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Interleukin-2 (IL-2) in flounder (*Paralichthys olivaceus*): Molecular cloning, characterization and bioactivity analysis

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ABSTRACT

Interleukin-2 (IL-2) is mainly produced by CD4$^+$ T helper lymphocytes, which is an important immunomodulatory cytokine that primarily promotes activation, proliferation and differentiation of T cells. In the present study, flounder (*Paralichthys olivaceus*) interleukin 2 homologue (poIL-2) was identified for the first time, and its expression patterns were characterized in healthy, virus- or bacteria-infected flounder. The full-length cDNA sequences of poIL-2 was 989 bp with an open reading frame of 423 bp coding a polypeptide of 140 amino acids (aa). The deduced aa sequences shared low similarities (< 53%) with other known fish IL-2s. Multiple alignment of aa sequences revealed that poIL-2 own the classical IL-2 family signature of "C-X(3)-EL-X(2)-(T/V)-(V/M/L)-(K/T/R)-X-EC" and "DS-X-(F/L)Y(A/T/S)P". In healthy flounder, IL-2 mRNA was highly expressed in PBLs, spleen and hindgut, and moderately expressed in gill, trunk kidney and stomach. PHA, LPS and Con-A could effectively induce poIL-2 expression in primary cultured peripheral blood leukocytes *in vitro*. poIL-2 transcripts were significantly up-regulated in spleen, kidney, gill and hindgut post infections with *Edwardsiella tarda* and Hirame novirhabdovirus (HIRRV). The eukaryotic expression vector encoding poIL-2 (pcIL-2) was constructed and intramuscularly injected, which could be successfully expressed in flounders and induced significantly higher expressions of six immune related genes including poIL-2, *β*-defensin, CD4-1, CD8α, IFN-γ and TNF-α compared with the injection with control plasmid. Moreover, pretreatment with pcIL-2 could markedly increase the survival rate of flounder challenged with HIRRV. Our results demonstrated that poIL-2 plays an important role in the induction of immune responses and immune defense against bacterial and virus infection, which indicated its potential use as an immunopotentiator to prevent diseases in flounder.

1. Introduction

Interleukins (IL) are important immune cell cytokines that exerts a wide spectrum of effects on the immune system, including regulating immune activation and homeostasis, hematopoiesis and inflammation [1]. To date, increasing interleukin genes have been cloned and characterized within teleost fish, however, characterized interleukin genes that produced by T cells and play key roles in lymphocyte-specific responses are still very limited. IL-2 was produced by T helper (Th) cells and originally described as a T-cell growth factor (TCGF) that found in mitogen-stimulated lymphocyte cultures [2]. It is synthesized and secreted primarily by Th (CD4$^+$) lymphocytes upon activation of resting T cells by antigens or certain mitogens like concanavalin A (ConA) or phytohemagglutinin (PHA), or as a result of interaction of the T-cell receptor with MHC complex on the surface of antigen-presenting cells [2–4]. Although the majority of IL-2 is derived from activated CD4$^+$ T cells that in response to TCR stimulation, transient induced IL-2 mRNA transcript and protein generation was also detected in CD8$^+$ T lymphocytes, dendritic cells, thymic regulatory T (Treg) cells and murine dendritic cells by gram-negative bacteria [5–7]. Besides, IL-2 also plays vital roles in the regulation of other immune cells, including activation of natural killer (NK) cells [8], translation of immunoglobulin (Ig) J chain that indirectly for Ig synthesis and secretion by B cells [9] and activation cytotoxic cell clones following antigen stimulation inducing lymphatic factors production [4,10].

The pleiotropic effects of IL-2 on lymphocytes including T, B and NK cells are mediated by IL-2 receptor which consist of an α, β and γ chain. IL-2 binds to the receptor complex would elicit intracellular signaling events that ultimately lead to cell proliferation [11]. The three subunits can be expressed individually or in various combinations, resulting in

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receptors that bind IL-2 with markedly different affinities. Low affinity intermediate receptors were first formed by the β and γ chain subunits, and then co-expressed with the α chain subunits to form highly affinity IL-2R. The α chain enhances the affinity of IL-2R for ligand, it does not participate in signal transduction in any way. Whereas, both the β and γ chains belong to a type I cytokine receptor superfamily, they are responsible for the signaling. Following IL-2 bind to IL-2R form ligand-receptor complex triggering phosphorylation of transcription factors and leading to induction of gene expression [12]. It is known that cytokines such as anti-inflammatory cytokines IL-4 and IL-10 strongly influence IL-2 responses [13,14]. The Th2- or macrophage-derived IL-10 can directly inhibit CD4$^+$ T-cells growth and then restrain IL-2 production [15]. In addition, IL-4 can affect the transcript of IL-2 receptor Alpha (IL-2Rα) in T cells, and inhibit the expression of the IL-2 receptor Beta (IL-2Rβ) chain in B cells [16,17]. Recently, it was also reported that B lymphocyte-induced maturation protein (Blimp-1) acts as the negative regulator of IL-2 functions in T and B cells and suppresses IL-2 expression in the way of negative feedback loop [18,19].

