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Functional characterisation of interleukin 34 in grass carp *Ctenopharyngodon idella*

Yujie Xue[a,b,c], Xinyu Jiang[a,b,c], Jingduo Gao[a,b,c], Xia Li[a,b,c], Jiawen Xu[a,b,c], Junya Wang[a,b,c], Qian Gao[a,b,c,*], Jun Zou[a,b,c,d,**]

[a] Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, Shanghai Ocean University, Shanghai, 201306, China
[b] International Research Center for Marine Biosciences at Shanghai Ocean University, Ministry of Science and Technology, China
[c] National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai, China
[d] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

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ABSTRACT

Interleukin (IL) 34 plays an important role in regulating macrophage functions and inflammation process. IL-34 homologues have recently been discovered in fish but the functions have not been studied. In this study, an IL-34 homologue was identified in grass carp *Ctenopharyngodon idella* and its bioactivities were investigated. The grass carp IL-34 was constitutively expressed in tissues, with the highest expression detected in spleen. It could be up-regulated in spleen after infection with *F. cloumnare* and grass carp reovirus II, and in primary head kidney leucocytes by recombinant IL-4/13B. The recombinant IL-34 produced in bacteria and HEK293T cells showed stimulatory effect on the expression of IL-1β, IL-6 and IL-8 but inhibited expression of IL-10 and TGF-β1 in primary head kidney macrophages. The results demonstrate that IL-34 is a proinflammatory cytokine in grass carp.

1. Introduction

Interleukin (IL) 34 is a multifunctional cytokine that plays a central role in regulating proliferation and differentiation of myeloid cells [1]. Despite lack of sequence similarity, IL-34 and colony stimulating factor 1 (CSF1) are structurally related and have a four-helix bundle core structure [2]. Functionally, they activate the same receptor (CSF1R) and possess mostly overlapping but distinct physiological functions [3,4]. IL-34 is required for the development of Langerhans cells and microglia and is associated with several inflammatory diseases in humans [5,6]. A recent study demonstrates that IL-34 induces expression of antiviral genes in Xenopus primary macrophages and enhances antiviral resistance to viral infection [7].

IL-34 is a secreted glycoprotein and forms homodimer or heterodimer with CSF1 to exert functions [2,8]. Many cell types including myeloid cells, epithelial cells, endothelial cells, fibroblasts, neurons, and cancer cells expressing CSF1R can be targeted by IL-34. Unlike CSF1 which interacts with CSF1R through hydrogen bonds, IL-34 and CSF1R exploit hydrophobic contact to form an active ligand/receptor complex which consists of an IL-34 homodimer and 2 copies of CSF1R [8]. IL-34 engages with the *N*-terminal immunoglobulin D2 and D3 domains of CSF1R, with D4 domain facilitating oligomerization. Upon activation of CSF1R, IL-34 triggers phosphorylation of Tyr^{723} of CSF1R and subsequent activation of extracellular signal-regulated kinase (ERK) 1/2 Erk 1/2 to regulate gene expression [9]. It induces expression of proinflammatory cytokines and chemokines such as IL-6, IL-8 and chemokine (C–C motif) ligand (CCL) 2 in humans [10]. Stimulation of peripheral blood mononuclear cells isolated from human Rheumatoid Arthritis patients enhances production of IL-17 [5]. It is suggested that IL-34 and CSF1 plays complementary roles in activation of the CSF1R in animals.

IL-34 is widely distributed in tissues and is highly abundant in brain, skin and spleen [11]. High levels of IL-34 is predominantly expressed in the cortex, olfactory nucleus, and the hippocampus in the brain and is believed to be required for the maintenance of microglia [3]. Inflamed tissues from patients with chronic inflammatory diseases display sustained high amount of IL-34 which is suggested to be associated with disease pathogenesis. In addition, IL-34 can be up-regulated during inflammation and infections. In human osteoblasts, proinflammatory cytokines such as IL-1β and TNF-1α are potent inducers to enhance IL-34 expression through activation of JNK and p44/42 MAPK dependent pathway [12].

* Corresponding author. National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai, China.
** Corresponding author. National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai, China.
E-mail addresses: qgao@shou.edu.cn (Q. Gao), jzou@shou.edu.cn (J. Zou).

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Table 1
Primers used in this study.

Primers	Sequence (5′ to 3′)	Application
F1	ACTGTTATCGGACCATGCTTTGCC	3′-RACE
F2	CTCAACTGGAGGAAAGGGAAGA	3′-RACE
R1	TCTGCTGATGTTGCGTAGTC	5′-RACE
R2	GAGTTTAGGCTTTCCCGTAC	5′-RACE
F3	TTCGAGTGGAGAAGGCTGTAG	Verify the full length
R3	AATAGTGGGAGATTTATTTC	Verify the full length
IL-34-qF	TCAACAGGGTATAAAGAGGGTT	Real-time PCR
IL-34-qR	ATCCAGTAATGACTTGGGTGTA	Real-time PCR
IL-6-qF	CAGCAGAATGGGGGAGTTATC	Real-time PCR
IL-6-qR	CTCGCAGAGTCTTGACATCCTT	Real-time PCR
IL-1β-qF	TCTCCTCGTCTGCTGGGTGT	Real-time PCR
IL-1β-qR	CAAGACCAGGTGAGGGGAAG	Real-time PCR
IL-8-qF	TCTACCCTCCTAGCCCTCACTG	Real-time PCR
IL-8-qR	TCATGGTGCTTTGTTGGCAAGGA	Real-time PCR
IL-10-qF	GCAACAGAACATCAATAGTCCTT	Real-time PCR
IL-10-qR	CACCCTTTTCCTTCATCTTTTCA	Real-time PCR
TGF-β1-qF	TTGGGACTTGTGCTCTAT	Real-time PCR
TGF-β1-qR	AGTTCTGCTGGGATGTTT	Real-time PCR
UPM-Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
UPM-Short	CTAATACGACTCACTATAGGGC	
NUP	AAGCAGTGGTATCAACGCAGAGT	
EF-1α-qF	CAGCACAAACATGGGCTGGTTC	Real-time PCR
EF-1α-qR	ACGGGTACAGTTCCAATACCTCCA	Real-time PCR
r*Ci*IL-34-F	CATGCCATGGCGAGCACCAGATCTCTGTGGC	Plasmid construction
r*Ci*IL-34-R	CCCAAGCTTTTATGTTGTTTTCTTCCCTTTCCTC	Plasmid construction
r*Ci* IL-4/13B–F	CCGGAATTCTCACAACCTGATCTTAGGAAG	Plasmid construction
r*Ci* IL-4/13B-R	CCCAAGCTTTCATTTCACTCTTTTTGGACG	Plasmid construction
pcDNA3.1-IL-34-F	CTAGCTAGCCACCATGATCCAGTCTGAATGCCGC	Plasmid construction
pcDNA3.1-IL-34-R	CCGCTCGAGCTATGTTGTTTTCTTCCCTTT	Plasmid construction

Both IL-34 and CSF1 exist in fish. Whilst two copies of CSF1 genes are common in teleosts, only a single copy of IL-34 gene has been found in the genome of trout, zebrafish, fugu, grouper and large yellow croaker [13–15]. The gene synteny of IL-34 is well conserved in vertebrates since the clustering of IL-34 gene with the SF3B3 gene remains unchanged in fish, birds and humans. Besides, fish and mammalian IL-34 genes are organized with the same genomic structure of 7 exons and 6 introns. It is believed that IL-34 and CSF1 have evolved independently.

