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Production and functional insights into the potential regulation of three isoforms of grass carp p40 subunit in inflammation

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ABSTRACT

The p40 subunit is known as a component of Interleukin (IL)-12 and IL-23. In mammals, p40 can be secreted as a monomer or homodimer and acts independently to mediate cellular responses. Recently, three p40 paralogues were isolated and identified from grass carp and other fish species, but whether they exist independently as well as their functional consequences and significance remain unclear. In the present study, using grass carp as the model, we for the first time demonstrated the existence of natural fish p40a, p40b and p40c (gcp40a, gcp40b and gcp40c) mainly as a monomer in culture supernatant of head kidney leukocytes (HKLs). Particularly, their excessive secretion induced by various immune stimuli suggests possible involvement of free p40s in fish immune responses. To define their functions, recombinant grass carp p40a/b/c (rgcp40a, rgcp40b and rgcp40c) were prepared by *Pichia pastoris* expression system, and they possessed the activities to enhance the secretion of pro-inflammatory cytokines including Il-1β and tumor necrosis factor-α (Tnf-α) in grass carp HKLs. These pro-inflammatory properties of p40 isoforms prompted us to investigate their roles during the inflammatory process. In line with this, *in vivo* study revealed the pathogenic effect of rgcp40a on intestinal inflammation, whereas gcp40a polyclonal antibodies remarkably ameliorated *Aeromonas hydrophila*-induced intestinal histopathological changes. Taken together, our results uncover the biological significance of free p40s in teleost, and provide new clue for targeting fish intestinal inflammation.

1. Introduction

In mammals, all the members of interleukin (IL)-12 family including IL-12, IL-23, IL-27 and IL-35 are heterodimeric cytokines [1]. Each of them consists of a heavy chain (p40 or EBI3) and a light chain (p19, p28 or p35), and is known to have diverse functions in terms of cell-mediated immunity [1–4]. The p40 component of IL-12 (p35/p40) is also a subunit of IL-23 (p19/p40) [5], and unlike p35 and p19, the p40 subunit (also named as IL-12p40) can be secreted as monomer and homodimer $(p40)_2$ [6–8]. In response to a variety of pathogenic or inflammatory agents, large amounts of p40 are produced primarily by monocytes, macrophages, dendritic cells or neutrophils [9]. Furthermore, a pathogenic role of p40 has been delineated in view of the fact that p40 levels are significantly increased in many inflammatory diseases [2,10,11]. For functionality, p40 is well known to provide a negative feedback loop by competitively binding to the IL-12Rβ1 and preventing IL-12/23-mediated shock in murine model [12,13]. Besides, p40 might have a pivotal and independent agonistic activity as well [14]. For example, p40 can act as a macrophage chemoattractant and promote the migration of the activated dendritic cells [15,16]. Moreover, p40 functions as an inflammatory cytokine which can induce the release of immune-related molecules, such as tumor necrosis factor-α (TNF-α) and nitric oxide (NO) via p38 mitogen-activated protein kinase (MAPK) and extracellular signal-related kinase (ERK) signaling pathways in mouse microglia and macrophages [8]. These findings strongly suggest that the p40 subunit has a unique role involving in immune regulation.

Compared with mammalian p40 encoded by a single gene, there are three distinct p40 genes (*p40a, p40b* and *p40c*) in some fish species, including common carp (*Cyprinus carpio*) [17], amberjack (*Seriola dumerili*) [18] and grass carp (*Ctenopharyngodon idellus*) [19]. In rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), *p40b* and *p40c* have been found while *p40a* is thought to be lost [20,21]. It is generally accepted that multiple p40 genes are generated by the fish-specific whole genome duplication (WGD) events [19–22]. To date, most efforts have been focused on isolating these three p40 paralogues

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and revealing their constitutive and inducible expression patterns following bacterial or viral infection [17,22] and immunostimulant challenge in different tissues [17,19,20]. However, a few important issues are needed to be addressed: Firstly, whether fish p40 subunit is independently secreted as seen in mammals is unknown; Secondly, functional characterization of three fish p40 isoforms in secreted forms is lacking; Thirdly, the role of the secreted fish p40 in inflammation has not been elucidated. Therefore, the biological significance of p40 in fish immunity so far remains obscure.

In the present study, using grass carp as the model, we firstly revealed the inducible expression of grass carp *p40a*, *p40b* and *p40c* (*gcp40a*, *gcp40b* and *gcp40c*) mRNA and proteins in head kidney leukocytes (HKLs) in response to various immune stimuli, particularly providing evidence of their inductive secretion in culture supernatant. Accordingly, recombinant grass carp p40a/b/c (rgcp40a, rgcp40b and rgcp40c) were prepared by using a *Pichia pastoris* expression system, and their effects on the protein release of some inflammatory-related cytokines were evaluated and compared in grass carp HKLs, prompting us to examine their regulatory effects on inflammation *in vivo*. In this regard, grass carp infected by *Aeromonas hydrophila* (*A. hydrophila*) were injected intraperitoneally (i.p.) with rgcp40a protein or gcp40a polyclonal antibodies (*anti*-gcp40a pAb), and the role of gcp40a in bacteria-induced intestinal inflammation was uncovered. Taken together, these results for the first time highlight the role of free fish p40 isoforms as new intrinsic regulators in inflammation.

2. Materials and methods

2.1. Fish

Chinese grass carp with an average body weight of 0.75–1.0 kg or 60 g were obtained from Chengdu Tongwei Aquatic Science and Technology Company (Chengdu, China) and were acclimatized in laboratory tanks at room temperature for 2 weeks before experiments. All animal experiments were conducted according to the Regulation of Animal Use in Sichuan province, China and were approved by the ethics committee of the University of Electronic Science and Technology of China.