To date, the information and function of IL-2 in teleost fish was limited, and only three IL-2 homologues in *Oncorhynchus mykiss* [20], *Fugu rubripes* [21] and *Larimichthys crocea* [22] were found so far. In this study, the IL-2 gene in flounder (*Paralicthys olivaceus*) was cloned, and its gene expression profile was analyzed in primary cultured leucocytes and flounder after various stimulations. Meanwhile, the expression profiles of six immune-related genes including antimicrobial peptides (*β*-defensin), T-cell surface markers (CD4-1 and CD8α), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and poIL-2 were investigated post stimulation with the eukaryotic expression plasmid encoding poIL-2 (pcIL-2). Moreover, immune enhancement effect of pcIL-2 on HIRRV infection was determined in flounder. This is the first report to demonstrate biological activities of IL-2 in flounder, which would promote better understanding of its function in the immune system in teleosts.

2. Materials and methods

2.1. Fish and sampling

Five hundred healthy flounders with body weights of 30 ± 5 g were obtained from a fish farm in Rizhao city of Shandong province, China. The fish were quarantined in an oxygen-supplying laboratory aquaria (500 L) using electric air compressors. Optical water conditions were controlled as follows: temperature at 20 ± 1 °C, dissolved oxygen 6.0 ± 0.5 mg/L, pH = 7.0, total ammonia < 0.2 mg/L and nitrite < 0.02 mg/L, and monitored by the YSI multi-probe system (YSI 556). Fish were fed with commercial diet twice daily. All fish were acclimatized for at least 2 weeks prior to treatment.

For poIL-2 cloning and tissues distribution detection, the peripheral blood leucocytes (PBLs), gill, skin, spleen, head kidney, trunk kidney, liver, foregut, midgut, hindgut, muscle, stomach and heart were randomly collected from three healthy flounder. For the Poly (I: C) (50 μg/mL), lipopolysaccharide (LPS, 500 μg/mL), PHA (10 μg/mL) or Con-A stimulation, the PBLs were randomly sampled from five individuals at 1, 3, 6 and 12 h post stimulation. For quantitative real-time PCR (qRT-PCR), the spleens were randomly sampled from three fish in each group at 0, 24, 48, 72 and 120 h post pcIL-2 treatment. For poIL-2 expression detection post challenge, the spleens, gills, kidney and hindgut were randomly sampled from three fish in each group at 0, 6, 12, 24, 48, 72 h, 5, 7 and 14 d post vaccination. For detection of the plasmid pcIL-2 distribution and transcription *in vivo*, spleens and muscles were randomly collected from three fish of each group at 5 d post vaccination. For detection of the pcIL-2 expression *in vivo*, muscles were randomly collected from three fish of each group at 5 d post vaccination. Samples were kept in Sample Protector (Baosheng, Dalian, China) and stored at −80 °C until use. Fish were anaesthetized with MS-222 before manipulations.

Table 1
Primers used for gene cloning and qPCR in this study.

Primer names	Oligonucleotide sequence (5′→3′)
RACE PCR	
3′-RACE 1st	TCCAACAAATGTGAAGGTAG
3′-RACE nested	TGAGAGAACTGAAAGGAACA
5′-RACE 1st	TGTTCCTTTCAGTTCTCTCA
5′-RACE nested	GCATTCTACCTTCACATTTG
UPM	CTAATACGACTCACTATAGGGCA AGCAGTGGTATCAACGCAGAGT
NUP	AAGCAGTGGTATCAACGCAGAGT
IL-2-cDNA cloning	
IL-2-cDNA-F	CACCTGCTCTAAACAACTTCCTTATC
IL-2-cDNA-R	ACACAGCATATTATAGACAATAAATA
IL-2 ORF cloning	
IL-2-F	CGGGGTACCAGATGTTTACCCATAGAGGATG
IL-2-R	CCGGAATTCTGGAGAAGCTTTGCATGGTTG
IL-2 qRT-PCR	
qIL-2-F	AGAGGATGCCAGTATCGGTTT
qIL-2-R	ACATTCTGCGGAGGTCGTTA
EF-1α- F	ATGTGTGACGACGAAGTAGC
EF-1α- R	GAAGCACTTCCTGTGAACGA
18S rRNA-F	GGTCTGTGATGCCCTTAGATGTC
18S rRNA-R	AGTGGGGTTCAGCGGGTTAC
Construction of pcIL2	
IL-2eF	TAATACGACTCACTATAGGGGATGGAGCACTT
IL-2eR	TAGAAGGCACAGTCGAGGTGGAGAAGCTTTG
T7	TAATACGACTCACTATAGGG
T3	ATTAACCCTCACTAAAGGGA
β-defensin-F	ACGCAGTTTCAGACCCAACA
β-defensin-R	CGGAACAGCCAAGAGCTCCA
CD4-1-F	CCAGTGGTCCCCACCTAAAA
CD4-1-R	CACTTCTGGGACGGTGAGATG
CD8α-F:	ATTAGTTGTGAAAGAGGGGGC
CD8α-R	TGAGGAATCAATGTATGGGGA
IFN-γ-F	TGTCAGGTCAGAGGATCACACAT
IFN-γ-R	GCAGGAGGTTCTGGATGGTTT
TNFα-F	AGGAGGCAGCGGAAAAACA
TNFα-R	TAGGCGTCCTCCTGACTCTTCT