Information on the expression of fish IL-34 is limited. The only available data are from rainbow trout and orange spotted grouper [13,14]. Trout IL-34 is constitutively expressed in a broad range of tissues at comparable levels and can be up-regulated in the cell lines including RTL, RTG-2, RTGill and RTS-11 cells, and primary head kidney macrophages after stimulation with polyI:C, LPS or IFN-γ [13]. Contrasting the tissue expression pattern in trout, grouper IL-34 is most abundant in the brain [14]. IL-1β, a central proinflammatory cytokine, is shown to enhance IL-34 expression in head kidney macrophages of trout. Moreover, in trout infected with parasitic *Tetracapsuloides bryosalmonae*, the IL-34 expression is elevated in kidney, the primary organ involved in pathogenesis of proliferative kidney disease. Similarly, in grouper infected with *Cryptocaryon irritans*, gills and skin (infection sites) exhibit high levels of IL-34. These data strongly suggest that IL-34 is involved in immune response to parasite infection.

The putative receptors for IL-34 have also been identified in teleost fishes including fugu [16], grass carp [17], grouper [18], gilthead seabream [19], goldfish [20], and rainbow trout [21]. Unlike mammals, teleost fish possess two copies of CSF1R which are widely expressed in tissues including head kidney and spleen [19,20]. Interestingly, in goldfish, a soluble form of CSF1R has been described and its expression is increased in macrophages in the presence of apoptotic cells [22]. The soluble CSF1R is important in regulating immune responses, including inhibiting proliferation of self-renewing macrophages, leucocyte migration into the infection site, bacterial killing and inflammatory response [22–24].

In this study, an IL-34 homologue was identified in grass carp *Ctenopharyngodon idella* (*Ci*) and its biological activity analysed in vitro using recombinant proteins generated from bacteria and HEK293T cells. In addition, its expression was examined in fish challenged with bacterial and viral pathogens. The results demonstrate that *Ci*IL-34 is an important cytokine in modulating macrophage functions.

2. Materials and Methods

2.1. Fish

Grass carp (*Ctenopharyngodon idella*) (120 ± 10 g) were obtained from a commercial farm in Shanghai, China. The fish were kept in freshwater tanks at 25 ± 2 °C in a recirculating system for 10 days prior to use in experiments. Fish were anesthetized with MS-222 (100 mg/L, Sigma-Aldrich) before injection and sampling. All experiments were conducted under the national regulations on use of laboratory animals of China and approved by the ethics committee of laboratory animals of Shanghai Ocean University.

2.2. Cloning and identification of CiIL-34

Total RNA was extracted from spleen of healthy grass carp using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesised from total RNA using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa). The synthesised cDNA was stored at −20 °C until use. Partial cDNA sequence of *Ci*IL-34 was obtained from the whole-genome sequence database of grass carp (http://www.ncgr.ac.cn/grasscarp/) [25]. The full-length sequence of *Ci*IL-34 was amplified using 5′ and 3′ RACE PCR according to the instructions for the kit of Rapid Amplification of cDNA Ends (Life Technology) with the gene specific primers listed in Table 1.

2.3. Sequence analysis of CiIL-34

The nucleotide sequences were assembled and analysed using the DNAMAN program (Version 8.0). Prediction of open reading frame

(ORF) and nucleotide/protein translation were performed using the Gene Finding and Translate Tool tools on the ExPASy (http://expasy.pku.edu.com) website. The BLASTN and BLASTP program (http://www.ncbi.nlm. Nih.gov/BLAST/) were used for homologous sequence search and the SignalP program (Version 3.0) (http://www.cbs.dtu.dk/services/SignalP/) was used for the prediction of protein signal peptide. The *N*-glycosylation sites and molecular weight were predicted using the software listed on the ExPASy server (https://www.expasy.org/). Known IL-34 sequences were retrieved from the NCBI GenBank database for sequence alignment using the ClustalW program. The phylogenetic tree was constructed using the Neighbor-Joining method (bootstrap replication = 10,000) of MEGA program (Version 5.0).

2.4. Tissue expression analysis of CiIL-34

Eight tissues (liver, spleen, head kidney, intestine, skin, gills, brain and thymus) were collected from 6 healthy grass carp and homogenized in 1 mL TRIzol reagent. Total RNA was extracted for cDNA synthesis using the premix 2 × HifairTM II SuperMix plus kit (Yeasen) according to the manufacturer's instructions. The cDNA samples were stored at −20 °C until use.

Quantitative real-time PCR (qPCR) was carried out using the iTaqTM Universal SYBR® Green Supermix (Bio-Rad) according to the manufacturer's instructions and the LightCycler 96 Real Time PCR System (Roche). The primers used for qPCR analysis were given in Table 1 and the elongation factor-1α (EF-1α) was used as the internal control to normalize gene expression. A standard curve for each gene was established using a 10-fold serial dilutions of the plasmid DNA containing gene fragment of interest for quantification of transcript levels. The expression levels of each gene were calculated as arbitrary units normalised to that of EF-1α. Fold changes of expression were calculated by comparing the average expression level of experimental group with that of the corresponding control group.

2.5. Bacterial challenge

A pathogenic G4 strain of *F. columnare*, provided by Dr Haixia Xie, the State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences [26], was used for the challenge experiment. The bacteria were cultured in Shieh medium at 28 °C for 48 h with constant shaking (200 rpm), centrifuged at 4200×*g* for 4 min and resuspended in PBS buffer. The bacterial suspension was adjusted to 1×10^7 colony-forming units (CFU)/mL. For the challenge experiment, 60 grass carp were randomly divided into 2 groups, each containing 30 fish. The challenge group were intraperitoneally (i.p.) injected with bacterial suspension (1 μL/g body weight), and the control group with PBS (1 μL/g body weight). At 12 h, 24 h, 48 h, 72 h and 5 d after injection, spleen and head kidney tissue were collected and homogenized in 1 mL TRIzol reagent, and reverse transcribed into cDNA as described above for expression analysis of *Ci*IL-34.

2.6. Viral infection

The Grass carp reovirus II (GCRV-II) was provided by Dr Qin Fang, State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences (Wuhan, China). Grass carp were randomly divided into two groups (16 fish each group), and each fish was i.p. injected with 200 μL GCRV (resuspended in DMEM). Control fish were i.p. injected with an equal volume of DMEM. Spleen, kidney, intestine and gills were collected at 1, 3, 7 and 14 d for total RNA extraction and reverse transcribed into cDNA as described above for expression analysis of *Ci*IL-34.

2.7. Expression of recombinant CiIL-34 (rCiIL-34) in HEK293T cells

The entire *Ci*IL-34 ORF was amplified with primers pcDNA3.1-IL-34-F/R (Table 1) which contained two restriction enzyme sites *Nhe*I and *Xho*I, digested with *Nhe*I and *Xho*I and inserted into the pcDNA3.1 plasmid (Invitrogen). The resultant plasmid was transformed into *E. coli* DH5α competent cells, and the positive plasmid extracted using the E.Z.N.A. Endo-free Plasmid Mini Kit (Promega) according to the manufacturer's instructions. The expression plasmid was verified by sequencing (Sangon Biotech). HEK293T cells were maintained in DMEM supplemented with 10% FBS (Gibco), 100 U/mL penicillin (P), and 100 U/mL streptomycin (S) at 37 °C in a 5% CO_2 incubator. The empty vector and IL-34 expression plasmid were transfected into HEK293T cells using the Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instructions. Cells and culture media were collected 48 h after transfection, diluted with DMEM medium (1:1, 1:2, 1:4, v/v) and stored at −80 °C.