2.2. Isolation of grass carp HKLs and primary cell culture

Grass carp HKLs were isolated using a discontinuous density gradient (Histopaque 1083, Sigma-Aldrich, Louis, USA) [23]. Briefly, head kidney was removed from the fish under sterile condition and squeezed gently to release the cells into RPMI-1640 medium (Thermo Scientific, Waltham, MA, USA). The cell suspension was passed through a 200-gauge stainless steel mesh. Subsequently, the cells in the medium were layered on a Histopaque 1083 and centrifuged at 2800 r/min for 30 min at room temperature with no brake. The cells at the interface were collected, washed twice and seeded at the density of 2×10^6 cells per well (in a volume of 400 μL RPMI-1640 medium with 10% FBS) in a 24-well cell culture plate (Corning, NY, USA) or seeded at 1×10^7 cells per well (in a volume of 1.5 mL RPMI-1640 medium with 10% FBS) in a 6-well cell culture plate (Corning). After incubation overnight at 28 °C under 5% CO_2 and saturated humidity, the HKLs were treated with the drugs as indicated in individual experiments.

2.3. Gene expression analysis by real time-quantitative PCR

Total RNA was extracted from the HKLs by using TriPure Isolation Reagent (Roche, Basel, Switzerland), and subjected to reverse transcription using M-MLV Reverse Transcriptase (Promega, Madison, USA) with Oligo $(dT)_{18}$ as the primer. The gene-specific intron-spanning primers for real-time quantitative PCR (RT-qPCR) were designed and listed in Supplementary Table 1. RT-qPCR was performed on the Bio-Rad CFX96™ System. To estimate the amplification efficiency, the standard curve for each target molecule was generated by 10-fold serial dilutions (from 10^{-1} to 10^{-6} fmol/μL) of the plasmid containing the target gene sequences as the PCR template. The mRNA levels of target genes and *bactin* were detected by RT-qPCR according to our previous study [24]. The *bactin* expression was detected and used as the normalization.

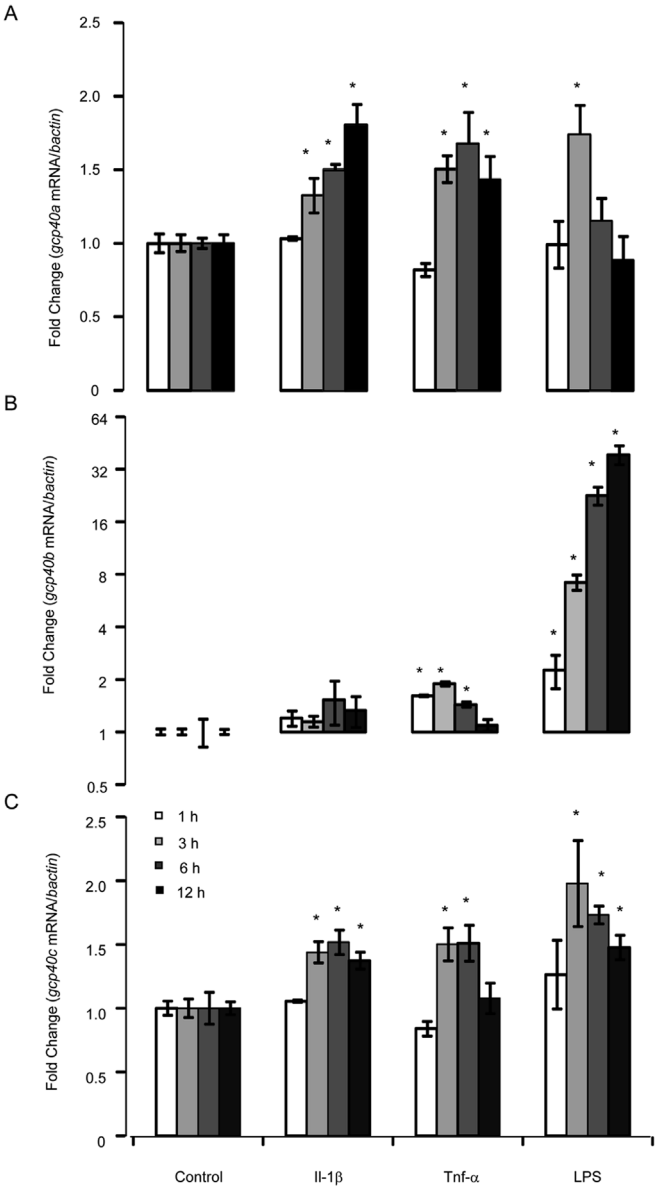

Fig. 1. Differential expression of the three *p40* paralogues upon immune stimuli in grass carp HKLs. The cells were exposed to LPS (30 μg/mL), rgcIl-1β (100 ng/mL) or rgcTnf-α (200 ng/mL) for different times (1–12 h). Transcript levels of three genes and *bactin* were determined by RT-qPCR. The mRNA levels of each gene were normalized by *bactin* and expressed as fold changes of the mean value in the control group at the same time point. Data are shown as mean ± SEM (N = 4). The *p*-values generated by paired sample *t*-tests between the stimulated and time-matched control samples were shown above the bars. *, $P < 0.05$.

2.4. Construction of expression plasmid and yeast transformation

The coding sequences of gcp40a, gcp40b and gcp40c without signal peptide were amplified by PCR using the primers with restriction enzyme cutting sites of Kpn1 and Not1 (Supplementary Table 1). The PCR products and pPICZα A vector (Thermo Scientific) were digested by

