Fish and Shellfish Immunology 89 (2019) 301–308

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Molecular and functional characterization of the indoleamine 2,3-dioxygenase in grass carp (*Ctenopharyngodon idella*)

Zheng-Wei Cui[a,d], Xiang-Yang Zhang[a,d], Xu-Jie Zhang[b], Nan Wu[a], Long-Feng Lu[a], Shun Li[a], Dan-Dan Chen[a], Yong-An Zhang[a,b,c,*]

[a] Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China
[b] State Key Laboratory of Agricultural Microbiology, College of Fisheries, Huazhong Agricultural University, Wuhan, China
[c] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China
[d] University of Chinese Academy of Sciences, Beijing, China

ARTICLE INFO

Keywords:
Indoleamine 2,3-dioxygenase (IDO)
Tryptophan
TGF-β1
Immunoregulation
Grass carp

ABSTRACT

Indoleamine 2,3-dioxygenase (IDO) is a kind of dioxygenase that can catalyze the degradation of levo-tryptophan (L-Trp) and plays key roles in immune tolerance. In this study, the IDO gene was cloned and functionally characterized from grass carp (gcIDO). The results showed that gcIDO overexpressed in GCO cells could catalyze the degradation of L-Trp through the L-Trp - kynurenine pathway, and this activity could be promoted by δ-aminolevulinic acid (ALA) while inhibited by levo-1-methyl tryptophan (L-1MT). Moreover, gcIDO was constitutively expressed in various tissues, and its expression could be significantly up-regulated by LPS and Poly (I:C) in peripheral blood leukocytes (PBLs). Furthermore, recombinant TGF-β1 of grass carp could up-regulate the expression of IDO, TGF-β1, CD25, and Foxp3 in PBLs, indicating that the TGF-β1/IDO pathway is present in fish. In the soybean meal induced enteritis (SBMIE) model, the expression of gcIDO in the intestine was up-regulated significantly, demonstrating that gcIDO may play an immunoregulatory role in SBMIE. Taken together, these data suggest that the IDO plays multiple roles in the immunity of fish.

1. Introduction

Levo-tryptophan (L-Trp), the least abundant essential amino acid, is important for protein synthesis and roles as a precursor of niacin, serotonin and other metabolites [1]. Indoleamine 2,3-dioxygenase (IDO) is a kind of cytosolic heme-containing dioxygenase that catalyze the first and also the rate-limiting steps in L-Trp catabolism via the kynurenine pathway, which is the major pathway of L-Trp catabolism in mammals [2].

In addition to its recognized function as an enzyme, IDO acts as an intracellular signal transducer of transforming growth factor-β1 (TGF-β1) [3]. This activity is mediated by its two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that are located at the sites distant from the catalytic domain [3,4]. In the TGF-β1/IDO pathway, TGF-β1 generates a positive feedback loop for sustained production of TGF-β1 and IDO through a phosphatidylinositol-3-OH kinase (PI(3)K)-dependent mechanism [3,5].

The IDO genes are constitutively expressed in various tissues of mammals [6–8], and their expression can be induced by LPS and Poly (I:C) [9–12]. The induction of IDO and the subsequent depletion of L-Trp in the tissue microenvironment exert a variety of physiological effects such as inhibition of pathogen growth, reduction in T-cell proliferation and induction of T cell apoptosis [13–16]. In mammals, the expression of IDO in the homeostatic intestine is low and mostly occurs in the cells of the lamina propria [17], however, the expression increases in inflammatory states [17–22], indicating that IDO plays an immunoregulatory role in intestinal inflammations.

Thus far, two IDO genes (IDO1 and IDO2) have been found in mammals, which are located tandemly on the same chromosome with conserved genomic structures [6,8,23], however, there is only one IDO homologue in lower vertebrates. Studies have proved that IDO1 arose from IDO2 by gene duplication that occurred before the divergence of marsupials and placentals [23,24]. In teleost, the IDO gene has been discovered in zebrafish (*Danio rerio*) [23] and rainbow trout (*Oncorhynchus mykiss*) [25]. Studies have revealed that zebrafish IDO can degrade L-Trp [23,26], and the expression of rainbow trout IDO can be induced by recombinant IFN-γ *in vitro* [25]. However, whether the TGF-β1/IDO pathway is present in fish and whether the IDO participates in fish intestinal inflammations are still unknown. In aquatic feeds, fishmeal has been replaced partly with plant protein sources, such as

* Corresponding author. Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.
E-mail address: yzhang@ihb.ac.cn (Y.-A. Zhang).

https://doi.org/10.1016/j.fsi.2019.04.005
Received 9 December 2018; Received in revised form 20 March 2019; Accepted 2 April 2019
Available online 06 April 2019
1050-4648/ © 2019 Elsevier Ltd. All rights reserved.

soybean meal (SBM), which often causes enteritis. To help understand the mechanism of enteritis in herbivorous fish and help prevent and treat enteritis, research on the roles of IDO in SBM induced enteritis (SBMIE) is necessary.

In the present study, the IDO gene was identified and characterized from grass carp (*Ctenopharyngodon idella*) (gcIDO). The enzymatic activity of gcIDO and the regulatory effects of LPS, Poly (I:C) and grass carp TGF-β1 (gcTGF-β1) on the expression of gcIDO were tested. Furthermore, the involvement of gcIDO in SBMIE was examined.

2. Materials and methods

2.1. Experimental fish and cell line

Healthy grass carp (weighing 200 ± 20 g) purchased from Xiantao Hatchery (Hubei, China) were maintained and acclimated to recirculating tanks (1000 L, 28 ± 1 °C) containing filtered and oxygenated water for at least two weeks before experiments. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Institute of Hydrobiology, Chinese Academy of Sciences.

Grass carp ovary (GCO) cells were grown at 28 °C in 5% CO_2 in medium 199 (Invitrogen) supplemented with 10% (w/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.2. Cloning of the cDNA of gcIDO

Total RNA was extracted from the tissues or cells by using the Trizol reagent (Invitrogen) and the cDNA was synthesized by using the PrimeScript TM RT reagent Kit with gDNA Eraser (Takara) to eliminate the contaminated genomic DNA. The cDNA fragment of gcIDO was obtained by PCR amplification with primers IDO-pF and IDO-pR (Table 1). The PCR products were cloned into the pMD19-T vector (Takara) for sequencing. Based on the obtained cDNA fragment of gcIDO, 5′-RACE was performed using the reverse primers 5′-R1 and 5′-R2, while the 3′-RACE was performed using the forward primers 3′-F1 and 3′-F2. All PCRs were performed in 25 μl mixtures containing 2.5 μl of 10 × PCR buffer, 1.5 mM $MgCl_2$, 1 mM dNTPs, 0.4 mM primers, 1 μl of cDNA template, and 1 U of ExTaq DNA polymerase (Takara) on a thermal cycler (Bio-Rad). The cycling conditions were as follows: an initial denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min, and an additional extension step at 72 °C for 5 min. The PCR products were sequenced as described above.