2.2. Cloning of the full-length poIL-2 cDNA

Total RNA was extracted from kidney of healthy flounder with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into first-strand cDNA using Super Script Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). A partial sequence was obtained from previously constructed cDNA library of flounder and Rapid amplification of cDNA ends (RACE) methods was performed to clone the full-length cDNA of poIL-2 with specific primers by SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Mountain View, CA) [23]. For 3′ RACE, the first round of PCR was performed using the primer pair of 3′-RACE 1st/UPM (adaptor primer). The resultant products were diluted (1:10) and re-amplified in the second round PCR using the primer pair of 3′-RACE nested/NUP. For 5′ RACE, the primers of the first round and second round were 5′-RACE 1st/UPM and 5′-RACE nested/NUP, respectively. The full-length of poIL-2 cDNA were checked by the pair of primers IL-2-cDNA-F/IL-2-cDNA/R (shown in Table 1). The PCR products were electrophoresed by 2% agarose gel and purified with a commercial gel extraction kit (Tiangen, Beijing, China). The purified PCR products were ligated into a pMD19-T Vector (TaKaRa, Otsu, Japan) and then transformation into competent *Escherichia coli* DH5 cells. The positive clones were screened by Ampicilin selection and colony PCR, and then sequenced on an ABI 3130xl Genetic analyzer (Applied Biosystems, Waltham, USA).

2.3. Sequence and phylogenetic analyses

The amino acid (aa) sequences of poIL-2 were analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast), and the molecular weights, the isoelectric point (pI) and the net charge of the

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1    AAGTATACCAACACAACACCTGCTCTAAACAACTTCCTTATCACACTGACAAACCTTGAAGCTGAACATGGAGCACTTTATTGGGATTCT
1                                                                        M  E  H  F  I  G  I  L
91   CTTTCAGATTGCTGCTTTTTTTCTTTGTCTCCAAGCCAGATGTTTACCCATAGAGGATGCCAGTATCGGTTTCATACGGACAAATGTCAC
9     F  Q  I  A  A  F  F  L  C  L  Q  A  R  C  L  P  I  E  D  A  S  I  G  F  I  R  T  N  V  T
181  ATGTCTAAAAGATTCTAAATTCTACGCTCCAACAAATGTGAAGGTAGAATGCATTCCCGCAGCAATGGACTGTGTCATGAGAGAACTGAA
39    C  L  K  D  S  K  F  Y  A  P  T  N  V  K  V  E  C  I  P  A  A  M  D  C  V  M  R  E  L  K
271  AGGAACAGTCAGGCCGGAATGCGATGGCCTTATGACATACGTACTTCAAGCTATCGAATTCTTCGAACTCAAGACAGAAGAAAAAGACCC
69    G  T  V  R  P  E  C  D  G  L  M  T  Y  V  L  Q  A  I  E  F  F  E  L  K  T  E  E  K  D  P
361  TGCACCAACGACCTCCGCAGAATGTACATGTGAGAAATGGCCTCAAACTGGTTTCAGTGATTTTCTGGACCAAGTGGAATCTTTACTTCA
99    A  P  T  T  S  A  E  C  T  C  E  K  W  P  Q  T  G  F  S  D  F  L  D  Q  V  E  S  L  L  Q
451  GCTCGAGAAATCAGCCCAACCATGCAAAGCTTCTCCATAAGCTGCACTGAAAAAATTATATTACTAAAATTCATCCCGGATATATGAGAG
129   L  E  K  S  A  Q  P  C  K  A  S  P  *
541  CCAGCCAATGACTCCGAAGCAAAAGTAAAAGTATTCGAGTATCTGCACATGTTGAGCGGGATGATCTCAGGTATATCTTGGTTTTTAATC
631  ATACTATTTGTTATGATGTTGAGCACTGCTCTACCATATCTGCATTAAAAAAAACAATATGGGGATAAGGGGACTATTCATATTGAATAT
721  TTTGAGTTTTATTTTTATTTATTGTTACAATTATTCTTTTTATCATTTTACCACATAACTGCTGTGAAATAAATGTTAAAGTTCTCGGTT
811  TATTTAAAATGAGAATGTTATGTAATGTAAGCTCCAGTACAGCTCTGAATATCTATATATTTATTGTCTATAATATGCTGTGTGTTGGTG
901  CCATAGGCCTGAATGTATAACTTAATGAACGAAGAAAAATTAAACAAACATAAAACTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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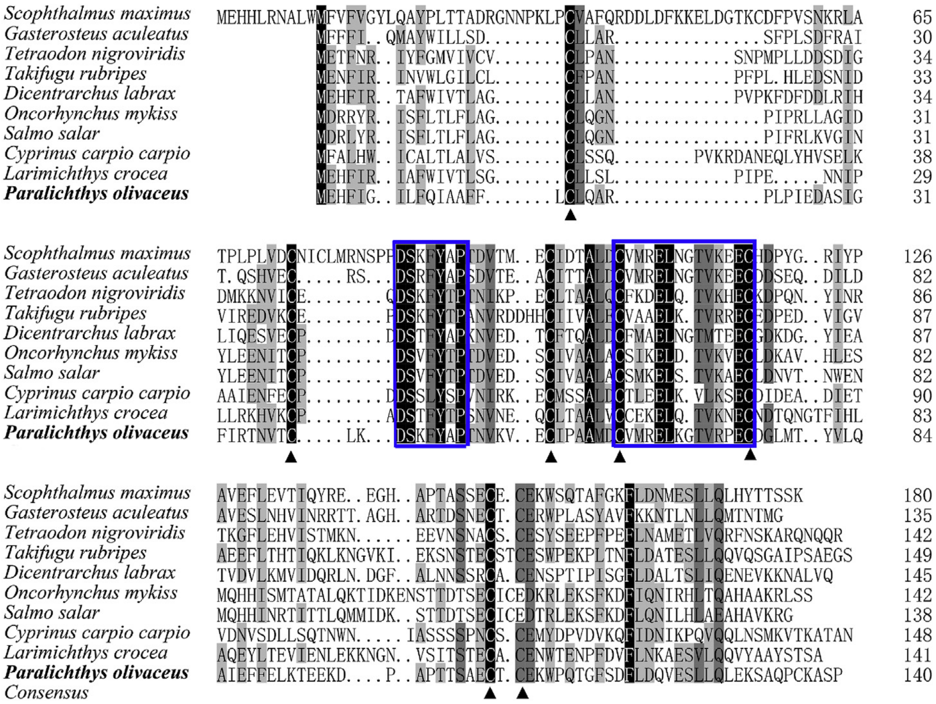
Fig. 1. Nucleotide and cDNA-derived aa sequences of poIL-2 (GenBank accession nr. KY307833). Nucleotides shown in lower cases represent the UTRs, while capital letters represent the ORF. The start codon is marked in red bold and the stop codon is marked in black bold. The 3′ UTR and the RNA instability motifs (ATTTA) are double underlined, and the polyadenylation signal (AATTAAA) is shown in a black box. The predicted signal peptide of poIL-2 is in marked red, and the conserved IL-2 family signature motif is shaded. The conserved cysteine residues are marked by black bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