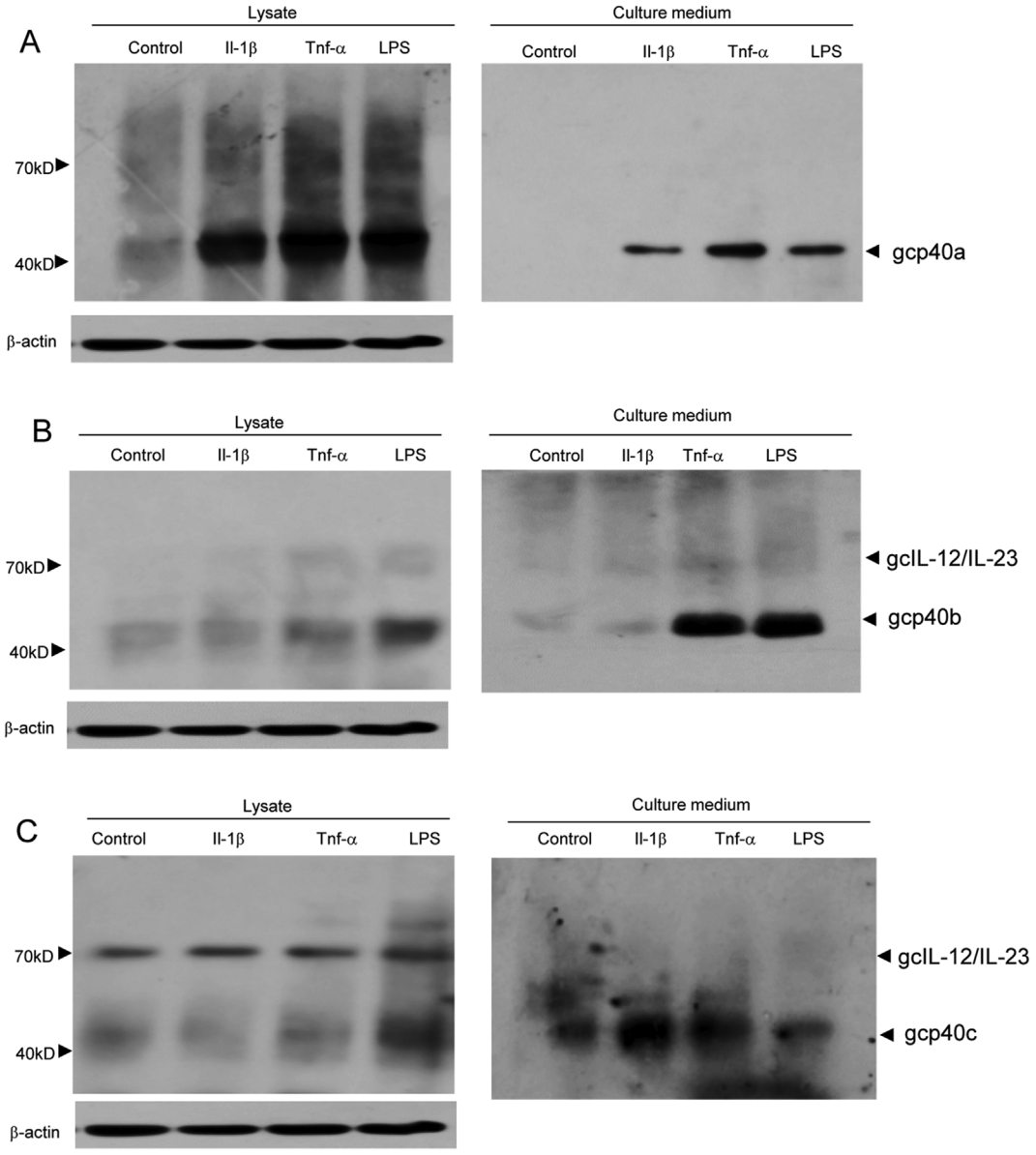

Fig. 2. Differential protein synthesis and secretion patterns of gcp40a, gcp40b and gcp40c in grass carp HKLs. The density of 1×10^7 cells were stimulated with/without LPS (30 μg/mL), rgcIl-1β (100 ng/mL) or rgcTnf-α (200 ng/mL) in 6-well cell culture plate (Corning). Twenty-four hours later, the cells were lysed and the culture medium were harvested and the protein concentrations of samples were quantified by BCA assay kit. All samples were separated on non-reducing SDS-PACE, and analyzed by Western blotting with three antibodies against p40a (A), p40b (B) and p40c (C), separately.

Kpn1 and Not1 (NEB, MA, USA), then the PCR fragments encoding gcp40a, gcp40b or gcp40c were ligated into the vector by T4 DNA ligase (Promega) to generate pPICZα A/gcp40a, pPICZα A/gcp40b or pPICZα A/gcp40c expression plasmid, respectively. The integrity of the inserted DNA was verified by sequencing. The expression plasmid was transformed into competent cells and positive single colony was selected and cultured. Then the plasmid was extracted by using a TIANGEN prep Mini Plasmid Kit (Tiangen, Beijing, China) and linearized by restriction endonucleases: *Sac*I (NEB) for p40a, p40c, and *Pme*I (NEB) for p40b. Subsequently, the linearized plasmid was transformed into *P. pastoris* X33 strain (Thermo Scientific) by electroporation according to the instruction of the manufacturer (Thermo Scientific). *P. pastoris* transformant was grown on YPDS plates (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol and 2% agar) containing 100 μg/mL of zeocin. The single colonies were picked and analyzed by PCR. The positive transformant was selected and used for producing recombinant proteins.

2.5. Expression and purification of rgcp40a, rgcp40b and rgcp40c

The positive single colony was picked into 5 mL BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% YNB, 4×10^{-5}% biotin and 1% glycerol) and grown at 29 °C with shaking at 260 rpm for approximately 16 h. The culture was inoculated in 200 mL of BMGY medium in a 2-L baffled flask and grown at 29 °C with shaking at 260 rpm until the $OD_{600\ nm}$ of the culture reached 2–6. The cells were harvested by centrifugation at 3000*g* for 5 min at room temperature. The supernatant was decanted and cell pellet was resuspended with 200 mL BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% YNB, 4×10^{-5}% biotin, and 1% methanol). Every 24 h, 100% methanol was added into the culture (final 1%) to induce the expression of recombinant protein for 48 h. Recombinant protein was purified from the culture medium by a His Trap affinity column (GE Healthcare, Lafayette, USA). The purified protein was desalted by a Superdex G25 prep grade column (GE Healthcare) and