The cDNA and deduced amino acid sequences of gcIDO were analyzed using the BLAST program from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov), and the ExPASy Molecular Biology server (http://us.expasy.org). Multiple sequence alignment was performed using the CLUSTAL X version 2.1 with default parameters according to the IMGI (the International ImMunoGeneTics information system) scientific chart and then manually adjusted. Phylogenetic tree was constructed with the MEGA 7.0 package using the neighbor-joining algorithm.

2.3. Construction of expression plasmids

The open reading frame (ORF) of gcIDO was cloned into the *Kpn* I and *Not* I sites of the pCMV-HA vector for over-expression in GCO cells and cloned into the *Nhe* I and *Xho* I sites of the pET23b vector for over-expression in *Escherichia coli* BL21 (DE3) cells (Stratagene). The sequence of the mature peptide of gcTGF-β1 (accession number EU099588) [27] was cloned into the *Eco*R I and *Xho* I sites of the pGEX-4T-1 vector for over-expression in *E. coli* BL21 (DE3) cells.

2.4. Analysis of the enzymatic activity of gcIDO in GCO cells

GCO cells were seeded in a 6-well microplate and transfected with plasmid pCMV-HA-gcIDO by using the X-treme GENE HP DNA Transfection Reagent (Roche), according to the manufacturer's instructions. After incubation for 20 h, the medium was replaced with 2 ml of phenol red-free medium 199 (Invitrogen) supplemented with 10% (w/v) FBS and different concentrations of L-Trp (0, 200, 400, 800, and 1600 μM, respectively). After incubation for 2 h, the medium was replaced with 2 ml of fresh medium and incubated for another 2 h. Then the medium was collected for measuring the concentration of kynurenine and the cells were collected for Western blot. After incubated at 65 °C for 15 min to hydrolyze *N*-formylkynurenine to kynurenine, the medium was centrifuged at 16,000 *g* for 10 min, then 125 μl of the clear supernatant was mixed with an equal volume of 2% (w/v) *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) in acetic acid in a 96-well microplate. The absorbance of each well was measured in a precision microplate reader (BioTek) at 480 nm to measure the concentration of kynurenine.

To determine the role of heme in the enzymatic activity of gcIDO,

Table 1
Primers used in this study.

Name	Sequence (5′→3′)	Application
IDO-pF	TATGTAGATCCATCCATATTCTAC	cDNA cloning
IDO-pR	TCCAGTACCTCTTTCCTCCAG	cDNA cloning
5′-R1	TCATCCGCTTGCTGGAGCAC	cDNA cloning
5′-R2	CCTAGCAGCTCATCAAAGGAG	cDNA cloning
3′-F1	GTGCTCCAGCAAGCGGATGA	cDNA cloning
3′-F2	ACAGCTCCGTGCAAACAAGCA	cDNA cloning
pET23b-IDO-F	CTAGCTAGCATGGGCACTGAAGTGAGAAATA	Prokaryotic expression
pET23b -IDO-R	CCGCTCGAGTTCTATTGTGAGCTCAGATGCA	Prokaryotic expression
pGEX-4T-1-TGF-β1-F	CCGGAATTC AAACGACAAACTGACGGAGTTT	Prokaryotic expression
pGEX-4T-1-TGF-β1-R	CCGCTCGAGTCAGGAACACTTGCAGTTCTTC	Prokaryotic expression
pCMV-HA-IDO-F	CGGGGTACCATGGGCACTGAAGTGAGAAAT	Eukaryotic expression
pCMV-HA-IDO-R	AAATATGCGGCCGCTCATTCTATTGTGAGCTCAGATG	Eukaryotic expression
IDO-QF	CGTGTACGAGGGAGTTCAGG	Real-time PCR
IDO-QR	CCGCTGTCACCTTTATGTTGG	Real-time PCR
β-actin-QF	AGCCATCCTTCTTGGGTATG	Real-time PCR
β-actin-QR	GGTGGGGCGATGATCTTGAT	Real-time PCR
TGF-β1-QF	CCACTGTAGAACTAAACCAGGAG	Real-time PCR
TGF-β1-QR	CTGTGATGTTGAACCATATGTGC	Real-time PCR
Foxp3-QF	GCTACCAAGGTATCTGTTGCTA	Real-time PCR
Foxp3-QR	CATGAACACCACATCCAGTAAC	Real-time PCR
CD25-QF	CGTGAGGAAAGCAGGAACATC	Real-time PCR
CD25-QR	GGCTTTTTGGGATCAGGTATGC	Real-time PCR

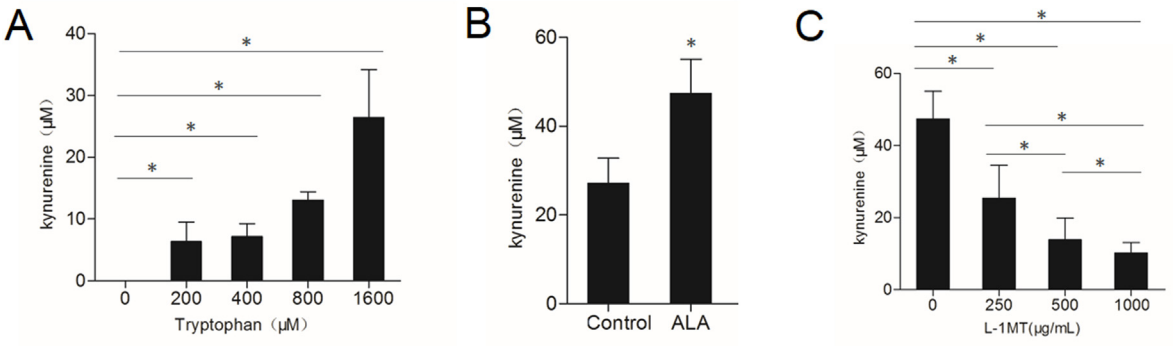

Fig. 1. Enzymatic activity of gcIDO in GCO cells, and the effect of ALA and L-1MT on the enzymatic activity of gcIDO. GCO cells were transfected with plasmid pCMV-HA-gcIDO, incubated for 20 h, then the medium was replaced with phenol red-free medium 199 supplemented with different concentrations of L-Trp. After incubation for 2 h, the medium was replaced with 2 ml of fresh medium and incubated for another 2 h. Then the concentration of kynurenine in the medium was measured using Ehrlich's reagent. (A) Enzymatic activity of gcIDO in GCO cells. (B) The effect of ALA on the enzymatic activity of gcIDO. (C) The effect of L-1MT on the enzymatic activity of gcIDO. The value represents the mean ± SEM of three replicates. An asterisk indicates a statistically significant difference ($p < 0.05$).

