c (1000 ng/mL) (C) or their heat inactivated (HI) proteins (1000 ng/mL) for 36 h. The protein levels of gcIl-17a/f1 in medium were detected by ELISA as described in materials and methods. Data are shown as Mean ± SEM (N = 3). The asterisk denotes a significant difference at $P < 0.05$ relative to the HI group. "**" means $P < 0.01$, "***" means $P < 0.001$.

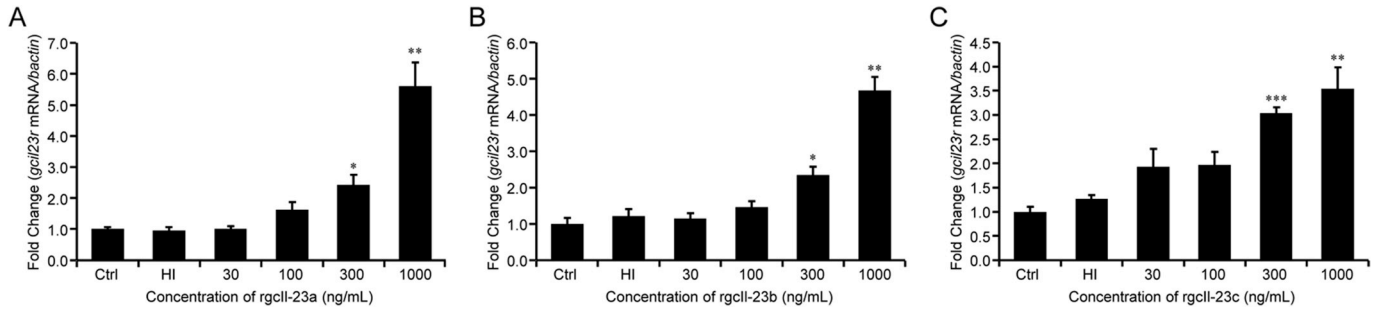

Fig. 4. Effect of three rgcIl-23 isoforms on mRNA expression of *il23r*. Freshly prepared HKLs were seeded at the density of 2×10^6 cells/well in 24-well plates and were stimulated with 30–1000 ng/mL rgcIl-23a (A), rgcIl-23b (B), rgcIl-23c (C) or their heat inactivated (HI) proteins (1000 ng/mL) for 12 h. The transcript levels of *bactin* and *il23r* were determined by RT-qPCR. The mRNA levels of each gene were normalized by *bactin* and expressed as fold changes of the mean value in the control group. Data are shown as mean ± SEM (N = 3). The "*" denotes significant differences ($P < 0.05$) relative to the HI group. "**" means $P < 0.01$, "***" means $P < 0.001$.

all of three rgcIl-23 isoforms (300–1000 ng/ml) significantly upregulated the mRNA expression of *il23r* in a dose-dependent manner after 12-h treatment (Fig. 4).

3.5. Regulation of three gcIl-23 isoforms on il17a/f1 and il23r mRNA expression via STAT3 pathway

To elucidate the signaling pathway of gcIl-23 isoforms, grass carp HKLs were exposed to 300 ng/mL of rgcIl-23a, rgcIl-23b or rgcIl-23c or combined with 30 μM of STAT3 inhibitor (S3I-201) for 12 h, and then the transcript levels of *il17a/f1* and *il23r* were examined by RT-qPCR. Results showed that the stimulatory effects of three rgcIl-23 isoforms on the mRNA expression of *il17a/f1* and *il23r* were impeded by the STAT3 inhibitor (Fig. 5).