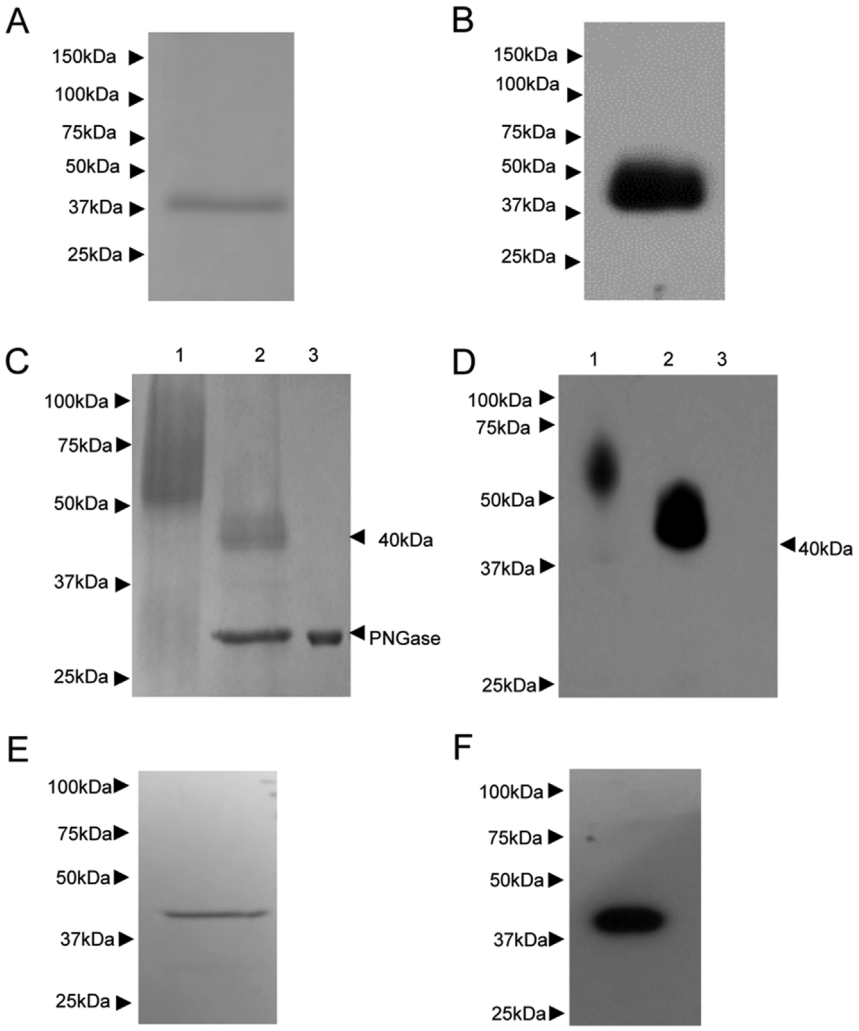

Fig. 3. Purification and validation of rgcp40a, rgcp40b and rgcp40c. SDS-PAGE analysis of rgcp40a (A), rgcp40b (C) and rgcp40c (E) after purification. Western blotting analysis of rgcp40a (B), rgcp40b (D) and rgcp40c (F) with *anti*-gcp40a pAb, *anti*-gcp40b pAb and *anti*-gcp40c pAb, respectively.

identified by SDS-PAGE and Western blotting. Finally, the purified protein was lyophilized and then stored at −80 °C for further use after its concentration was determined by a BCA assay kit (Beyotime Biotechnology, Shanghai, China).

2.6. Glycosylation analysis of rgcp40b

To verify glycosylation of rgcp40b, PNGase F (NEB) was used following the manufacturer's instruction. Briefly, 10 μg of rgcp40b was denatured in 10 × Glycoprotein Denaturing buffer (5% SDS and 0.4 M DTT) for 10 min in a boiling water bath. Then 2 μL of 10 × G7 Reaction buffer, 2 μL of 10% NP-40, 2 μL of PNGase F and distilled water were added into the denatured rgcp40b to give a final volume of 20 μL. The reaction mixture was incubated at 37 °C for 1 h. In control group, the enzyme was replaced by distilled water.

2.7. Validation of anti-gcp40 pAb specificity by transient transfection

Transient transfection was performed as described previously [25]. In brief, HEK293 cells (2×10^5 cells) were seeded in 35 mm dish with 1.5 mL DMEM/F12 medium (Thermo Scientific) before transfection and incubated at 37 °C overnight. Subsequently, the cells were transiently transfected with C-terminal His-tagged gcp40a/b/c (p40a/b/c-His) expression plasmids (constructed in our previous study [19]) or blank vector, pcDNA3.1/myc-His (−) by Lipofectamine 2000 (Thermo Scientific). Forty-eight hours after transfection, the cells were lysed and Western blotting assay was performed.

2.8. Western blotting (WB) assay

In protein sample preparation, the supernatant of cell culture of HKLs was collected and the cells were lysed in ice-cold RIPA lysis buffer (Beyotime) with EDTA-free Protease Inhibitor Cocktail Tablets (Roche). The protein samples were separated on a 10% SDS-PAGE or non-reducing SDS-PAGE, and electrophoretically transferred to a PVDF membrane (Millipore, Billerica, MA). After they were blocked in Tris-buffered saline (pH 7.4) containing Tween-20 (0.05%) (Sigma-Aldrich) with 8% (w/v) nonfat milk, the membranes were incubated in either 5% milk or 5% BSA with the antibodies specific for the proteins (*anti*-gcp40a pAb, 1:1000, *anti*-gcp40b pAb, 1:1000, *anti*-gcp40c pAb, 1:1000, Biogot Technology, Nanjing, China; mouse *anti*-β-actin mAb, 1:1000, Boster, Wuhan, China), separately, overnight at 4 °C. After that, the membranes were exposed to horseradish peroxidase-conjugated goat anti-rabbit/mouse secondary antibody (1:5000, ZSGB-BIO, Beijing, China) for 2 h at room temperature. Finally, the signals were detected using an ECL kit (Roche) according to the manufacturer's instruction.