2.8. Production and purification of rCiIL-34 in bacteria

The cDNA fragment encoding the predicted mature *Ci*IL-34 (73–633 bp) was amplified by Phusion High-Fidelity DNA polymerase (New England Biolabs) using r*Ci*IL-34-F and r*Ci*IL-34-R gene-specific primers (Table 1). The PCR product was cloned into the pMD19-T vector, digested with *Nco*I and *Hind*III and cloned into the pEHISTEVb expression vector (kindly provided by Dr Hai Deng, University of Aberdeen, UK). The recombinant plasmid pEHISTEVb-IL34 was verified by sequencing and transformed into *E. coli* BL21 (DE3) bacteria [27]. The recombinant protein was purified by chromatography on a Superdex 75 column (GE Healthcare). The purity and size of the product were analysed by SDS-PAGE. Protein concentration was determined by the Bradford method and then stored at −80 °C.

2.9. Isolation of primary head kidney macrophages and stimulation with rCiIL-34

Grass carp head kidney macrophages were prepared by discontinuous density gradient centrifugation [28]. Briefly, head kidney tissue was collected from healthy fish, placed on a 70 μm nylon mesh and gently pushed through the mesh with constantly dripping of cold L-15 medium containing 0.1% FBS, 0.1% heparin and P/S. The cell suspension was carefully loaded on a 51% Percoll (GE Healthcare) density gradient and centrifuged at 400 × *g* for 30 min at 4 °C without breaking. Cells were harvested at the Percoll/medium interface using a Pasteur pipette, resuspended in L-15 medium containing 0.1% FBS, 0.1% heparin and P/S, and washed twice at 400 × *g* for 10 min at 4 °C. The supernatant was discarded and the cells were resuspended in 5 mL RPMI/DMEM (1:1, v/v) medium containing 10% FCS and P/S. Lastly, the cells were seeded at a density of 1×10^6 cells/well in a 6-well plate (Corning) and cultured in a 5% CO_2 incubator at 28 °C. After overnight culture, non-adherent cells were carefully washed off and the adherent cells (enriched macrophages) exposed to r*Ci*IL-34 protein purified from bacteria or expressed in HEK293T cells. After 12 h, cells were harvested for RNA preparation and qPCR analysis of expression of IL-1β, IL-6, IL-8, IL-10 and TGF-β1.

2.10. Expression analysis of CiIL-34 in primary head-kidney cells after stimulation with recombinant CiIL-4/13B

Based on the published sequence, the nucleotide fragment encoding the mature peptide of grass carp IL-4/13B (13.8 kDa) was amplified by PCR and cloned into the pETHISTEVb expression vector (Novagen) [29]. The expression plasmid was transformed into *E. coli* BL21 (DE3) competent cells and cultured in LB medium containing 50 μg/mL kanamycin at 37 °C. When the absorbance of the bacterial solution at 600 nm reached 0.6–0.8, IPTG was added to the final concentration of

1 mM and cultured for 12 h. One mL of the cells was lysed for SDS-PAGE analysis to check the expression of the recombinant *Ci*IL-4/13B (r*Ci*IL4/13B) protein (17.1 kDa, 13.8 kDa *Ci*IL-4/13B plus 3.3 kDa HISTEV tag). The recombinant protein was then purified by chromatography on a Superdex 75 preparative column (GE Healthcare). The purified IL-4/13B protein was validated by SDS-PAGE.

To confirm the purified r*Ci*IL-4/13B, a polyclonal antibody against the polypeptide sequence of IL-4/13B (AVNGSQPDLRKTLLKDIIVF) was generated in rabbit for Western blotting analysis (Huamei Biotech). The r*Ci*IL-4/13B antibody was purified by affinity chromatography and diluted 1:100 (v/v, 4 μg/mL) for Western blotting.

The head kidney cells were isolated as described above and adjusted to a concentration of 5×10^6 cells/mL, seeded into culture flasks, and cultured at 28 °C, 5% CO_2 for 4 h. The r*Ci*IL-4/13B was added to cells at a final concentration of 2, 20, 200 ng/mL and cultured for 12 h; The control group was incubated with protein buffer (50 mM sodium chloride, 20 mM Tris, pH8.0). The adherent cells together with cells in suspension were collected for RNA extraction for reverse transcription.

2.11. Statistical analysis

The qPCR data were analysed using the SPSS package 20.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA and the LSD post hoc test was used to analyze the expression data, with "$p < 0.05$" or "$p < 0.01$" between treatment groups and control groups considered significant.

3. Results

3.1. Sequence identification of CiIL-34

The cDNA sequence of *Ci*IL-34 gene (NCBI accession number: MK297321) is 1792 bp in length with an ORF of 633 bp encoding a protein of 210 aa (Fig. 1). It is transcribed from a gene with 6 exons and 5 introns. The genomic organization of *Ci*IL-34 is identical to that of zebrafish homologue, with comparable size of exons in both species. A putative signal peptide of 24 aa is present and four potential *N*-glycosylation sites are found (Supplementary Fig. 1). Multiple sequence alignment reveals that four cysteine residues known to form the intramolecular disulfide bonds and stability of IL-34 in humans are well conserved in fish homologues [8]. However, fish IL-34s have additional cysteine at the C-terminal region. One striking difference between human and fish IL-34s is that the C-terminal tail of fish IL-34s is 38–39 aa shorter than human counterpart which comprises a region of flexible mucin like O-linked glycosylation-rich amino acid residues [8]. Furthermore, the C-terminal tail of fish IL-34s contain a conserved motif with multiple cationic aa residues (RKx[R/K]K) which are absent in human IL-34.

The identity of aa sequence between IL-34s is shown in Table 2. The aa identity of *Ci*IL-34 with other known homologues is 25.7–77.6%, with the highest identity seen between grass carp and zebrafish IL-34. Known fish IL-34s are moderately conserved, sharing 45.0%–77.6% aa identity.

A phylogenetic tree was constructed using the CSF1 molecules as an outgroup (Fig. 2). As shown in Fig. 2, all the IL-34s from fish, birds and mammals grouped to form a clade with a high bootstrap value (100%), which was separated well from the CSF1 group. The results suggest that the IL-34 and MCSF genes have evolved independently in vertebrates during evolution.

3.2. Tissue distribution

Quantitative real time PCRanalysis was used to assess the gene expression of *Ci*IL-34 in various tissues including head kidney, liver, spleen, intestine, skin, gills, brain and thymus. As shown in Fig. 3, the *Ci*IL-34 was constitutively expressed in all the eight tissues but the expression levels varied considerably among the tissues. The expression