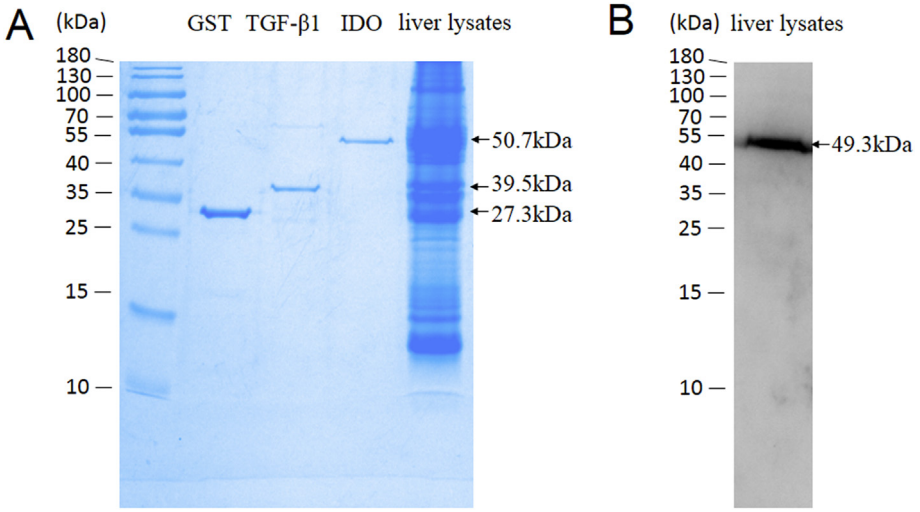

Fig. 2. Detection of the purified recombinant GST, gcTGF-β1, and gcIDO and native gcIDO. (A) SDS-PAGE analysis of the recombinant GST, gcTGF-β1, and gcIDO. (B) Western blot analysis of the native gcIDO in liver by rabbit *anti*-gcIDO pAb.

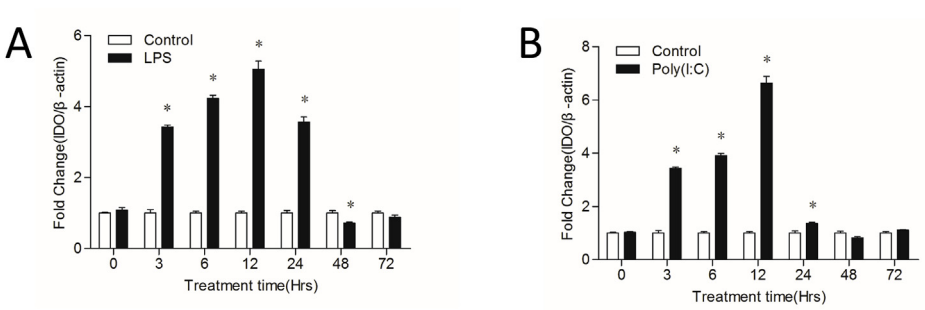

Fig. 3. Induced expression of gcIDO in grass carp PBLs by LPS and Poly (I:C). PBLs (1×10^6 cells/ml) were seeded in a 24-well plate at a cell density of 1×10^6 cells/well and incubated at 28 °C in 5% CO_2. After 24 h, the cells were treated by 10 μg/ml LPS and 10 μg/ml poly (I:C), respectively. The cells treated by 10 μl PBS were used as the control. Treated cells were collected at 0, 3, 6, 12, 24, 48, and 72 h for qPCR. (A) Induced expression of gcIDO in PBLs treated by LPS. (B) Induced expression of gcIDO in PBLs treated by Poly (I:C). The value represents the mean ± SEM of three individual fish. An asterisk indicates a statistically significant difference ($p < 0.05$).

the natural biosynthesized δ-aminolevulinic acid (ALA), precursor of heme, was added to the cells transfected with pCMV-HA-gcIDO for 6 h to a final concentration of 0.5 mM. PBS was added to the control cells. The concentration of kynurenine was measured as above described.

To test the inhibition effect of levo-1-methyl tryptophan (L-1MT) to the enzymatic activity of gcIDO, L-Trp and L-1MT were added to the cells transfected with pCMV-HA-gcIDO. The final concentration of L-Trp was 1600 μM, while that of L-1MT was 0, 250, 500, and 1000 μg/ml, respectively. The concentration of kynurenine was measured as above described.

2.5. Purification of recombinant proteins

The expression plasmids were transformed into *E. coli* BL21 (DE3) cells. The expression of recombinant proteins was induced by isopropyl β- D-thiogalactopyranoside (IPTG) and purified from the supernatants of cell lysates under native conditions. The His-tagged recombinant gcIDO was bound to the Ni-NTA Superflow resin (Qiagen) by rocking at 4 °C overnight. The resin was in turn washed with wash buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), wash buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 40 mM imidazole, pH 8.0),