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

Grass carp HKLs were maintained in RPMI-1640 medium. The cells

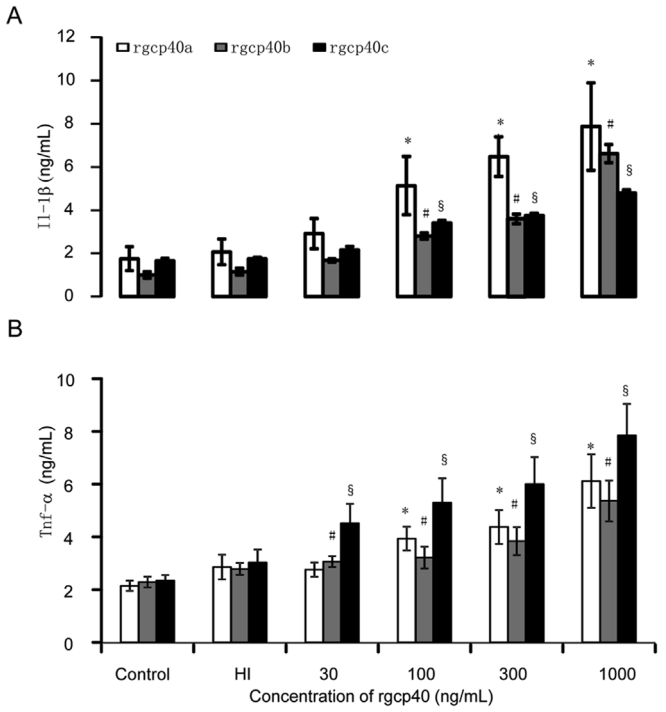

Fig. 4. Effects of rgcp40a, rgcp40b and rgcp40c on the release of gcIl-1β and gcTnf-α in grass carp HKLs. The cells were treated with rgcp40a, rgcp40b and rgcp40c (30–1000 ng/mL) or heat inactivated (HI) rgcp40a, rgcp40b and rgcp40c (1000 ng/mL) for 24 h, separately. The protein levels of gcIl-1β (A) and gcTnf-α (B) in medium were detected by ELISA as described in Materials and methods. Data are expressed as Mean ± SEM (N = 4). Data shown are representative of three independent experiments. *: $p < 0.05$ in comparison with control group of rgcp40a. #: $p < 0.05$ in comparison with control group of rgcp40b. §: $p < 0.05$ in comparison with control group of rgcp40c.

treated with culture medium, rgcp40a, rgcp40b or rgcp40c (30–1000 ng/mL) for 24 h at 28 °C under 5% CO_2. The culture medium was collected, centrifuged to remove precipitation and stored at −80 °C for later use. Two hundred nanograms per well of rgcIl-1β [26]/rgcTnf-α [27] were coated into 1 × 8 Stripwell high-binding plates (Costar, Cambridge, MA, USA) at 4 °C for 16 h. In the following day, coated wells were blocked with 5% nonfat milk plus 0.3% BSA in PBS for 2 h at room temperature. Subsequently, 100 μL of culture medium or the quantified recombinant protein and 100 μL of HRP-conjugated gcTnf-α (1:1000) pAb/gcIl-1β pAb (1:1000) were mixed and pre-incubated at room temperature for 2 h. The mixture was added into each well after these plates were washed with PBST (0.05% Tween-20 in PBS) for three times. After incubation for 2 h, 100 μL of the substrate buffer (1 mg/mL, 3, 3′, 5, 5′-Tetramethylbenzidine, TMD, Tiangen) was added into the wells and incubated at 37 °C for about 20min. The reaction was stopped by 2 M H_2SO_4 and the OD at 450 nm was measured with a Microplate Reader (Bio-Rad). The control groups were pre-coated with BSA following the same procedure as described above. All samples were run in quadruplicates. Absorbance reading was measured and the results for test samples were extrapolated from a standard curve for gcTnf-α/gcIl-1β inhibition.

2.10. Hematoxylin-eosin (HE) staining of grass carp intestine

The bacteria of *A. hydrophila* were cultured in 100 mL TSB medium (0.5% soya peptone, 1.5% tryptone, and 3% NaCl) at 28 °C with shaking at 180 rpm for 18 h. Subsequently, bacteria were collected and re-suspended in 10 mL of 10 mM PBS (pH 7.4). Finally, 1 mL of bacteria were diluted to different concentrations and spread on TSA plates (0.5% soya peptone, 1.5% tryptone, 3% NaCl, and 1.5% agar) to calculate the amount of *A. hydrophila*. Healthy grass carp with body weight about 60 g were maintained in lab for two weeks and then injected intraperitoneally (i.p.) with *A. hydrophila* (0.05 mL/fish; 2×10^8 CFU/mL in PBS) or with PBS (0.05 mL/fish). An hour later, the fish were injected with 5 μg of rgcp40a (0.05 mL), PBS (0.05 mL), 10 μg of *anti*-p40a pAb (0.05 mL) or isotype-matched rat IgG control (10 μg/0.05 mL) (ZSGB-BIO). After that, intestines of the fish were isolated at 1-day post-injection from the infection and control groups (N = 5), and then HE staining was performed. Briefly, tissue samples were fixed in 4% neutral buffered paraformaldehyde for 24 h and dehydrated in ascending concentrations of alcohol and cleaned in xylene. These tissues were embedded in paraffin and sectioned with a rotary microtome. The tissue slices were stained by using standard hematoxylin and eosin (H&E). The images were obtained and analyzed by using the BX51 system (OLYMPUS, Tokyo, Japan).