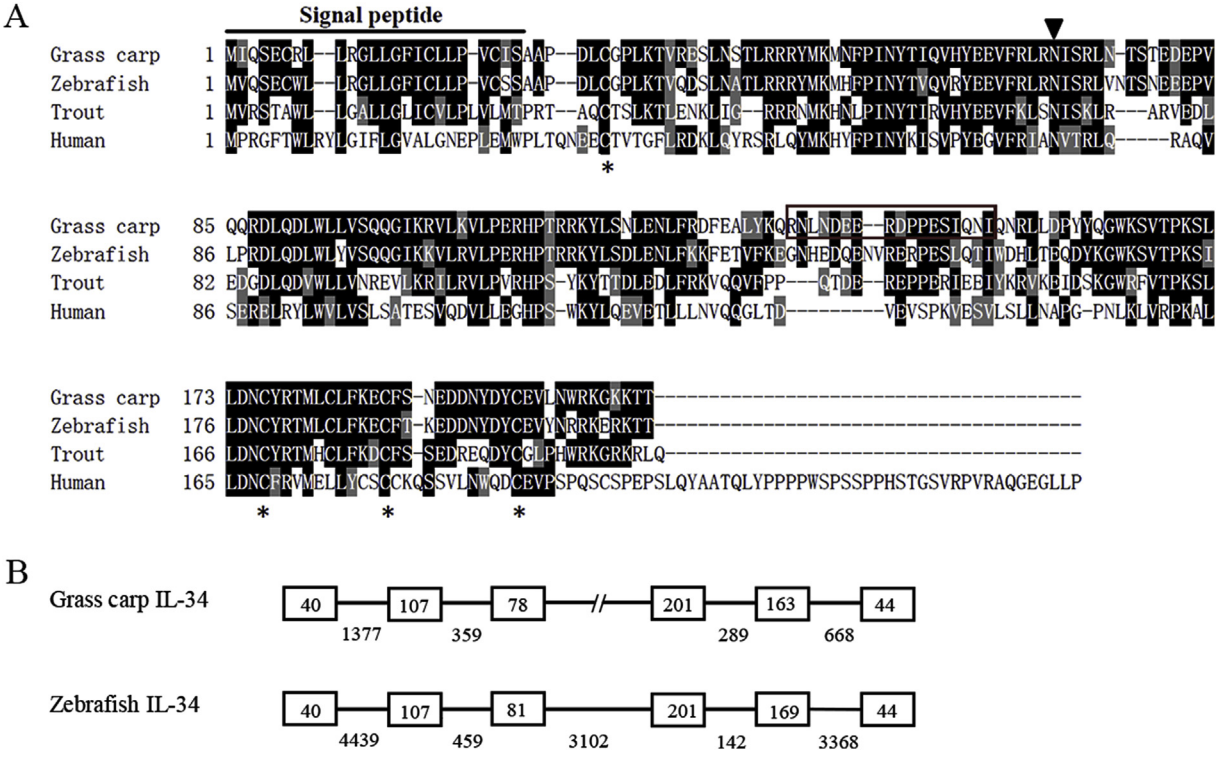

Fig. 1. Multiple sequence alignment and gene organization of *Ci*IL-34. (A) shows a signal peptide, predicted *N*-glycosylation sites and conserved cysteines. The conserved glycosylation site is indicated by a triangle above the alignment. The peptide used for generation of polyclonal antibody is boxed. (B) Exon-intron organization of IL-34 gene in grass carp and zebrafish. The size of the exon coding sequence (bp) is indicated in the box and the intron size is displayed below the line. Incomplete intron sequence is indicated by "//". Please note that the size of exons and introns is not proportional.

Table 2
Amino acid identities (%) of IL-34 molecules.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.Grass carp														
2.Zebrafish	77.6													
3.Trout	52.2	51.5												
4.Fugu	45.0	47.3	50.0											
5.Tilapia	47.3	46.0	51.5	69.5										
6.Catfish	51.0	51.0	48.7	42.4	43.4									
7.Salmon A	52.2	52.0	95.6	50.5	51.0	48.7								
8.Salmon B	52.0	49.3	83.3	51.0	52.0	48.7	84.2							
9.Chicken	32.8	31.0	29.6	31.0	33.5	34.1	30.8	29.0						
10.Zebra finch	36.0	35.4	32.9	32.9	34.3	33.9	34.7	32.4	77.0					
11.Human	31.6	29.1	30.7	25.1	27.0	32.3	31.8	30.3	41.7	44.0				
12.Cow	31.8	29.7	28.3	25.8	25.3	29.9	29.3	27.3	44.3	46.6	73.9			
13.Rat	30.8	28.7	27.7	23.8	24.6	29.4	28.8	27.3	42.0	45.4	69.7	69.0		
14.Mouse	25.7	24.1	23.0	21.4	21.5	24.9	24.1	23.2	37.6	40.6	54.6	54.6	69.1	

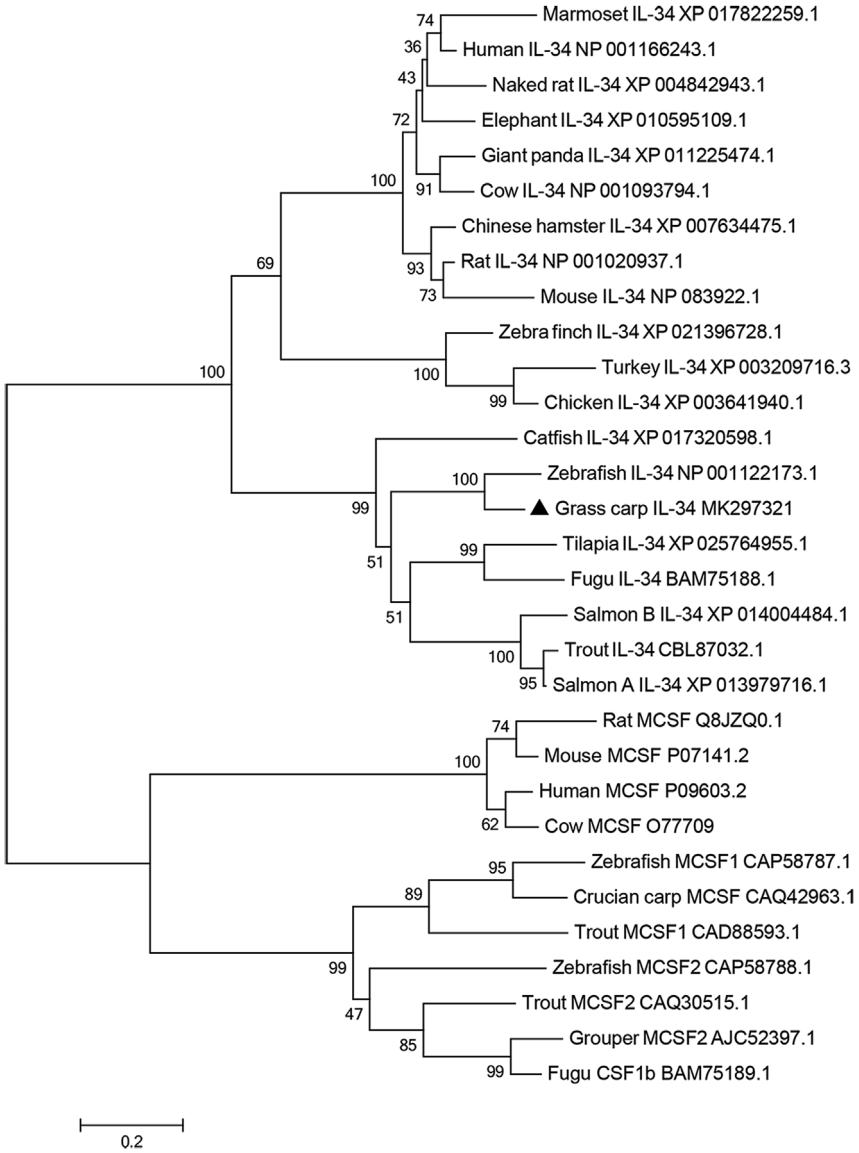

Fig. 2. Neighbour Joining (NJ) phylogenetic tree of IL-34 and CSF proteins. The tree was constructed using the NJ method within the MEGA programme (version 5.0). The JTT matrix-based model using pair-wise deletion option was chosen to construct the tree. The percentage of bootstrap values is shown next to the branches based on 10,000 bootstrap replications. The grass carp IL-34 is indicated by "▲".