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Scophthalmus maximus      MEHHLRNALWMFVFVGYLQAYPLTTADRGNNPKLPCVAFQRDDLDFKKELDGTKCDFPVSNKRLA   65
Gasterosteus aculeatus              MFFFI..QMAYWILLSD.......CLLAR...............SFPLSDFRAI   30
Tetraodon nigroviridis              METFNR..IYFGMVIVCV......CLPAN............SNPMPLLDDSDIG   34
Takifugu rubripes                   MENFIR..INVWLGILCL......CFPAN............PFPL.HLEDSNID   33
Dicentrarchus labrax                MEHFIR..TAFWIVTLAG......CLLAN............PVPKFDFDDLRIH   34
Oncorhynchus mykiss                 MDRRYR..ISFLTLFLAG......CLQGN...............PIPRLLAGID   31
Salmo salar                         MDRLYR..ISFLTLFLAG......CLQGN...............PIFRLKVGIN   31
Cyprinus carpio carpio              MFALHW..ICALTLALVS......CLSSQ........PVKRDANEQLYHVSELK   38
Larimichthys crocea                 MEHFIR..IAFWIVTLSG......CLLSL............PIPE.....NNIP   29
Paralichthys olivaceus              MEHFIG..ILFQIAAFF......LCLQAR...............PLPIEDASIG   31

Scophthalmus maximus      TPLPLVDCNICLMRNSPPDSKFYAPTDVTM..ECIDTALDCVMRELNGTVKEECHDPYG..RIYP  126
Gasterosteus aculeatus    T.QSHVEC.....RS...DSRFYAPSDVTE..ACITTALDCVMRELNGTVKEECDDSEQ..DILD   82
Tetraodon nigroviridis    DMKKNVICE........QDSKFYTPTNIKP..ECLTAALQCFKDELQ.TVKHECKDPQN..YINR   86
Takifugu rubripes         VIREDVKCE........PDSKFYTPANVRDDHHCIIVALECVAAELK.TVRRECEDPED..VIGV   87
Dicentrarchus labrax      LIQESVECP........DDSTFYAPKNVED..TCFTQALDCFMAELNGTMTEECGDKDG..YIEA   87
Oncorhynchus mykiss       YLEENITCP.........DSVFYTPTDVED..SCIVAALACSIKELD.TVKVECLDKAV..HLES   82
Salmo salar               YLEENITCP.........DSVFYTPTDVED..SCIVAALACSMKELS.TVKAECLDNVT..NWEN   82
Cyprinus carpio carpio    AAIENFECP........DDSSLYSPVNIRK..ECMSSALDCTLEELK.VLKSECDIDEA..DIET   90
Larimichthys crocea       LLRKHVKCP........ADSTFYTPSNVNE..QCLTAALVCCEKELQ.TVKNECNDTQNGTFIHL   83
Paralichthys olivaceus    FIRTNVTC.....LK...DSKFYAPTNVKV..ECIPAAMDCVMRELKGTVRPECDGLMT..YVLQ   84