2.11. Statistical analysis

Statistical analysis was conducted by ANOVA followed by Fisher's least significance difference (LSD) using the SPSS Statistics 19.0 software (SPSS Inc., Chicago, USA). For comparison between two groups, Student's *t*-test was performed. Data were expressed as mean ± SEM for at least three independent experiments, and differences were considered as statistically significant at $p < 0.05$.

3. Results

3.1. Different inducibility of three p40 paralogue mRNA expression in response to inflammatory stimuli

To test the inductive expression profiles of *gcp40a, gcp40b* and *gcp40c in vitro*, grass carp HKLs were treated with culture medium, LPS, rgcIl-1β or rgcTnf-α for 1–12 h in 24-well plate, separately. RT-qPCR assay showed both rgcIl-1β and rgcTnf-α significantly induced *gcp40a* gene expression from 3 h to 12 h, while LPS stimulated *gcp40a* expression only at 3 h (Fig. 1A). Meanwhile, rgcIl-1β had no effect on *gcp40b* expression, whereas rgcTnf-α significantly increased *gcp40b* mRNA levels from 1 to 6 h and LPS dramatically induced its expression from 1 to 12 h with a 38-fold up-regulation at 12 h (Fig. 1B). For the inductive expression of *gcp40c*, rgcIl-1β and rgcTnf-α significantly enhanced its mRNA levels from 3 to 12 h and from 3 to 6 h, respectively, while LPS treatment led to a small but significant induction of *gcp40c* mRNA from 3 to 12 h (Fig. 1C).

3.2. Distinct protein synthesis and secretion profiles of three p40 paralogues in response to inflammatory stimuli

After the HKLs were treated with rgcIl-1β, rgcTnf-α or LPS for 24 h in 6-well plate, WB assay was performed under non-reducing conditions to detect the protein levels of gcp40a, gcp40b and gcp40c in cell lysates and cell culture medium by using *anti*-gcp40a pAb, *anti*-gcp40b pAb, and *anti*-gcp40c pAb, respectively. In this case, the specificity of these antibodies were validated by the overexpression of p40a/b/c in HEK293. Moreover, three antibodies specifically recognized their target proteins, separately and no cross-reaction among them was observed (Supplementary Fig. 1). As shown in Fig. 2A, rgcIl-1β, rgcTnf-α and LPS markedly up-regulated monomeric gcp40a (40 kDa) under non-reducing conditions in both cell lysates and cell culture medium. Similarly, rgcTnf-α and LPS but not rgcIl-1β significantly enhanced gcp40b levels as monomer (40 kDa) in cell lysates and particularly in culture medium (Fig. 2B). Notably, both gcp40a and gcp40b were mainly presented as monomer, with a very few of them in a heterodimeric form (70 kDa) (Fig. 2A and B). Interestingly, large amounts of the gcp40c heterodimer (70 kDa) were detected in cell lysates although three immune stimuli did not alter its protein levels (left panel, Fig. 2C). In cell lysates, small amounts of gcp40c monomers were found, and LPS but not rgcIl-1β or

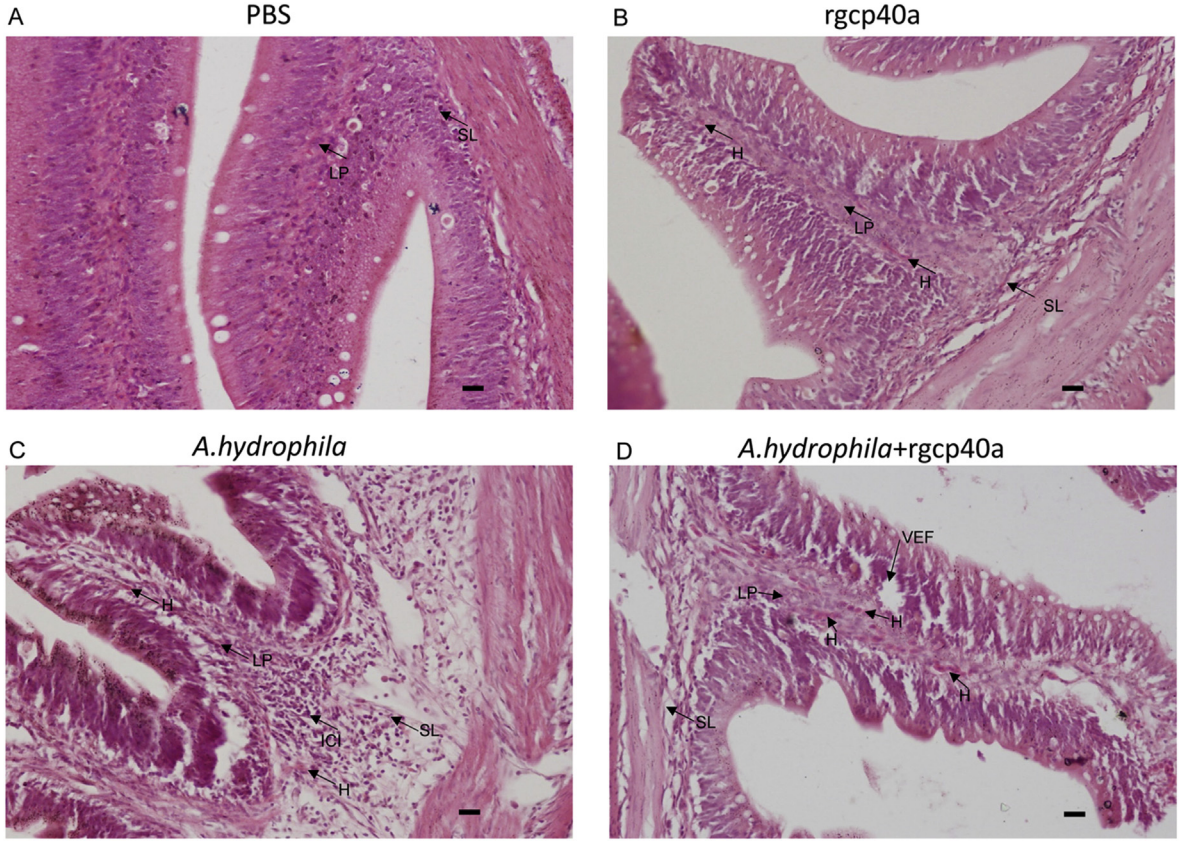

Fig. 5. The representative images of the histological changes in intestine at 1-day post-infection (magnification: × 400, scale bar: 50 μm). (A) Intestine of PBS-treated grass carp. (B) Intestine from rgcp40a-treated grass carp. (C) Grass carp intestine after *A. hydrophila* infection. (D) Intestine of bacteria combined with rgcp40a-challenged grass carp. Indicator arrows: H, Hemorrhage. ICI, Inflammatory cell infiltration. VEF, villus and epithelial cell fall off. LP: lamina propria. SL: submucosa layer.