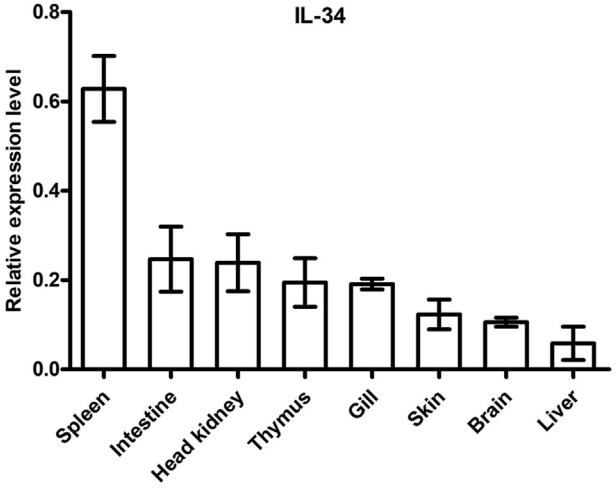

Fig. 3. Expression of *Ci*IL-34 in tissues. The mRNA levels of *Ci*IL-34 were determined by qPCR. The relative expression levels of *Ci*IL-34 were expressed as arbitrary units that were normalised against the expression levels of EF-1α. The results are expressed as mean ± SEM (N = 4).

of *Ci*IL-34 was found to be the highest in the spleen, much higher than that in other tissues.

3.3. Expression analysis of CiIL-34 during bacterial and viral infection

The expression of *Ci*IL-34 were examined in fish in response to *F. cloumnare* infection (Fig. 4). In spleen, the *Ci*IL-34 was significantly down-regulated at 24 h but showed up-regulation at 72 h. In contrast, in head kidney, the increase of *Ci*IL-34 expression was detected only at 12 h but not at other time points.

The expression of *Ci*IL-34 was also investigated in fish during a 14 days challenge trial after GCRV infection (Fig. 5). Four tissues including gills, head kidney, intestine and spleen were collected at 1, 3, 7 and 14 d for gene expression analysis. Up-regulation of *Ci*IL-34 was detected in spleen at 7 d and 14 d and in gills at 3 d. Head kidney and intestine did not show apparent alteration of expression.

3.4. Modulation of CiIL-34 expression in primary head kidney leucocytes by IL-4/13B

IL-4/13 has been shown to be involved in regulating monocyte/macrophage functions in fish [29,30]. In this study, the recombinant protein of *Ci*IL-4/13B was produced in bacteria and purified (Fig. 6). The SDS-PAGE analysis gave rise to a band of approx. 17 kDa, matching the expected size of the tagged fusion protein containing the mature *Ci*IL-4/13B peptide (13.8 kDa) and the HISTEV tag (3.3 kDa). The r*Ci*IL-4/13B protein was further verified using a polyclonal antibody raised against a *Ci*IL-4/13B peptide (AVNGSQPDLRKTLLKDIIVF). The r*Ci*IL-4/13B protein was then used to stimulate primary head kidney leucocytes for 12 h at doses of 2, 20, 200 ng/mL. The cells treated with 20 ng/mL r*Ci*IL-4/13B displayed a moderate increase of *Ci*IL-4/13B expression.

3.5. Analysis of gene expression in primary head kidney macrophages stimulated by recombinant CiIL-34 protein

To evaluate the biological activity of *Ci*IL-34, the recombinant *Ci*IL-34 protein was produced in bacteria and purified using size exclusion chromatography (Fig. 7). A protein of the *Ci*IL-34 protein was then used to stimulate the primary head kidney macrophages and the effect on expression of inflammatory genes including IL-1β, IL-6, IL-8, IL-10 and TGF-β1 was evaluated by qPCR (Fig. 8). After exposure to 2 ng/mL protein for 12 h, IL-8 and IL-10 were down-regulated, whilst the expression of IL-1β, IL-6 and TGF-β1 was unaltered (Fig. 8A). However, the mRNA expression levels of IL-1β and IL-6 were elevated after stimulation with 20 ng/mL and 200 ng/mL of r*Ci*IL-34, with decreases of IL-8 and TGF-β1 transcripts (Fig. 8B). In addition, IL-8 was induced by a higher dose of r*Ci*IL-34 protein (200 ng/mL) at 24 h protein (Fig. 8C).

To confirm the bioactivity of bacteria-derived r*Ci*IL-34, the r*Ci*IL-34 was also produced in HEK293T cells. For this, the entire *Ci*IL-34 coding region was inserted into the pcDNA3.1 vector, transfected into the HEK293T cells and cell lysate (containing r*Ci*IL-34) was collected at 48 h post transfection. The pcDNA3.1 vector was also transfected into the HEK293T cells as control. The expression of r*Ci*IL-34 was confirmed by Western blotting using a polyclonal antibody raised against *Ci*IL-34. A protein of approx. 31 kDa was detected (Fig. 9A), which was 4.5 kDa bigger than the theoretical molecular weight of pro-*Ci*IL-34 (26.5 kDa). The difference of molecular weight could be due to the glycosylation of *Ci*IL-34 expressed in the HEK293T cells. After protein validation, the r*Ci*IL-34 containing medium and control medium were used to incubate the primary head kidney macrophages for 12 h. After incubation with undiluted r*Ci*IL-34 containing medium, the expression of IL-1β, IL-6 and IL-8 was induced whilst IL-10 was down-regulated (Fig. 9B). The TGF-β1 expression was not affected. Dilution of r*Ci*IL-34 containing medium (1:1, v/v) resulted in induction of IL-8 expression and inhibition of IL-10 and TGF-β1 and further dilution of medium (1:4, v/v) did not exhibit any effects on gene expression.

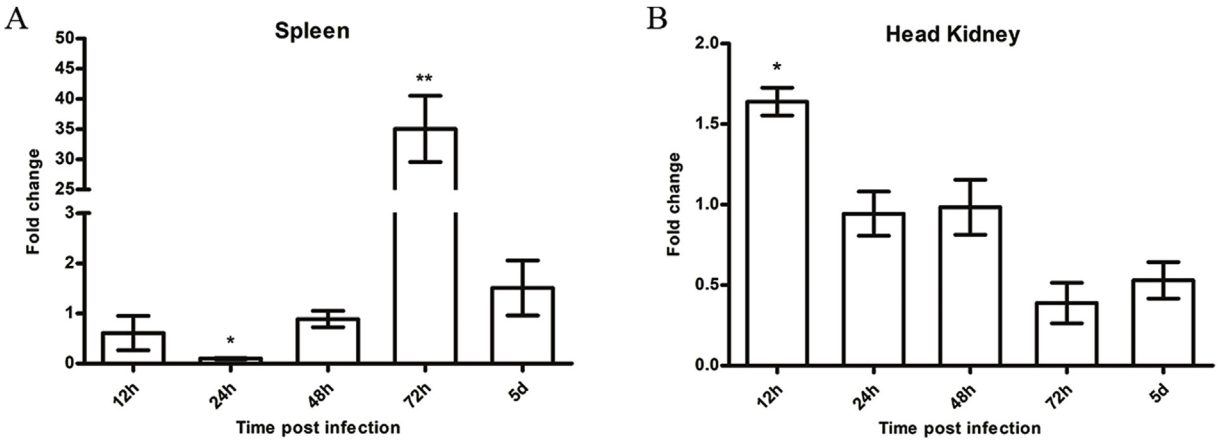

Fig. 4. Expression analysis of *Ci*IL-34 after infection of *F. cloumnare*. Grass carp were i.p. injected with 100 μL of *F. cloumnare* (10^7 CFU/mL in PBS) or equal volume of PBS and head kidney and spleen collected at 12 h, 24 h, 48 h, 72 h and 5 d after injection. The EF-1α gene was used as an internal control. Fold change was calculated by comparing the average levels of expression of infected tissues with that of corresponding control groups. Data are shown as mean ± SEM (N = 4). *$p < 0.05$ or **$p < 0.01$ are considered significant.