4. Discussion

The immunological role of mammalian IL-23 has been well characterized [1–4], but the function of fish Il-23 remains elusive. In particular, WGD events lead to the generation of three Il-23 isoforms in various fish species [12,13]. In our previous studies, we have proved the existence of three soluble Il-23 isoforms in grass carp [12], prompting us to identify their functions. To address this issue, we prepared rgcIl-23 in the form of inclusion bodies by a prokaryotic expression system, and the inclusion body proteins were refolded *in vitro* to obtain renatured rgcIl-23a, rgcIl-23b and rgcIl-23c. Given that IL-23 is a crucial cytokine in controlling the development and proliferation of Th17 cells which are the main cell source of IL-17 [11,17–19], the ability of gcIl-23 in mediating *il17a/f1* expression was assessed in grass carp HKLs. Results showed that all of three isoforms dramatically augmented *il17a/f1* gene expression and Il-17a/f1 release. These findings strongly supported the existence of Il-23/Il-17a/f1 axis in fish. In mammals, it is generally accepted that IL-23 and IL-17 form an axis through Th17 cells, which has evolved in response to autoimmunity and bacterial or viral infections [10,11]. We previously identified the proinflammatory role of grass carp Il-17a/f1 in host defense in which it could recruit immune cells through producing CXCL-8 [16]. Accordingly, the potential of gcIl-23 to modulate Il-17a/f1 production implies the importance of Th17-like response in fish.

Besides, Th17 cells are also characterized by the expression of IL-21, IL-22 and IL-26 in mammals [19,20]. As expected, three rgcIl-23 isoforms significantly elevated the mRNA expression of *il26* and *il21* (Fig. 2D–I). In addition, rgcIl-23a and rgcIl-23b but not rgcIl-23c were effective in enhancing *il22* mRNA expression (Fig. 2J–L). Importantly, these Th17 signature cytokines have unique immunoregulatory abilities in both mammals and fishes. For example, the regulatory role of Il-26 in inflammation has been characterized in grass carp [21], while human IL-26 exhibits its action on antibacterial host defense by triggering neutrophil migration [22]. Similarly, Il-22 plays an immunoregulatory role in host defense against *Edwardsiella ictaluri* invasion in yellow catfish [23], and mammalian IL-21 can participate in bacteria killing [24]. Collectively, regulation of Th17-related cytokines which have bactericidal activities by IL-23 suggests its importance in host defense against pathogens. Notably, our results also suggested that three gcIl-23 isoforms modulated the expression of these Th17 signature cytokines with different extents, indicating that the immunoregulatory role of three rgcIl-23 isoforms may be roughly the same while reserving differences. In accordance with the functional discrepancy of three