rgcTnf-α could induce its production (left panel, Fig. 2C). In contrast, only gcp40c monomers were tested in culture supernatant and rgcIl-1β and rgcTnf-α but not LPS were effective in stimulating gcp40c monomer production (right panel, Fig. 2C).

3.3. Recombinant expression and purification of gcp40a, gcp40b and gcp40c

To understand the role of three p40 isoforms, rgcp40a, rgcp40b and rgcp40c were prepared by *Pichia pastoris* expression system and their purity was evaluated by SDS-PAGE (Fig. 3). Among them, the purified protein of rgcp40a and rgcp40c have a MW of approximately 40 kDa, which was corresponding to the predicted size of them (Fig. 3A, E). However, the rgcp40b protein has a molecular weight of approximately 50 kDa (Fig. 3C) that is bigger than the predicted size (40 kDa). While rgcp40b was treated with N-glycosidase F (PNGase F), an amidase that cleaves N-linked glycans from proteins, a band of about 40 kDa was detected (Fig. 3C and D).

3.4. Stimulatory effects of rgcp40 on pro-inflammatory cytokine secretion in grass carp HKLs

To examine the regulatory effect of rgcp40a, rgcp40b and rgcp40c on the secretion of some proinflammatory cytokines, grass carp HKLs were exposed to different concentrations of rgcp40a, rgcp40b and rgcp40c for 24 h, separately. Secretion of gcIl-1β and gcTnf-α in culture supernatant was measured by ELISA, showing that rgcp40a, rgcp40b and rgcp40c (100–1000 ng/mL) were effective in inducing these two cytokine's releases in a dose-dependent manner (Fig. 4A and B). In contrast, 1000 ng/mL of heat inactivated (HI) rgcp40a, rgcp40b and rgcp40c did not alter these two proteins' production (Fig. 4A and B).

3.5. In vivo inflammatory action of rgcp40a in grass carp

To examine the regulatory function of gcp40 *in vivo*, the rgcp40a was injected i.p. into grass carp with PBS or *A. hydrophila* pre-infection. HE staining showed that the intestines from control group displayed organizational structural integrity and no signs of hemorrhage and inflammation (Fig. 5A). However, rgcp40a alone might cause the villi sparse and congestion (Fig. 5B), while bacterial infection led to swelling in submucosa layer and serious hemorrhage and inflammatory cell infiltration in lamina propria and submucosa layer of intestine (Fig. 5C). Moreover, rgcp40a enhanced bacterial challenge-induced inflammatory symptoms in intestine, showing more serious hemorrhage in lamina propria and shedding of villus and epithelial cell. (Fig. 5D).

3.6. Rescue effect of anti-gcp40a pAb against A. hydrophila infection in vivo

The *anti*-gcp40a pAb was injected i.p. into *A. hydrophila* pre-infected grass carp, HE staining of the intestine showed a continuous thin submucous layer under muscular layer in control group (Fig. 6A). In parallel, the intestine from *A. hydrophila*-treated group developed severe histological changes, characterized by the inflammatory cell infiltration in submucous layer, the increase of submucous layer thickness and hemorrhage in the lumen of villus (Fig. 6B). Notably, these symptoms were remarkably relieved by *anti*-gcp40a pAb administration, showing an extremely mild inflammatory hyperemia in submucous layer compared with *A. hydrophila*-challenged group (Fig. 6C).