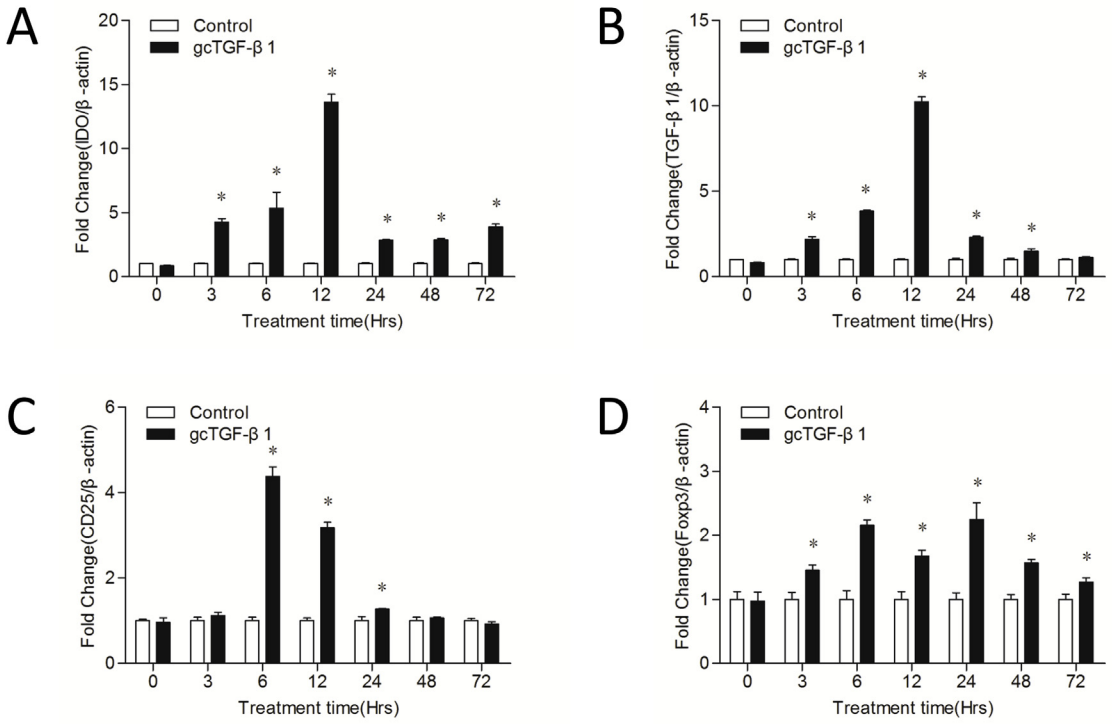

Fig. 4. Induced expression of gcIDO, TGF-β1, CD25 and Foxp3 in grass carp PBLs by gcTGF-β1. PBLs (1×10^6 cells/ml) were seeded in a 24-well plate at a cell density of 1×10^6 cells/well and incubated at 28 °C in 5% CO_2. After 24 h, the cells were treated by 6.4 μg/ml gcTGF-β1. The cells treated with 6.4 μg/ml GST were used as the control. Treated cells were collected at 0, 3, 6, 12, 24, 48, and 72 h for qPCR. (A) Induced expression of gcIDO in PBLs treated by gcTGF-β1. (B) Induced expression of TGF-β1 in PBLs treated by gcTGF-β1. (C) Induced expression of CD25 in PBLs treated by gcTGF-β1. (D) Induced expression of Foxp3 in PBLs treated by gcTGF-β1. The value represents the mean ± SEM of three individual fish. An asterisk indicates a statistically significant difference ($p < 0.05$).

and wash buffer 3 (50 mM NaH_2PO_4, 300 mM NaCl, 60 mM imidazole, pH 8.0). The bound His-gcIDO was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The GST and GST-tagged recombinant gcTGF-β1 was purified by using the Glutathione-Sepharose 4 Fast Flow resin (GE Healthcare) according to the manufacturer's instructions. The purified proteins were further dialysed in PBS.

The purity and concentration of the purified proteins were determined by SDS-PAGE and BCA Protein Assay Kit (Thermo Scientific), respectively. The GST and GST-tagged recombinant gcTGF-β1 were used for cell stimulation, while the His-tagged recombinant gcIDO was applied to immunize white rabbits to raise antisera. The polyclonal antibody (pAb) against gcIDO was purified using Protein G Column (GE Healthcare) from the antisera. Briefly, 5 ml antisera diluted with 5 ml PBS-EDTA (20 mM EDTA, pH 7.4), filtered with 0.2 μm filter, loaded onto Protein G Column, and washed with 30 ml PBS-EDTA. Then the bound pAb was eluted with 0.1 M glycine (pH 2.5) and desalted on the equilibrated PD-10 column (GE Healthcare) with PBS immediately. The pAb was stored at −20 °C.

2.6. Expression patterns of gcIDO

To determine the expression levels of gcIDO in different tissues, total RNA was isolated from the brain, heart, head kidney, trunk kidney, gill, liver, spleen, intestine, skin, muscle, and blood of three healthy grass carp. The cDNA was obtained by reverse transcription as described above, and real-time quantitative PCR (qPCR) was performed using the specific primers IDO-QF and IDO-QR (Table 1) in a CFX real-time PCR detection system (Bio-Rad). The reaction was conducted as follows: 2 μl cDNA template, 5 μl SsoAdvanced™ SYBR Green Supermix (Bio-Rad), and 0.25 μl of each forward and reverse primer (10 μM) in 10 μl reaction volume. The amplification condition was as follows: 95 °C for 3 min, followed by 45 cycles of 95 °C for 20 s, 60 °C for 20 s, and then 72 °C for 20 s. Three independent qPCR experiments were performed. The expression level of gcIDO gene in tissues was determined by the cycle threshold (Ct) method and normalized against the internal control β-actin using the $2^{-\Delta Ct}$ method.

2.7. Stimulation of grass carp PBLs with LPS, poly (I:C) or recombinant gcTGF-β1

Peripheral blood leukocytes (PBLs) were isolated from grass carp as described previously [28]. Briefly, peripheral blood was diluted (1/5) with RPMI-1640 medium (Invitrogen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 U/ml heparin (Invitrogen). The cell suspensions were carefully layered onto a 51/34% discontinuous Percoll (GE Healthcare) density gradient and centrifuged, with zero accelerate and brake, at 400 *g* for 30 min. The PBLs lying at the interfaces were collected, and then washed twice with RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 U/ml heparin. The PBLs were resuspended in RPM-1640 medium supplemented with 10% (w/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Then, 1×10^6 cells/1 ml/well were seeded in a 24-well plate and incubated at 28 °C in 5% CO_2. After 24 h, the cells were treated by 10 μg/ml LPS (from *E. coli* 0111:B4; Sigma-Aldrich), 10 μg/ml poly (I:C) (Sigma-Aldrich), and 6.4 μg/ml recombinant gcTGF-β1, respectively. Treated cells were collected at 0, 3, 6, 12, 24, 48 and 72 h for qPCR. Fold change of gene expression was calculated by comparing the stimulated group with the control group (defined as 1) using the $2^{-\Delta\Delta Ct}$ method [29].