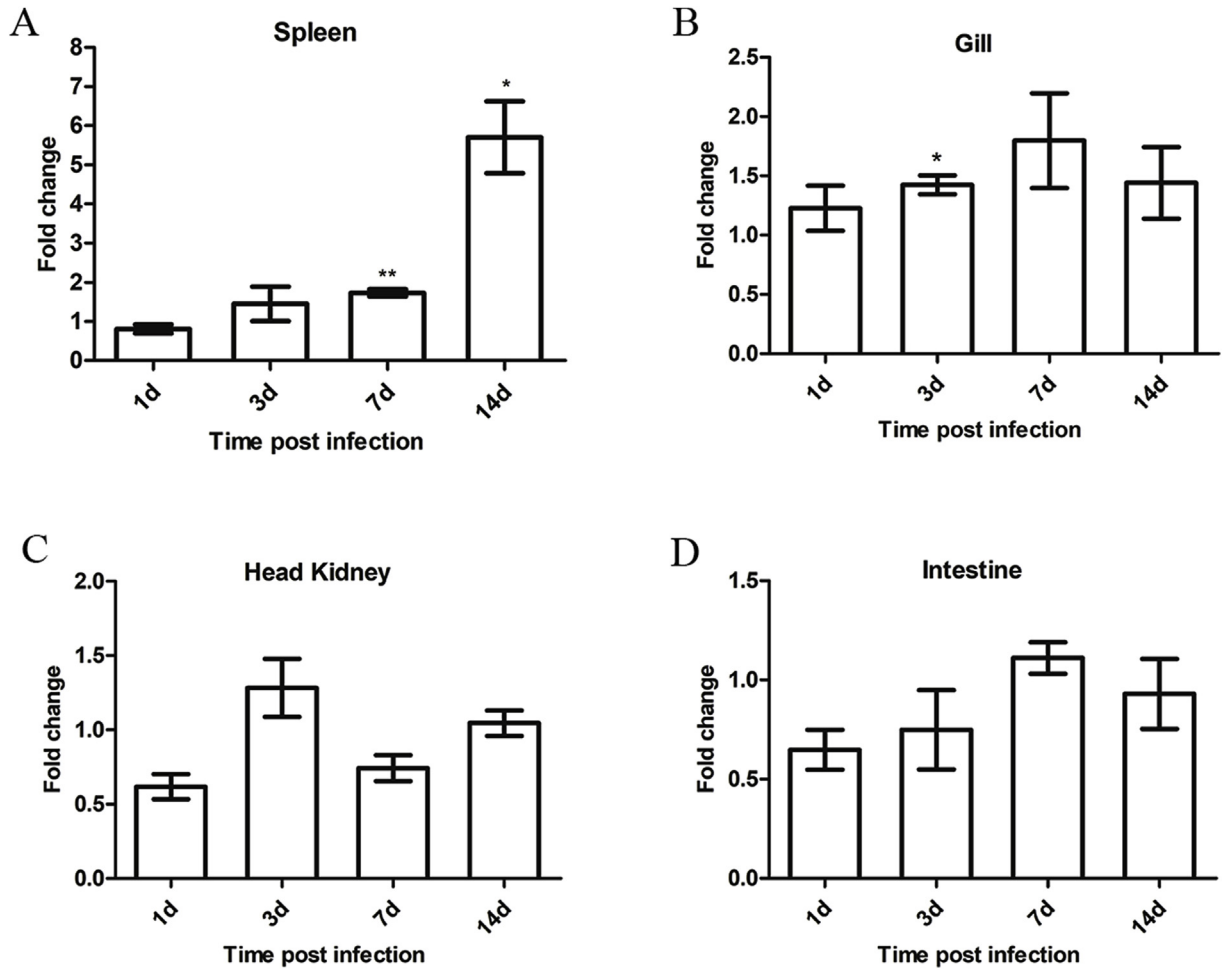

Fig. 5. Expression analysis of *Ci*IL-34 after infection of GCRV. Grass carp were i.p. injected with 200 μL GCRV (in DMEM medium) and control fish received an equal volume of DMEM medium. The EF-1α gene was used as an internal control. Fold change was calculated by comparing the average levels of expression of infected tissues with that of corresponding control groups. Data are shown as mean ± SEM (N = 4). $*p < 0.05$ or $**p < 0.01$ are considered significant.

4. Discussion

In this study, an IL-34 homologue (termed *Ci*IL-34) was identified in grass carp and functionally characterized. It shares a conserved genomic organization of 6 exons and 5 introns and synteny with vertebrate IL-34s. The grass carp and other fish IL-34 proteins possess a cationic lysine/arginine rich motif (RKx[R/K]K) near the C-terminus, which is absent in the mammalian IL-34s. The importance of this motif

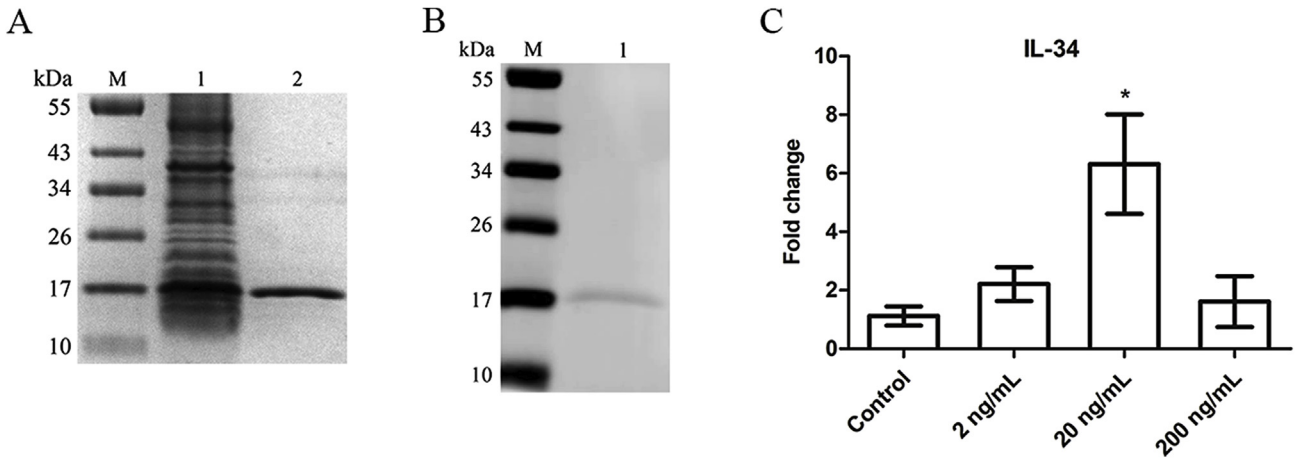

Fig. 6. Expression analysis of *Ci*IL-34 in primary head kidney leucocytes after stimulation with rIL-4/13B. (A) SDS-PAGE analysis of purified rIL-4/13B. (B) Western blotting analysis. (C) Expression analysis of *Ci*IL-34 in primary head kidney leucocytes after stimulation with rIL-4/13B. The head kidney leucocytes were stimulated with rIL-4/13B for 12 h and subject to qPCR analysis of *Ci*IL-34 expression. The EF-1α gene was used as an internal control. Fold change was calculated by comparing the average levels of expression of infected tissues with that of corresponding control groups. Data are shown as mean ± SEM (N = 4). $*p < 0.05$ or $**p < 0.01$ are considered significant.