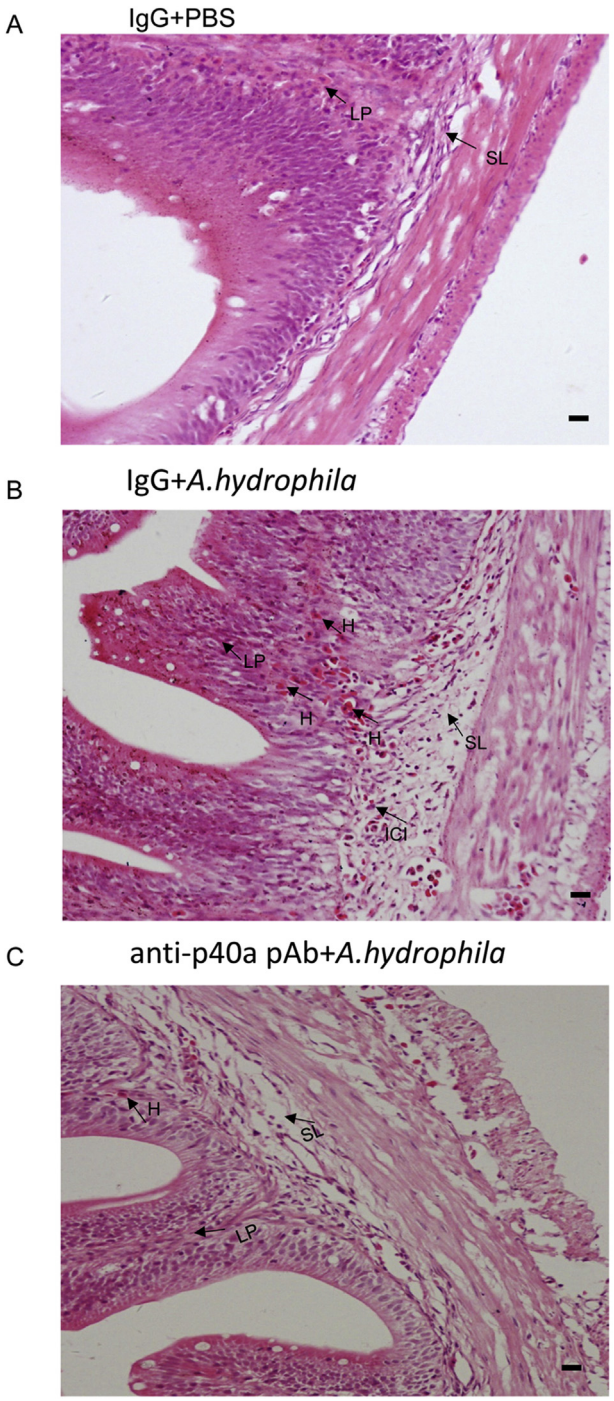

Fig. 6. Effect of *anti*-p40a pAb on morphological changes of fish intestines with *A. hydrophila* challenge. (magnification: × 400, scale bar: 50 μm). (A) Intestine of PBS together with IgG-treated grass carp. (B) Intestine from *A. hydrophila* combined with IgG-treated grass carp. (C) Intestine from *A. hydrophila* together with *anti*-p40a pAb-treated grass carp. H, Hemorrhage. ICI, Inflammatory cell infiltration. LP: lamina propria. SL: submucosa layer.

4. Discussion

Two or three of p40 genes present in teleost that likely arose from the teleost-wide WGD events [17–19,21]. WGD is an especially important event that may provide teleost with diversification potential and evolutionary and environmental adaptation [28]. If this is the case, the information about expression profiles and functions of three p40s may enlarge our knowledge of fish immunity. In this context, increasing evidence reveals the inductive expression of p40 paralogue genes in response to different immune challenges in various fish species [19–22]. However, these expression patterns at transcriptional level cannot clarify the possibility and preference of these paralogues being a heterodimeric subunit for IL-12/IL-23 and/or an independent subunit. To address this issue, it is required to detect the protein synthesis and secretion of fish p40 isoforms. In this study, rgcIl-1β, rgcTnf-α and LPS possessed the ability to induce *gcp40a, gcp40b* and *gcp40c* mRNA expression in grass carp HKLs at different time points (Fig. 1), and were used as stimulators to investigate the intracellular and extracellular protein expression of three isoforms in HKLs by WB analysis under non-reducing conditions. As shown in Fig. 2, 24-h treatment of three immune stimuli triggered distinct protein synthesis and release patterns of three isoforms. Notably, possible heterodimeric Il-12 or Il-23 with the corresponding size (70 kDa) were mainly detected in cell lysates, but their amounts were much lesser than that of free gcp40a and gcp40b except for gcp40c, suggesting that gcp40c is likely the dominant subunit for heterodimeric assembly which is consistent with the hypothesis that the p35/p40c is the predominate form of IL-12 in rainbow trout [21]. It is interesting that three isoforms could be secreted in response to different immune stimuli after 24 h stimulation, suggesting that the response of fish p40 isoforms secretion to rgcIl-1β, rgcTnf-α and LPS differed from each other. These secretion patterns might indicate distinct roles of three isoforms in immune responses. In particular, gcp40a, gcp40b and gcp40c mainly existed as a monomer in large amounts in the supernatant fluids from cell culture, which was agreement with the findings in mammals in which monocytes secrete 5–500-fold excess of free p40 relative to IL-12 [2]. These findings highlighted the large-scale rapid release of three p40 monomer in response to inflammatory signals in fish, prompting us to address their function and the biological significance.

Accordingly, rgcp40a, rgcp40b and rgcp40c were prepared by using *Pichia pastoris* expression system and their activities in mediating protein release of grass carp Il-1β and Tnf-α in HKLs were revealed (Fig. 3). In rock bream and orange-spotted grouper, the recombinant p40 proteins have the ability to augment the mRNA expression of several inflammatory cytokines in kidney [29] and peripheral blood lymphocytes (PBLs) [30]. Our data provided evidence at protein secretion level for the pro-inflammatory property of free p40 in fish, thereby supporting its role in fish inflammatory responses.

In line with this hypothesis, we investigated the *in vivo* effect of free p40 in grass carp challenged by *A. hydrophila* which is the most common pathogen in water environments and causes great economic loss in aquaculture [31,32]. Given that rgcp40a, rgcp40b and rgcp40c showed similar functions as described above, here rgcp40a was chosen to define the *in vivo* function of free p40. In this context, from the morphology observation after HE staining, rgcp40a alone displayed a pathogenic effect in intestine but not in other tissues, such as liver, gill and spleen (data not shown). As expected, rgcp40a also enhanced *A. hydrophila*-induced intestinal inflammation in grass carp (Fig. 5). Furthermore, gcp40a neutralization by *anti*-gcp40a pAb was effective in ameliorating *A. hydrophila* infection-triggered damage and inflammation symptoms (Fig. 6). In agreement with this, the *anti*-p40 therapies are widely used in autoimmune diseases, such as psoriasis [33,34], giant-cell arteritis lesions [35] and intestinal inflammation [36].

In summary, our study uncovered that multiple p40 isoforms of grass carp might to be independently released. Intriguingly, the secretion pattern of three isoforms were varied when they subjected to the discrepancy of immune stimuli, indicating their possible diverse roles in regulating fish immune responses. In accordance with these functions, a pathogenic role of p40 was delineated in fish intestinal inflammation. Together these results strongly suggest the potential of teleost p40 subunits as independent pro-inflammatory regulators, thereby highlighting a new protective and pathogenic immune pathway in fish.

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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.2019.04.015.

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