Scophthalmus maximus      AVEFLEVTIQYRE..EGH..APTASSECE.CEKWSQTAFGKFLDNMESLLQLHYTTSSK        180
Gasterosteus aculeatus    AVESLNHVINRRTT.AGH..ARTDSNECT.CERWPLASYAVFKKNTLNLLQMTNTMG          135
Tetraodon nigroviridis    TKGFLEHVISTMKN......EEVNSNACS.CESYSEEPFPEFLNAMETLVQRFNSKARQNQQR    142
Takifugu rubripes         AEEFLTHTIQKLKNGVKI..EKSNSTECSTCESWPEKPLTNFLDATESLLQQVQSGAIPSAEGS   149
Dicentrarchus labrax      TVDVLKMVIDQRLN.DGF..ALNNSSRCA.CENSPTIPISGFLDALTSLIQENEVKKNALVQ     145
Oncorhynchus mykiss       MQHHISMTATALQKTIDKENSTTDTSECICEDKRLEKSFKDFIQNIRHLTQAHAAKRLSS       142
Salmo salar               MQHHINRTITTLQMMIDK..STTDTSECICEDTRLEKSFKDFLQNILHLAEAHAVKRG         138
Cyprinus carpio carpio    VDNVSDLLSQTNWN.....IASSSSPNCS.CEMYDPVDVKQFIDNIKPQVQQLNSMKVTKATAN   148
Larimichthys crocea       AQEYLTEVIENLEKKNGN..VSITSTECA.CENWTENPFDVFLNKAESVLQQVYAAYSTSA      141
Paralichthys olivaceus    AIEFFELKTEEKD....P..APTTSAECT.CEKWPQTGFSDFLDQVESLLQLEKSAQPCKASP    140
Consensus
```

Fig. 2. Multiple alignment of the deduced amino acid sequences of poIL-2 with other known vertebrate IL2s. The family signature of vertebrate IL-2s referred to as "C-X(3)-EL-X(2)-(T/V)-(V/M/L)-(K/T/R)-X-EC" and "DS-X-(F/L)Y(A/T/S)P" were boxed, and the conserved cysteines (C) were marked in "▲".

peptide were calculated using the ProtParam tool, both available on the ExPASy molecular biology server (http://www.expasy.org/tools). The signal peptide of the protein was identified using the SignalP program (version 3.0) (www.cbs.dtu.dk/services/SignalP). The ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) was used to create the multiple sequence alignment, and annotated using GeneDoc software (http://www.psc.edu/biomed/genedoc). The identities between each pair of the aa sequences were calculated using the MatGAT package (version 2.02). The phylogenetic tree was constructed using the Neighbor-Joining (N-J) method in the MEGA 5.0 software with 10,000 bootstrap replicates. The secondary structure of IL-2 was predicted by YASPIN (http://www.ibi.vu.nl/programs/yaspinwww/).

2.4. Distribution of poIL-2 mRNA in healthy flounder tissues

Total RNAs were extracted from the peripheral blood leucocytes (PBLs), gill, skin, spleen, head kidney, trunk kidney, liver, Foregut, Midgut, Hindgut, muscle, stomach and heart of healthy flounder using the Trizol reagent (Takara, Ost, Japan). The RNAs were incubated with RNase-free DNase I (Takara) for 20 min at 37 °C, and then quantified with a Nanodrop 8000 Spectrophotometer (Thermo Scientific, MA, USA). Then, the single-strand cDNA was synthesized from 1 μg total RNA using PrimeScript™ RT-PCR Kit (Takara, Ost, Japan). qRT-PCR was performed with