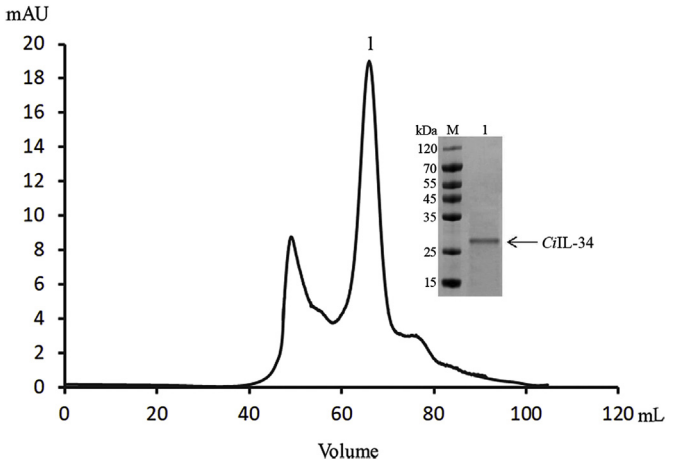

Fig. 7. Purification of r*Ci*IL-34 in bacteria. The r*Ci*IL-34A was expressed in *E. coli* BL21 (DE3) cells as inclusion bodies, refolded and purified by size exclusion chromatography. Fraction 1 was checked by SDS-PAGE.

is yet to be determined. However, it has been shown that a similar cationic motif, termed nuclear translocation site (NLS), is present in the C-terminal region of fish interferon gamma (IFN-γ) and IFN-γ related proteins known to be essential for the biological activities [31,32]. Removal of the NLS abrogates the activities of trout IFN-γ [31].

It has been reported that in mammals IL-34 is a glycosylated protein [33] The O-linked glycosylation occurs at the C-terminal tail where multiple potential aa residues responsible for glycosylation are predicted. However, this region is absent in fish homologues (Fig. 1). Glycosylation results in the higher molecular weight of protein than the theoretical size when analysed by the SDS-PAGE. Akin the observation in the humans, the r*Ci*IL-34 (approx. 31 kDa) expressed in HEK293T cells differs by approx. 4.5 kDa from the theoretical MW of the mature *Ci*IL-34 (26.5 kDa) (Fig. 9), which is likely to be resulted from glycosylation. It is probable that glycosylation of r*Ci*IL-34 could be mainly via *N*-linked mode since four potential *N*-linked glycosylation sites (NxT/S) are predicted in the *N*-terminal region of *Ci*IL-34. Interestingly, one of the glycosylation sites is well conserved among fish and human IL-34 (Fig. 1). Besides, fish have an additional conserved *N*-linked glycosylation site. The results of bioactivity analyses show that the r*Ci*IL-34 proteins derived from bacteria and HEK293T cells are equally bioactive in modulating gene expression in primary macrophages, suggesting glycosylation may not be essential for all the activities of *Ci*IL-34. The differences of protein features between fish and humans could provide insight into the importance of glycosylation in unveiling IL-34 functions in vertebrates from evolution perspectives.

Analysis of gene expression in tissues reveals that *Ci*IL-34 is constitutively expressed (Fig. 3). The highest expression was detected in spleen. These results are in line with previous findings in rainbow trout and large yellow croaker but contrasts with that in grouper where brain exhibits the highest level of expression [13,14]. In Xenopus, a notable high level of IL-34 expression is also reported in the spleen [7]. Spleen is regarded as the major primary lymphoid organ in fish, where

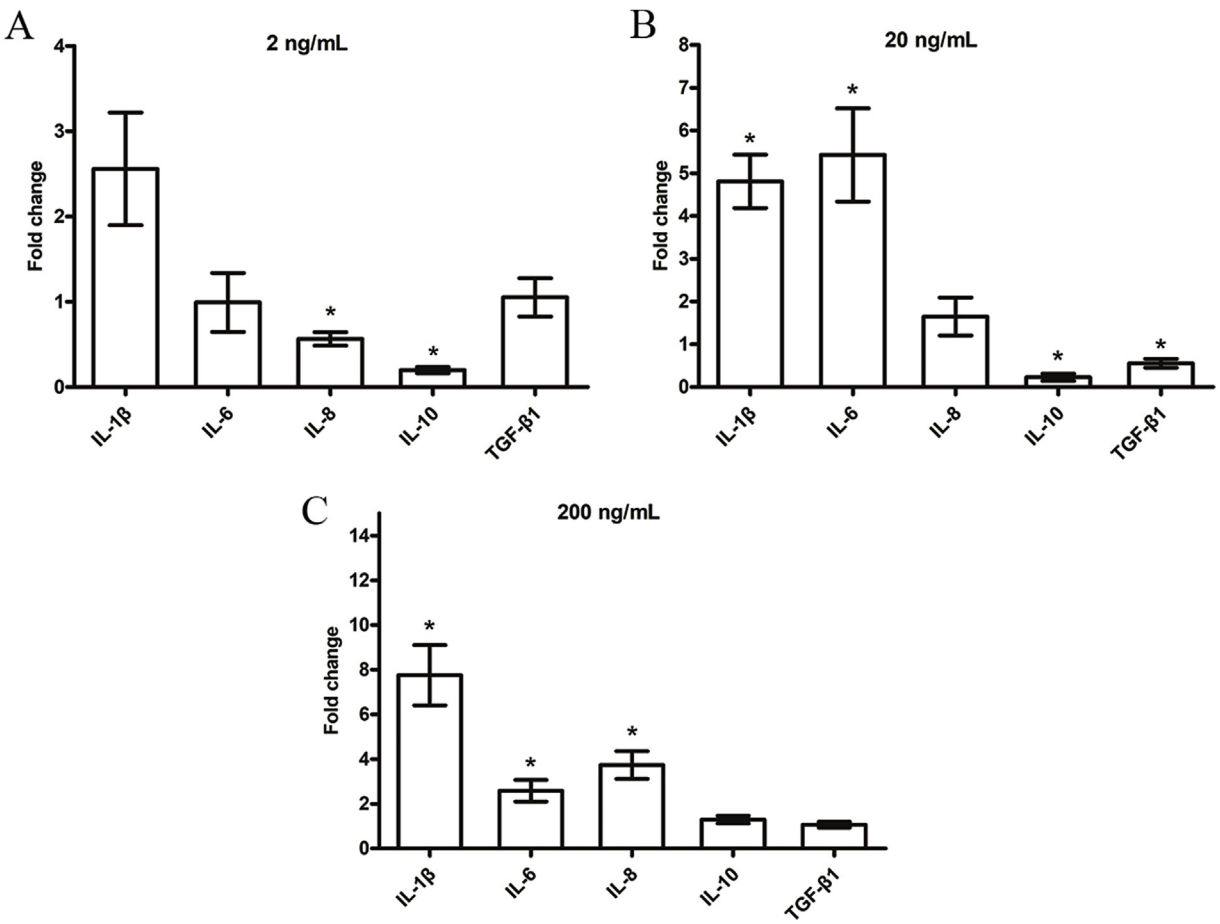

Fig. 8. The effects of bacteria-derived r*Ci*IL-34 on cytokine expression in primary head kidney macrophages. The primary head kidney macrophages were prepared as described in Materials and Methods and stimulated with r*Ci*IL-34 for 12 h. The mRNA expression of IL-1β, IL-6, IL-8, IL-10 and TGF-β1 was analysed by qPCR. The EF-1α gene was used as an internal control. Fold change was calculated by comparing the average levels of expression of infected tissues with that of corresponding control groups. Data are shown as mean ± SEM (N = 4). $*p < 0.05$ or $**p < 0.01$ are considered significant.