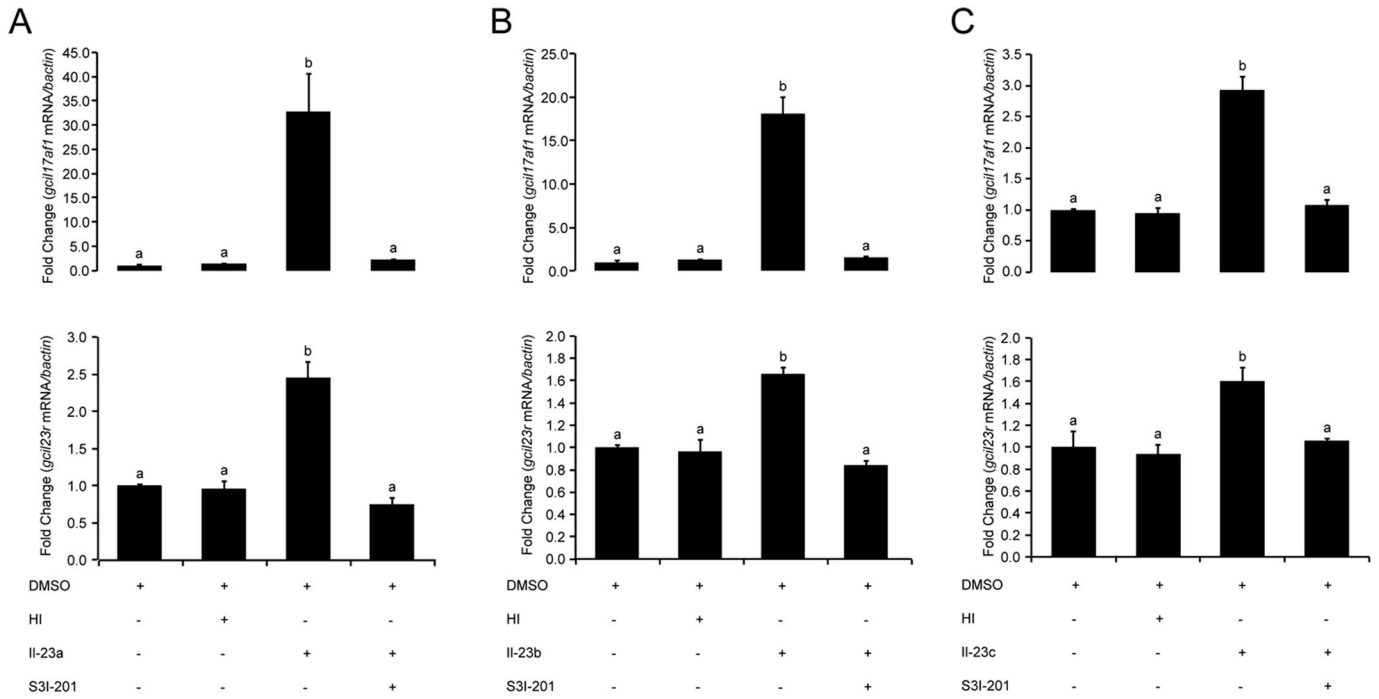

Fig. 5. Three rgcIl-23 isoforms regulated *il17a/f1* and *il23r* mRNA expression via STAT3 pathway. HKLs were seeded at the density of 2×10^6 cells/well in 24-well plates. The cells were incubated with or without 30 μM STAT3 inhibitor for 1 h, and then treated with rgcIl-23a (300 ng/mL, A) or rgcIl-23b (300 ng/mL, B) rgcIl-23c (300 ng/mL, C) or their heat inactivated (HI) proteins (300 ng/mL) for another 12 h. The transcript levels of *bactin*, *il17a/f1* and *il23r* were determined by RT-qPCR. The mRNA levels of each gene were normalized by *bactin* and expressed as fold changes of the mean value in the control group. Data are shown as mean ± SEM (N = 3). Different letters denote a significant difference at $P < 0.05$.

isoforms, three *p40* paralogues also exhibit divergent expression profiles in response to immune stimuli in fish [13].

In mammals, the inductive expression of IL-23R is thought as a regulatory point for Th17 cell development. For example, IL-23 and TGF-β3 promote the Th17 phenotype by increasing expression of IL-23R, respectively [25,26]. In support of this notion, it has been documented that full differentiation of effector Th17 cells requires IL-23R *in vivo* [27]. Along this line, we determined the effects of three isoforms on *il23r* gene expression in grass carp HKLs, finding their stimulatory actions on the mRNA expression of *il23r* in a dose dependent manner (Fig. 4). In agreement with our findings, IL-23 induces IL-23R expression in human CD4 T cells [28], and chicken IL-23 also significantly elevates the mRNA expression of *IL-23R* in macrophages and T cell lines [29]. Moreover, in mice lymph node cells, the mRNA expression of *IL-23R* was induced by IL-23, and the expression levels of *IL-23R* correlate with the *IL-17A* mRNA levels [30]. These findings support the possibility that gcIl-23-induced its own receptor expression might be a regulatory point to modify its cellular signaling as seen in mammals. Furthermore, we observed that the upregulation of rgcIl-23 isoforms on both *il17a/f1* and *il23r* expression was impeded by STAT3 inhibitor in grass carp HKLs (Fig. 5), suggesting the requirement of STAT3 pathway for rgcIl-23 signaling, which is consistent with findings in mammals in which IL-23 transduces its signal predominantly through STAT3 pathway [3,6].

In conclusion, the conserved functional roles of Il-23 were revealed in terms of specific modulation and signaling pathway in grass carp, particularly for the first time uncovering the existence of Il-23/Il-17a/f1 axis and Th17-like responses in fish as seen in mammals.

Acknowledgments

This work was supported by the grants from the National Natural Science Foundation of China (31572650, 31471181) and Applied Basic Research Program of Sichuan Province (19YYJC0036).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.06.028.

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