SYBR GreenIMaster Mix (Roche, Basel, Switzerland) in a LightCycle® 480II Real Time PCR System (Roche, Basel, Switzerland). Each assay was carried out in triplicate using β-actin as the control gene [24]. Each reaction contained 1 μL of reverse and forward primers (10 μM), 10 μL of SYBR GreenIMaster, 2 μL of cDNA and RNase-free water to a total volume of 20 μL. The amplification process consisted of an initial denaturation at 95 °C for 30 s, 45 cycles of denaturation at 95 °C for 5 s and extension at 60 °C for 30 s. All data were given in terms of relative mRNA using the $2^{-\triangle\triangle Ct}$ method [25]. The specific primers used for qRT-PCR were listed in Table 1.

2.5. Expression patterns of poIL-2 in PBLs post different stimulations

The PBLs were isolated from healthy flounder by discontinuous

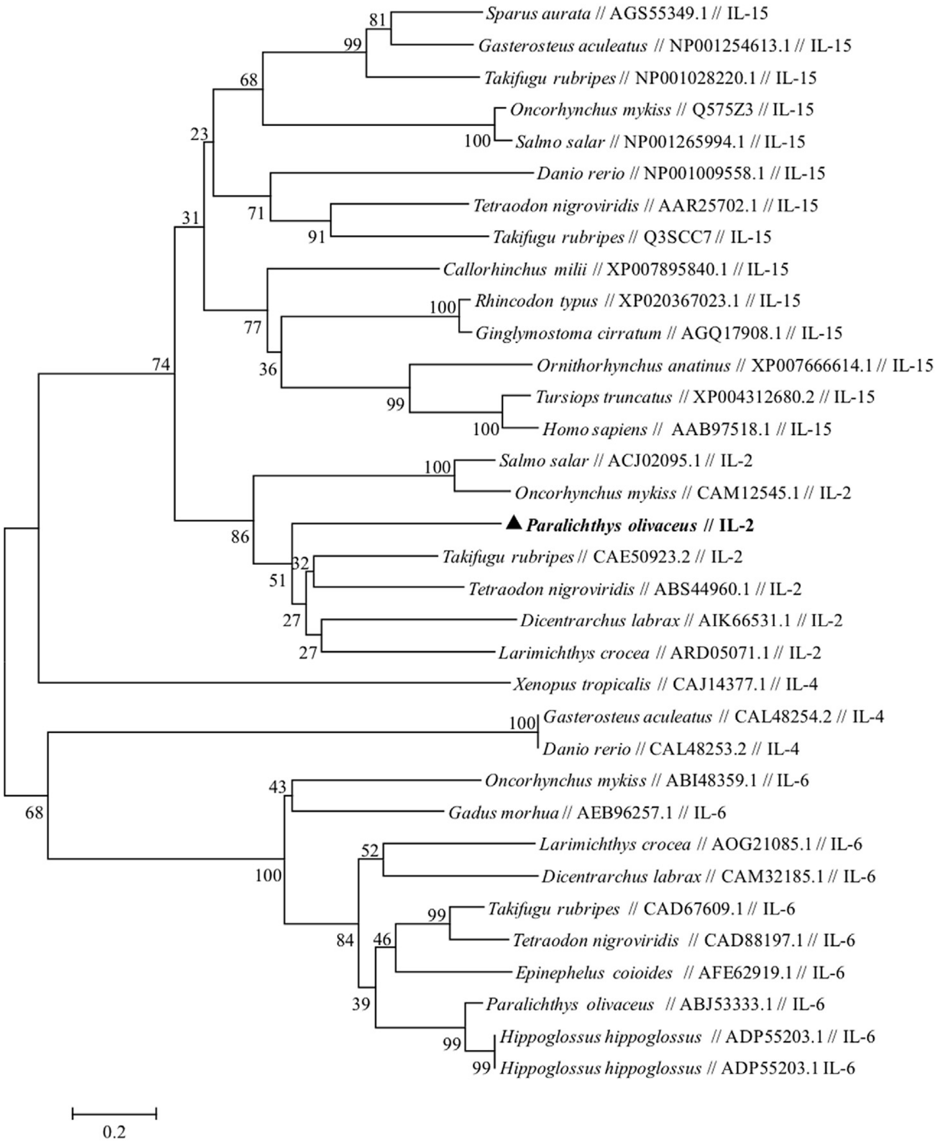

Fig. 3. Phylogenetic tree analysis of the relationship of the poIL-2 with other known IL-4, IL-6 and IL-15 family members. The tree was constructed using MEGA 5 software by the N-J method.

percoll (GE Healthcare China, Beijing, China) gradient (1.020/1.070) according to the method described previously [26]. Subsequently, the isolated PBLs were primary cultured at 24 °C in Leibovitz's L-15 medium (ThermoFisher, MA, USA), supplemented with 20% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin. The cell density was adjusted to 5×10^7 per well in 6-well plate and the cells were randomly divided into five groups. Four groups of them were stimulated with Poly (I: C) (50 μg/mL), LPS (500 μg/mL), PHA (10 μg/mL) or Con-A (10 μg/mL) [27]. The last one group was maintained as before as the control group. Each treatment group was performed in triplicate. The PBLs were randomly sampled at 1, 3, 6 and 12 h post stimulation. Total RNA isolation, single-strand cDNA synthesis and qRT-PCR operation was conducted as described above. The specific primers used for qRT-PCR were listed in Table 1.

2.6. Expression patterns of poIL-2 after E. tarda and HIRRV infection

Healthy flounders were intraperitoneal injected (i.p.) with 200 μL of pathogenic *E. tarda* HC01090721 [28] and HIRRV [29] with concentrations of 1×10^7 CFU/mL and 1×10^5 $TCID_{50}$, respectively. The fish were i.p. with a same volume of PBS used as the negative control. The HIRRV- and *E. tarda*-infected fish were transferred to 200 L tanks at 13 °C and 22 °C, respectively. At 6 h, 12 h, 1 d, 2 d, 3 d, 5 d, 7 d and 14 d post infection, the spleen, head kidney, hindgut and gill were sampled from 3 fish in each group. Total RNA isolation, single-strand cDNA synthesis and qRT-PCR operation were conducted as described above.

2.7. Plasmid construction and vaccination