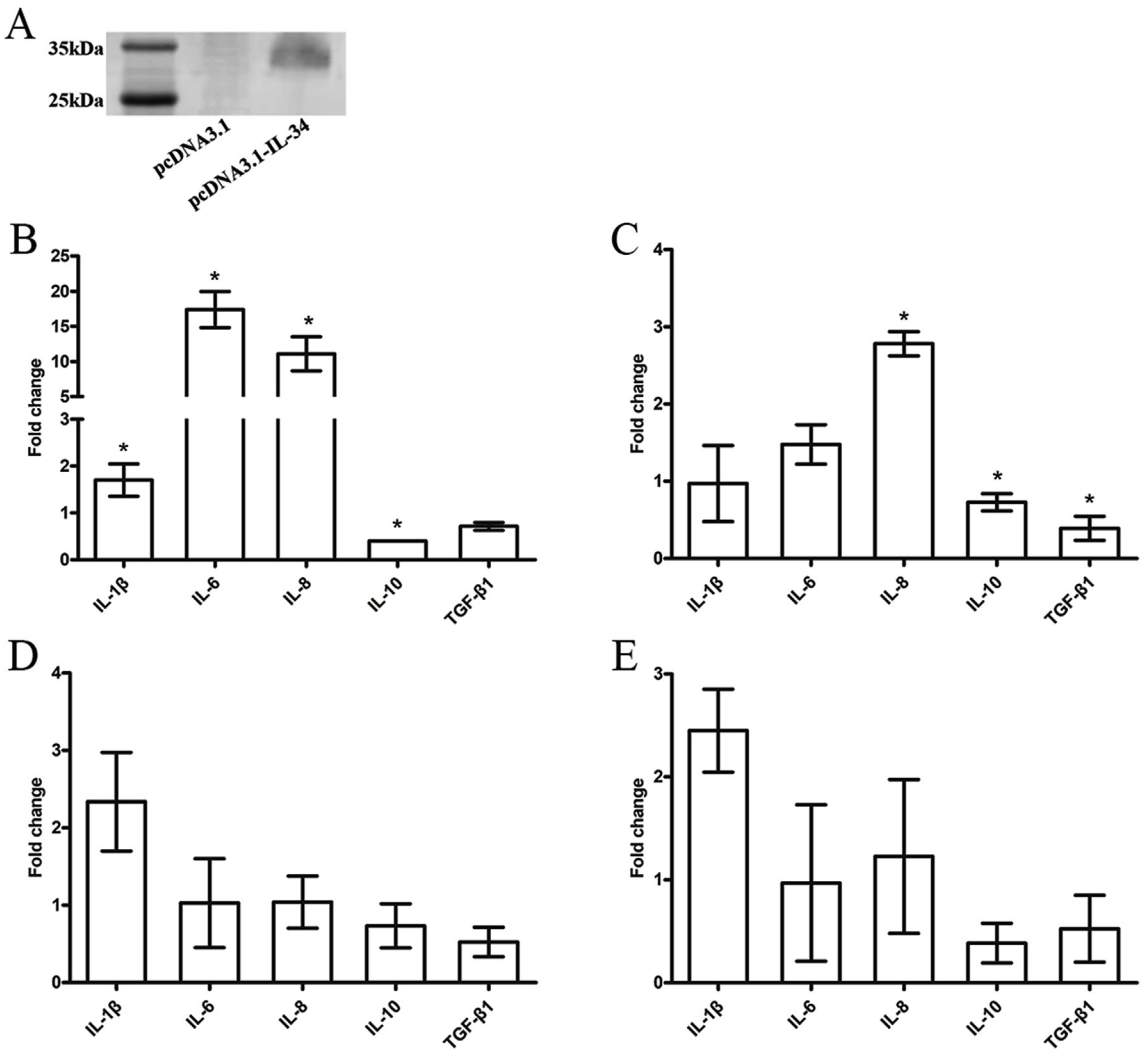

Fig. 9. The effects of HEK293T cell-derived r*Ci*IL-34 on cytokine expression in primary head kidney macrophages. (A) Western blot analysis of r*Ci*IL-34. The HEK293T cells were transfected with *Ci*IL-34 expression plasmid and collected at 48 h. The cell lysate was subject to Western blotting using the *anti-Ci*IL-34 polyclonal antibody raised. Undiluted medium (B) and diluted media (C, 1:1; D, 1:2; E, 1:4; v/v) were used for stimulation of primary macrophages for 12 h. The EF-1α gene was used as an internal control. Fold change was calculated by comparing the average levels of expression of infected tissues with that of corresponding control groups. (Data) are shown as mean ± SEM (N = 4). $*p < 0.05$ or $**p < 0.01$ are considered significant.

monocytes/macrophages and lymphocytes reside, differentiate and interact. It is possible that IL-34 could play a role in such events. Studies in rainbow trout and grouper demonstrate that parasite infection can lead to elevation of IL-34 transcript levels in tissues, especially the infected sites [13,14]. Besides, in large yellow croaker, IL-34 is shown to be up-regulated in response to polyI:C stimulation and *Vibrio anguillarum* infection [15]. The present study reveals that pathogenic bacteria and viruses can also modulate the IL-34 expression (Figs. 4 and 5). Curiously, in spleen, it is observed that at the early stage of infection with *F. cloumnare* (24 h), the *Ci*IL-34 expression is inhibited, whilst at the late stage of infection (72 h) markedly increased. Additionally, during GCRV infection, up-regulation of *Ci*IL-34 is also detected in the spleen, suggesting that IL-34 could be involved in antiviral response in fish. In fact, a recent study reports that primary Xenopus macrophages, when cultured with recombinant IL-34, display strong antiviral ability against Frog Virus 3 infection [7]. Furthermore, PAMPs and inflammatory cytokines such as LPS, polyI:C, IL-1β and IFN-γ induces IL-34 expression in trout primary head kidney macrophages [13]. These observations are line with the studies in humans showing that the osteoblasts and gingival fibroblasts display enhanced IL-34 expression after treatment of TNF-α and IL-1β [12,34]. The present study shows that IL-4/13B is able to stimulate IL-34 expression in the primary head kidney macrophages (Fig. 6), although at a moderate degree. IL-4/13B is a typical Th2 cytokine and has been shown to be involved in the modulation of immune response of Atlantic salmon post smolts after infection with amoeba *Paramoeba perurans* [35]. These findings imply that IL-34 is regulated by multiple immune factors and is an important cytokine regulating macrophage functions and disease pathogenesis in fish.

The biological effects of *Ci*IL-34 on the cytokine gene expression were evaluated in enriched primary head kidney macrophages. The bacteria-derived r*Ci*IL-34 is shown to induce expression of IL-1β, IL-6 and IL-8 whilst inhibit IL-10 and TGF-β1 (Fig. 8). These effects are confirmed using the r*Ci*IL-34 expressed in HEK293T cells. IL-1β, IL-6 and IL-8 are classical proinflammatory cytokines whilst IL-10 and TGF-β1 suppress inflammation process. In humans, it has been shown that high levels of IL-34 has been detected in several inflammation diseases such as rheumatoid arthritis, Sjogren's syndrome, and inflammatory bowel disease [5,36,37]. The results reported in this study indicate that IL-34 is an important proinflammatory cytokinein fish. To our knowledge, this is the first study reporting on the functions of IL-34 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.2019.05.059.

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