2.8. Western blot

The cell and tissue samples were lysed in 200 μl of

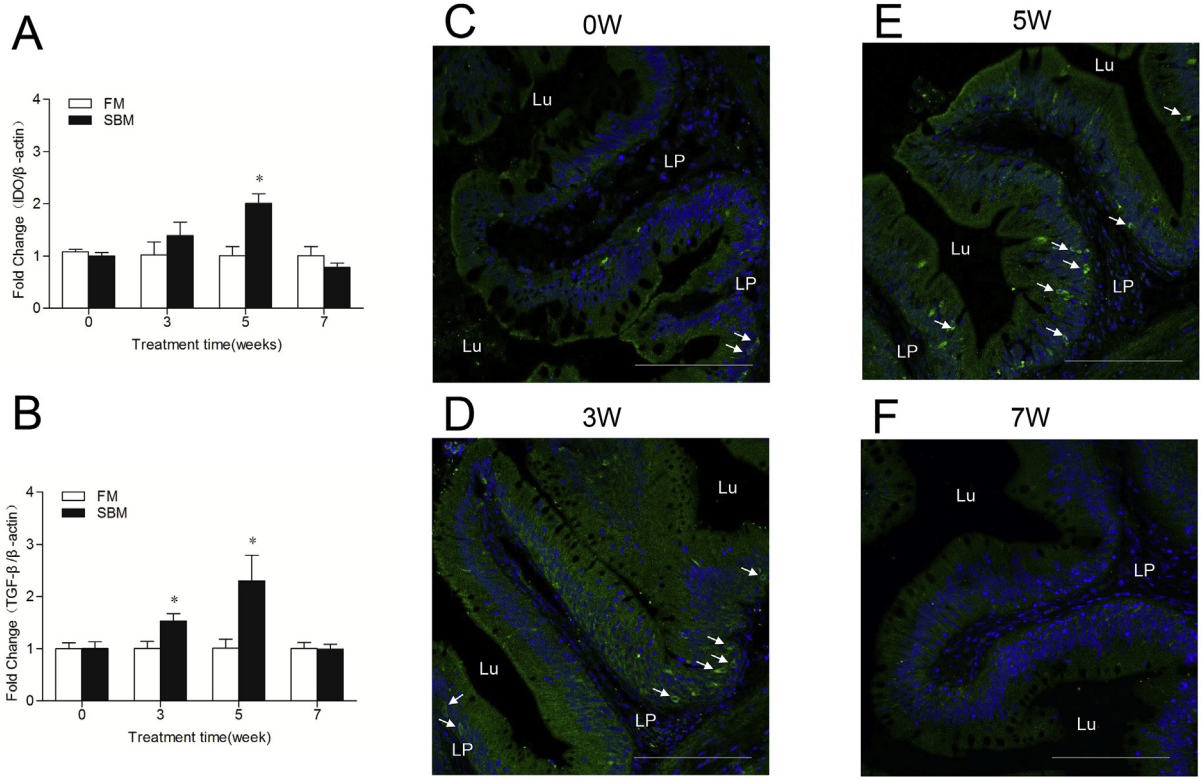

Fig. 5. Expression of gcIDO and TGF-β1 in the intestine of grass carp SBMIE. (A) The mRNA expression level of gcIDO in the intestine of grass carp SBMIE. (B) The mRNA expression level of TGF-β1 in the intestine of grass carp SBMIE. The value represents the mean ± SEM of six individual fish. An asterisk indicates a statistically significant difference ($p < 0.05$). (C) Immunofluorescence staining of gcIDO in 0 w intestine of grass carp SBMIE. (D) Immunofluorescence staining of gcIDO in 3 w intestine of grass carp SBMIE. (E) Immunofluorescence staining of gcIDO in 5 w intestine of grass carp SBMIE. (F) Immunofluorescence staining of gcIDO in 7 w intestine of grass carp SBMIE. Green: IDO; blue: Nuclei; LP: lamina propria; Lu: gut lumen. White arrows point to cells expressing gcIDO. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.).

radioimmunoprecipitation assay (RIPA) lysis buffer [1% NP-40, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.25% sodium deoxycholate, pH 7.5] containing protease inhibitor cocktail (Sigma-Aldrich) at 4 °C for 60 min on a rocker platform. The samples were centrifuged at 12,000 g for 15 min at 4 °C, and the supernatant (160 μl) was mixed with 5 × SDS sample buffer (40 μl), heated at 100 °C for 10 min, and stored at -80 °C for further use.

The same amount of protein samples was separated on SDS-PAGE and transferred to PVDF membrane (Bio-Rad). The membrane was blocked in TBST buffer (25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) containing 5% nonfat dry milk for 1 h at room temperature, then incubated with the primary antibody (Ab) at 4 °C overnight. After three washes with TBST, the membrane was incubated with the secondary Ab for 1 h at room temperature. After additional three washes with TBST, the membrane was stained with Immobilon TM Western Chemiluminescent HRP Substrate (Millipore) and examined using an ImageQuant LAS 4000 system (GE Healthcare). The Abs were diluted as follows: *anti*-gcIDO at 1:1000 (1 μg/ml), *anti*-β-actin (Cell Signaling Technology) at 1:1000, HRP-conjugated anti-rabbit IgG (Thermo Scientific) at 1:5000.

2.9. Experimental model of grass carp SBMIE

The experimental model of grass carp SBMIE was built as previously described [30]. Briefly, two fish groups were fed with normal diet for two weeks, then the control group was continued to be fed with normal diet, while the experimental group fed with the diet containing 40% soybean meal. Fish were sampled at 0, 3, 5 and 7 w.

2.10. Immunofluorescence staining

Immunofluorescence staining was performed as previously described [31] to analyze the expression of gcIDO in intestine. Briefly, cryosections of intestine were incubated with rabbit *anti*-gcIDO pAb (1 μg/ml), followed by FITC-conjugated goat anti-rabbit IgG Ab (diluted at 1:200, Jackson). After staining with 1 μg/ml 4′, 6′-diamidino-2-phenylindole (DAPI, Beyotime), the images were acquired using a confocal microscope (Leica).

2.11. Statistical analysis

The statistic p value was calculated by one-way ANOVA with a Dunnett post hoc test (SPSS Statistics, version 19, IBM). A p value < 0.05 was considered statistically significant.

3. Results

3.1. cDNA sequence of gcIDO

The full-length cDNA of gcIDO is comprised of 1787 bp, which includes a 78 bp 5′ untranslated region (UTR), a 1317 bp open reading frame (ORF), and a 392 bp 3′ UTR (Fig. S1). The 3' UTR includes a putative polyadenylation consensus sequence (AATAAA), but lacks RNA instable sequence (ATTTA).