Based on the genes of poIL-2 and Kozak consensus sequence, specific primers IL-2-eF/IL-2-eR were used to amplify the coding regions. The purified PCR products and expression vector pcDNA™3.1/His-myc C (pcN3, Invitrogen, NY, CA, USA) were both digested with KpnIand Eco*R*Ito construct the recombinant clones with T4 ligase. The linked products were then transformed into Trans 5α chemically competent cells and inoculated in LB plate with ampicillin at 37 °C for overnight. Then, the transformants were verified by double enzymatic digestion and sequencing. The correctly constructed recombinant plasmid was named pcIL-2. The concentrations of pcIL-2 were measured by NanoDrop 8000 spectrophotometer. For the further vaccination, the endotoxin in pcIL-2 was removed using EndoFree plasmid Kit (Tiangen, Beijing, China). Subsequently, the acclimated flounders were randomly

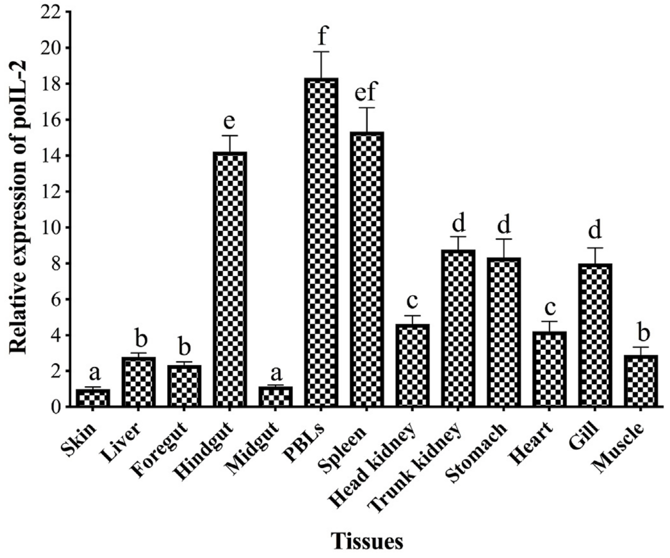

Fig. 4. Expression profiles of poIL-2 mRNA in various tissues of healthy flounder by qRT-PCR. Total RNA from flounder (n = 3) were isolated and subjected to DNase I treatment and then transcribed into cDNA. β-actin of flounder was employed as the internal reference gene.

divided into five groups containing 30 fish in each. The fish of two pcIL-2-vaccinated groups were injected intramuscularly with 100 μL of PBS containing 20 μg pcIL-2. Two groups of fish injected with 100 μL of PBS containing 20 μg pcN3 was set as the control group, and the fish only injected with 100 μL of PBS was set as the blank control group.

2.8. Detection of the distribution, transcription and expression of pcIL-2 in flounder tissues post vaccination

To examine the distribution, transcription and expression of pcIL-2 in immunized flounder, muscles and spleens were sampled from three fish of each group at 5 d post immunization. For detecting the distribution of pcIL-2, total DNA were extracted from spleens and kidneys post with TIANamp DNA Kit (Tiangen, Beijing, China). For detecting the transcription of pcIL-2, total RNA and cDNA synthesis were performed as described above. Specific primers were designed for detecting the distribution and transcription of pcIL-2 in spleens and kidneys based on the sequences of poIL-2 and pcN3 (Table 1). RT-PCR were carried out as described above using 18S rRNA as the internal control [30]. RT-PCR and PCR programs were carried out as follows: each reaction contained 1 μL of reverse and forward primers (10 μM), 2.5 μL of Taq buffer, 1 μL of cDNA, 2 μL of dNTP (2.5 mM), 0.5 μL of Taq DNA polymerase and RNase-free water to a total volume of 25 μL. The amplification process consisted of an initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 30s and extension at 60 °C for 30 s.