3.2. Phylogenetic analysis of the gcIDO

The gcIDO is predicted to encode a 49 kDa protein, which consists of 438 amino acid residues, with an isoelectric point (pI) of 6.2. The amino

acid sequence of gcIDO showed 78.5% and 63.9% identity to its homology in zebrafish and rainbow trout, respectively (date not shown). Multiple sequence alignment showed that gcIDO possesses two conserved ITIMs [(I/V/L/S)-X-Y-X-X-(L/V), X denotes any amino acid] and two conserved key residues Asp_{274} and His_{346} that coordinate the heme group with the enzyme (Fig. S2A). The phylogenetic tree revealed that all fish IDO form a subcluster, which is associated with amphibian IDO, bird IDO, and mammalian IDO2 (Fig. S2B).

3.3. Expression patterns of gcIDO in tissues

The mRNA expression of gcIDO was examined in eleven tissues by qPCR. As shown in Fig. S3, gcIDO transcripts could be detected in all selected tissues, with the highest expression in liver and trunk kidney, followed by intestine, muscle, and spleen.

3.4. Enzymatic activity of gcIDO in GCO cells

The enzymatic activity of gcIDO was detected in GCO cells. The results indicated that gcIDO could catalyze the degradation of L-Trp via the kynurenine pathway, and the concentration of kynurenine increased with the concentration of L-Trp (Fig. 1A). When ALA was added to the medium of GCO cells transfected with gcIDO for 6 h, the kynurenine concentration in medium was nearly threefold higher than that of the control without ALA (Fig. 1B). Meanwhile, the enzymatic activity of gcIDO could be inhibited by L-1MT in a concentration dependent manner. (Fig. 1C).

3.5. Detection of purified GST, gcTGF-β1 and gcIDO proteins and rabbit anti-gcIDO Ab

The purified recombinant GST, gcTGF-β1 and gcIDO were detected by SDS-PAGE (Fig. 2A), and the results showed that they were pure enough to be used in the subsequent experiments. The rabbit *anti*-gcIDO pAb could be used to detect gcIDO in liver lysates by Western blotting specifically (Fig. 2B).

3.6. Induced expression of gcIDO by LPS or Poly (I:C) in grass carp PBLs

To examine whether gcIDO is involved in immune response, its expression levels were evaluated by qPCR in PBLs stimulated with LPS or Poly (I:C). As shown in Fig. 3A, compared with the controls, the expression of gcIDO was up-regulated by LPS from 3 to 24 h, with the maximal increase occurred at 12 h. However, the expression of gcIDO was down-regulated at 48 h. Like LPS, Poly (I:C) could also induce the expression of gcIDO (Fig. 3B), with the maximal increase occurred at 12 h.

3.7. Induced expression of gcIDO by gcTGF-β1 in grass carp PBLs

The effect of gcTGF-β1 on the expression of gcIDO was determined by qPCR in grass carp PBLs. When compared with the controls, the expression of gcIDO was up-regulated by the recombinant gcTGF-β1 from 3 to 72 h, with the peak reached at 12 h (Fig. 4A). Meanwhile, the expression of TGF-β1 could be induced by itself from 3 to 48 h, with the peak reached at 12 h (Fig. 4B). Furthermore, the expression of CD25 and Foxp3 were also measured, both of them could be induced by gcTGF-β1 (Fig. 4C and D).

3.8. Induced expression of gcIDO in grass carp SBMIE model

The experimental model of grass carp SBMIE was built as previously described [30]. In order to investigate the roles of gcIDO in grass carp SBMIE, the expression levels of gcIDO in intestine were measured by qPCR and immunofluorescence staining. As shown in Fig. 5, the results of qPCR and immunofluorescence staining both indicated that the expression of gcIDO increased significantly at 5 w in the SBMIE model. Like gcIDO, the expression of TGF-β1 was also increased at 5 w in the SBMIE model.

4. Discussion

Although the molecular and functional features of IDO have been widely studied in mammals, research on fish IDO has been predominantly limited to gene identification. In this study, the full-length cDNA of gcIDO was obtained, which contains a 1317 bp ORF encoding 438 amino acid residues. The amino acid sequence identity between gcIDO and mammalian IDO2 is significantly higher than that between gcIDO and mammalian IDO1. Moreover, phylogenetic analysis revealed that fish IDO form a cluster with amphibian IDO, bird IDO, and mammalian IDO2, which is distinct from the cluster consisted of mammalian IDO1. The mammalian IDO1 and IDO2 genes are located on the same chromosome in a tandem arrangement with conserved genomic structures [23,26]. Considering that IDO1 only exists in mammals while IDO2 exists from fish to mammals [6,23], it is reasonable to speculate that IDO1 arose from IDO2 by gene duplication in mammals.

The enzymatic activity of lower vertebrate IDO is similar to that of mammalian IDO2, which is much lower than that of mammalian IDO1 [23,26]. In our study, low enzymatic activity of gcIDO was observed in GCO cells. Site-directed mutagenesis revealed that two residues Asp_{274} and His_{346} are essential for the enzymatic activity of mammalian IDO, because they are essential for the binding of IDO to heme [32]. Multiple sequence alignment showed that the two residues Asp_{274} and His_{346} are conserved between grass carp and mammalian IDO. In this study, the enzymatic activity of gcIDO could be promoted by ALA, indicating that heme is important for the activity of IDO. Like L-Trp, L-1MT has an indole ring structure, and can be degraded by IDO [33]. Our results proved that L-1MT can inhibit the enzymatic activity of gcIDO in a concentration dependent manner, which is consistent with the results conducted in zebrafish, frog, chicken, and mouse [34].

Expression analysis showed that gcIDO was constitutively expressed in all tissues analyzed, with the highest expression levels detected in liver, trunk kidney and intestine. Moreover, the expression of gcIDO could be rapidly and significantly up-regulated by LPS and Poly (I:C) in PBLs. Like gcIDO, the expression of rainbow trout IDO also can be induced by LPS [25]. These results indicated that IDO not only acts as an enzyme, but also plays roles in the immunity of fish. In mammals, TGF-β1 is a critical cytokine in the regulation of T cell–mediated immune responses and immune tolerance [35]. In this process, IDO acts as an intracellular signal transducer of TGF-β1. At first, TGF-β1 induces the Fyn-dependent phosphorylation of ITIMs of IDO by a PI(3)K-dependent while Smad-independent pathway [36]. Then, IDO recruits SHP-1 and SHP-2 to selectively activate the noncanonical NF-κB pathway, resulting in the induction of long-lasting IDO expression and autocrine TGF-β1 production in a positive feedback loop [3,5]. Further, TGF-β1 induces the differentiation of naive $CD4^+CD25^-$ T cells to $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs), which can initiate, maintain and amplify the immunoregulatory force to effect long-term immune tolerance [37–39]. Like mammalian IDO1 and IDO2, gcIDO also possesses two conserved ITIMs, suggesting that fish IDO can act as an intracellular signal transducer of TGF-β1. To prove this point, recombinant gcTGF-β1 was generated to stimulate grass carp PBLs, and the results showed that gcTGF-β1 could indeed induce the expression of endogenous IDO and TGF-β1. Moreover, the expression of CD25 and Foxp3 could also be induced by gcTGF-β1. All these results indicated that the TGF-β1/IDO pathway is present in teleost fish. However, the mechanisms that how gcTGF-β1 regulates the expression of endogenous IDO, what factors participate in the process, and what is the biological function of the TGF-β1/IDO pathway are still unknown.

In mammals, several gastrointestinal diseases, such as inflammatory bowel diseases (IBD) and malignancy, are associated with elevated expression of IDO [21,40]. With the addition of plant protein in aquatic

feeds, more and more enteritis had been caused in fish, which brought people heavy losses. However, there are few solutions in regards to the prevention or cures of foodborne enteritis. In order to investigate the mechanism of enteritis in herbivorous fish and give some guide to prevent or cure foodborne enteritis, the experimental model of grass carp SBMIE was built in this study and the process of SBMIE could be divided into three stages, including early (0–3 week), middle (3–5 week), and late (5–7 week) stages [30]. In the middle stage, the mRNA expression level of gcIDO and the number of cells expressing gcIDO both increased in intestine. Moreover, the mRNA expression of TGF-β1 in intestine was also increased. These results suggested that gcIDO may play an immunoregulatory role in SBMIE. There are two hypotheses to explain that gcIDO may play an immunoregulatory role in SBMIE. One is that IDO catalyzes the degradation of tryptophan into its metabolites, such as kynurenine, inhibiting T cell activation and decreasing T cell viability following activation. The other is that the TGF-β/IDO pathway mediates lasting regulatory functions in the generation and maintenance of Tregs, which is crucial to regulate immune responses. Whether gcIDO plays its role through tryptophan catabolism pathway or TGF-β1/IDO pathway or maybe both deserves further study.

In conclusion, the full-length cDNA of gcIDO was cloned and characterized, and the expression patterns in various tissues were analyzed. The gcIDO could catalyze the degradation of L-Trp, and this activity could be promoted by ALA and inhibited by L-1MT. Both LPS and Poly (I:C) could up-regulate the expression of gcIDO in PBLs, indicating that gcIDO is involved in the immune response. Our study first demonstrated that gcTGF-β1 could induce the expression of endogenous IDO, TGF-β1, CD25, and Foxp3 in PBLs, indicating that the TGF-β1/IDO pathway is present in fish. Moreover, gcIDO may play immunoregulatory roles in SBMIE. Taken together, these data suggest that IDO plays multiple roles in the immunity of fish.

Conflicts of interest

The authors have no conflicting commercial or financial interest in publishing this paper.

Acknowledgments

This work was funded by grants from the National Natural Science Foundation of China (31472307) and the National Basic Research Program of China (2014CB138601). We would like to thank the senior experimentalist Yun-Xia Yang, assistant professor Hao-Kun Liu, and professor Shou-Qi Xie (Institute of Hydrobiology, CAS) for suggestions on making diet and experimental design for the feeding trial.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.005.

References

[1] E.L. Sainio, K. Pulkki, S.N. Young, L-tryptophan: biochemical, nutritional and pharmacological aspects, Amino Acids 10 (1996) 21–47.
[2] O. Takikawa, Biochemical and medical aspects of the indoleamine 2,3-dioxygenase-initiated L-tryptophan metabolism, Biochem. Biophys. Res. Commun. 338 (2005) 12–19.
[3] W.J. Chen, Ido: more than an enzyme, Nat. Immunol. 12 (2011) 809–811.
[4] F. Fallarino, U. Grohmann, P. Puccetti, Indoleamine 2,3-dioxygenase: from catalyst to signaling function, Eur. J. Immunol. 42 (2012) 1932–1937.
[5] J.C. Mbongue, D.A. Nicholas, T.W. Torrez, N.S. Kim, A.F. Firek, W.H. Langridge, The role of indoleamine 2, 3-dioxygenase in immune suppression and autoimmunity, Vaccines 3 (2015) 703–729.
[6] H.J. Ball, A. Sanchez-Perez, S. Weiser, C.J. Austin, F. Astelbauer, J. Miu, et al., Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice, Gene 396 (2007) 203–213.
[7] N.J.C. King, S.R. Thomas, Molecules in focus: indoleamine 2,3-dioxygenase, Int. J. Biochem. Cell Biol. 39 (2007) 2167–2172.
[8] R. Metz, J.B. Duhadaway, U. Kamasani, L. Laury-Kleintop, A.J. Muller, G.C. Prendergast, Novel tryptophan catabolic enzyme Ido2 is the preferred biochemical target of the antitumor indoleamine 2,3-dioxygenase inhibitory compound D-1-methyl-tryptophan, Cancer Res. 67 (2007) 7082–7087.
[9] R. Yoshida, T. Oku, J. Imanishi, T. Kishida, O. Hayaishi, Interferon: a mediator of indoleamine 2,3-dioxygenase induction by lipopolysaccharide, poly(I) · poly(C), and pokeweed mitogen in mouse lung, Arch. Biochem. Biophys. 249 (1986) 596–604.
[10] S. Fujigaki, K. Saito, K. Sekikawa, S. Tone, O. Takikawa, H. Fujii, et al., Lipopolysaccharide induction of indoleamine 2,3-dioxygenase is mediated dominantly by an IFN-gamma-independent mechanism, Eur. J. Immunol. 31 (2001) 2313–2318.
[11] W. Li, B.P. Katz, S.M. Spinola, Haemophilus ducreyi lipooligosaccharides induce expression of the immunosuppressive enzyme indoleamine 2,3-dioxygenase via type I interferons and tumor necrosis factor Alpha in human dendritic cells, Infect. Immun. 79 (2011) 3338–3347.
[12] A.L. Mellor, D.H. Munn, Ido expression by dendritic cells: tolerance and tryptophan catabolism, Nat. Rev. Immunol. 4 (2004) 762–774.
[13] U. Grohmann, F. Fallarino, P. Puccetti, Tolerance, DCs and tryptophan: much ado about Ido, Trends Immunol. 24 (2003) 242–248.
[14] J.R. Moffett, M.A. Namboodiri, Tryptophan and the immune response, Immunol. Cell Biol. 81 (2003) 247–265.
[15] H. Soliman, M. Mediavilla-Varela, S. Antonia, Indoleamine 2,3-dioxygenase: is it an immune suppressor? Cancer J. 16 (2010) 354–359.
[16] F. Fallarino, U. Grohmann, C. Vacca, R. Bianchi, C. Orabona, A. Spreca, et al., T cell apoptosis by tryptophan catabolism, Cell Death Differ. 9 (2002) 1069–1077.
[17] B.J. Cherayil, Indoleamine 2,3-dioxygenase in intestinal immunity and inflammation, Inflamm. Bowel Dis. 15 (2009) 1391–1396.
[18] G.J. Gurtner, R.D. Newberry, S.R. Schloemann, K.G. McDonald, W.F. Stenson, Inhibition of indoleamine 2,3-dioxygenase augments trinitrobenzene sulfonic acid colitis in mice, Gastroenterology 125 (2003) 1762–1773.
[19] B.K. Dieckgraefe, W.F. Stenson, J.R. Korzenik, P.E. Swanson, C.A. Harrington, Analysis of mucosal gene expression in inflammatory bowel disease by parallel oligonucleotide arrays, Physiol. Genom. 4 (2000) 1–11.
[20] S. Barcelo-Batllori, M. Andre, C. Servis, N. Levy, O. Takikawa, P. Michetti, et al., Proteomic analysis of cytokine induced proteins in human intestinal epithelial cells: implications for inflammatory bowel diseases, Proteomics 2 (2002) 551–560.
[21] A.M. Wolf, D. Wolf, H. Rumpold, A.R. Moschen, A. Kaser, P. Obrist, et al., Overexpression of indoleamine 2,3-dioxygenase in human inflammatory bowel disease, Clin. Immunol. 113 (2004) 47–55.
[22] M.I. Torres, M.A. Lopez-Casado, P. Lorite, A. Rios, Tryptophan metabolism and indoleamine 2,3-dioxygenase expression in coeliac disease, Clin. Exp. Immunol. 148 (2007) 419–424.
[23] H.J. Yuasa, M. Takubo, A. Takahashi, T. Hasegawa, H. Noma, T. Suzuki, Evolution of vertebrate indoleamine 2,3-dioxygenases, J. Mol. Evol. 65 (2007) 705–714.
[24] G.C. Prendergast, R. Metz, A.J. Muller, L.M.F. Merlo, L. Mandik-Nayak, Ido2 in immunomodulation and autoimmune disease, Front. Immunol. 5 (2014) 1–6.
[25] J. Cortes, C. Alvarez, P. Santana, E. Torres, L. Mercado, Indoleamine 2,3-dioxygenase: first evidence of expression in rainbow trout (Oncorhynchus mykiss), Dev. Comp. Immunol. 65 (2016) 73–78.
[26] H.J. Yuasa, H.J. Ball, Y.F. Ho, C.J. Austin, C.M. Whittington, K. Belov, et al., Characterization and evolution of vertebrate indoleamine 2, 3-dioxygenases IDOs from monotremes and marsupials, Comp. Biochem. Physiol. B 153 (2009) 137–144.
[27] M. Yang, H. Zhou, Grass carp transforming growth factor-beta 1 (TGF-beta 1): molecular cloning, tissue distribution and immunobiological activity in teleost peripheral blood lymphocytes, Mol. Immunol. 45 (2008) 1792–1798.
[28] H. Boshra, T.H. Wang, L. Hove-Madsen, J. Hansen, J. Li, A. Matlapudi, et al., Characterization of a C3a receptor in rainbow trout and Xenopus: the first identification of C3a receptors in nonmammalian species, J. Immunol. 175 (2005) 2427–2437.
[29] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[30] N. Wu, B. Wang, Z.W. Cui, X.Y. Zhang, Y.Y. Chen, X. Xuan, et al., Integrative transcriptomic and microRNAomic profiling reveals immune mechanism for the resilience to soybean meal stress in fish gut and liver, Front. Physiol. 12 (2018) 1154.
[31] X.J. Zhang, X.Y. Zhang, N. Zhang, X. Guo, K.S. Peng, H. Wu, et al., Distinctive structural hallmarks and biological activities of the multiple cathelicidin antimicrobial peptides in a primitive teleost fish, J. Immunol. 194 (2015) 4974–4987.
[32] T.K. Littlejohn, O. Takikawa, R.J. Truscott, M.J. Walker, Asp274 and his346 are essential for heme binding and catalytic function of human indoleamine 2,3-dioxygenase, J. Biol. Chem. 278 (2003) 29525–29531.
[33] S.G. Cady, M. Sono, 1-Methyl-Dl-Tryptophan, beta-(3-Benzofuranyl)-Dl-Alanine (the oxygen analog of tryptophan), and beta-[3-Benzo(B)Thienyl]-Dl-Alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase, Arch. Biochem. Biophys. 291 (1991) 326–333.
[34] H.J. Yuasa, H.J. Ball, C.J. Austin, N.H. Hunt, 1-L-methyltryptophan is a more effective inhibitor of vertebrate Ido2 enzymes than 1-D-methyltryptophan, Comp. Biochem. Physiol. B 157 (2010) 10–15.
[35] L. Gorelik, R.A. Flavell, Transforming growth factor-beta in T-cell biology, Nat. Rev. Immunol. 2 (2002) 46–53.
[36] M.T. Pallotta, C. Orabona, C. Volpi, C. Vacca, M.L. Belladonna, R. Bianchi, et al., Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells, Nat. Immunol. 12 (2011) 870–878.
[37] E.M. Dons, G. Raimondi, D.K. Cooper, A.W. Thomson, Induced regulatory T cells:

mechanisms of conversion and suppressive potential, Hum. Immunol. 73 (2012) 328–334.
[38] E.N. Hadaschik, A.H. Enk, TGF-beta1-induced regulatory T cells, Hum. Immunol. 76 (2015) 561–564.
[39] S. Fu, N. Zhang, A.C. Yopp, D. Chen, M. Mao, D. Chen, et al., TGF-beta induces Foxp3^{+} T-regulatory cells from CD4^{+} CD25^{-} precursors, Am. J. Transplant. 4 (2004) 1614–1627.
[40] Y.F. Gao, R.Q. Peng, J. Li, Y. Ding, X. Zhang, X.J. Wu, et al., The paradoxical patterns of expression of indoleamine 2,3-dioxygenase in colon cancer, J. Transl. Med. 7 (2009) 71.