For detecting the expression of pcIL-2, total RNA extraction and cDNA synthesis were carried out as described above. qRT-PCR was carried out as described previously with 18S rRNA as the internal control. Specific primers for detection of poIL-2 expression derived from pcIL-2 were listed in Table 1. Each reaction contained 10 μL of SYBR GreenI Master, 2 μL of specific primers (10 μM) and 8 μL of RNase-free water. The amplification process contains an initial denaturation for 30 s at 94 °C and 45 cycles of denaturation for 5 s at 94 °C and extension for 40 s at 60 °C. All data were performed using the $2^{-\triangle\triangle Ct}$ method [25].

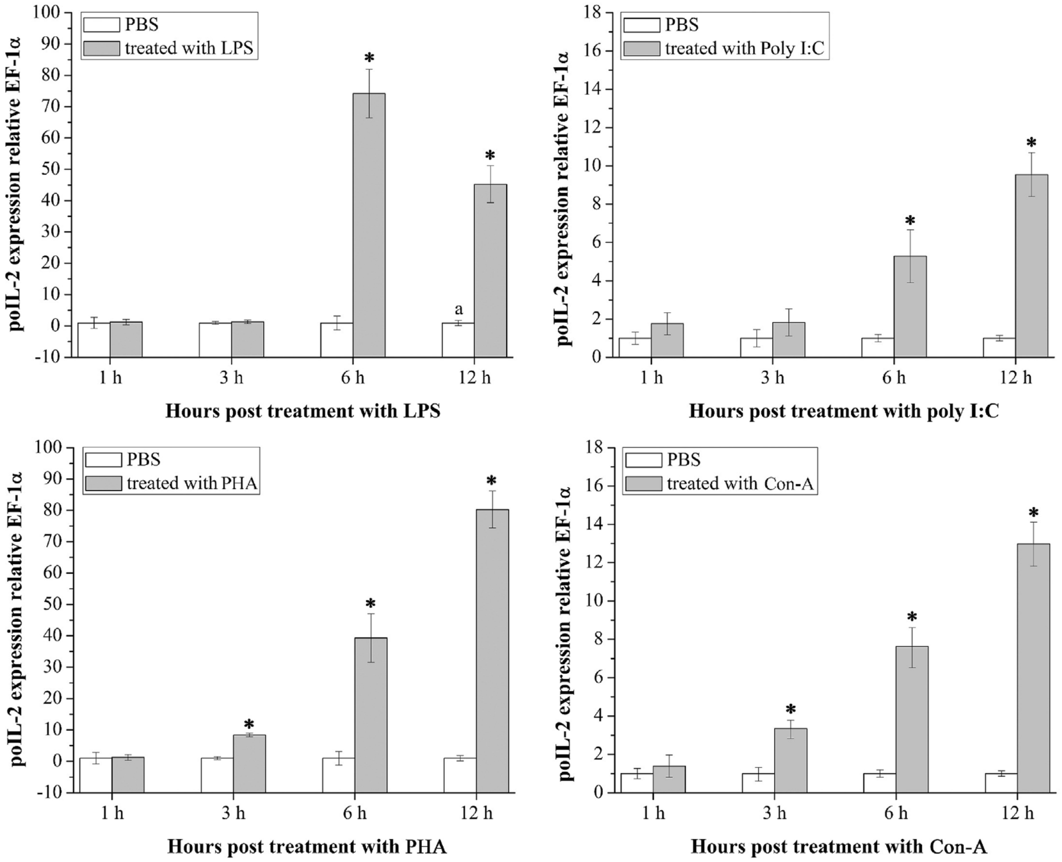

Fig. 5. The expression profiles of poIL-2 gene in primary cultured PBLs following stimulations with LPS, Poly (I: C), PHA or Con-A. All data were normalized to EF-1α and data are expressed as relative to the control. Error bars indicate standard deviation of the mean. Asterisks indicate significant difference compared with control groups ($p < 0.05$).

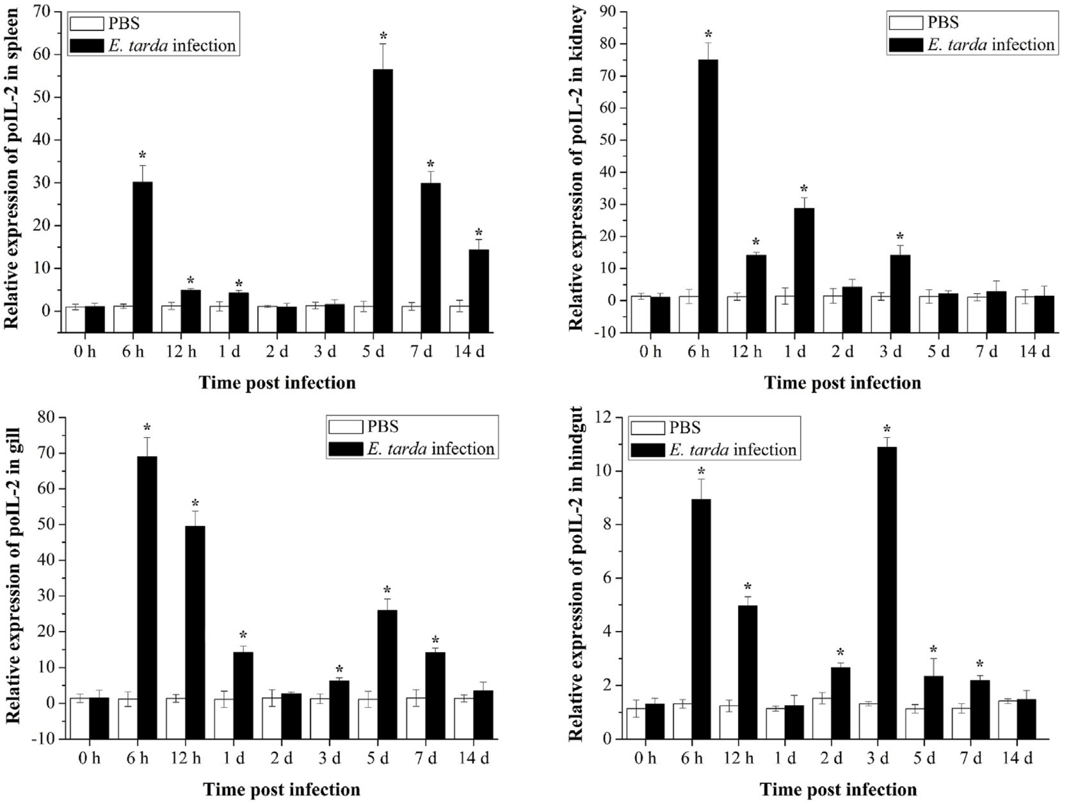

Fig. 6. The expression profiles of poIL-2 gene in spleen, kidney, hindgut and gill following infections with *E. tarda*. All data were normalized to EF-1α and data are expressed as relative to the control. Error bars indicate standard deviation of the mean. Asterisks indicate significant difference compared with control groups ($p < 0.05$).

The protein expression of the His/Myc-tagged pcIL-2 was also detected in muscles of vaccinated flounder by the indirect immunofluorescence assay (IIFA). Tissue cryosections were prepared based on the method described previously [31]. For IIFA, the cryosections of muscle tissues in PBS- and pcIL-2-immunized group were washed triple for 5 min with PBST (PBS containing 0.6% Triton-X 100), then pre-incubated with 3% bovine serum albumin solution for 30 min, and then incubated with the mouse anti-His monoclonal antibody (1:1000, Sigma-Aldrich, St. Louis, MO, USA) for 90 min at 37 °C. The normal mouse serum (1:1000) were used for the negative control. After washing triple with PBST, the sections of muscle tissues were incubated with FITC-conjugated goat anti-mouse IgG (1:256, Sigma-Aldrich, St. Louis, MO, USA) containing 1 μg/mL of Evans blue dye (Fluka, Lyon, France) as the counterstain. After incubation for 45 min at 37 °C, all the sections were washed in triple and then observed using the fluorescence microscope BX51 (Olympus, Japan).

2.9. Expression analysis of poIL-2, β-defensin, CD40, CD8α, TNF-α and IFN-γ after pcIL-2 treatment in vivo

Ninety flounders were randomly selected and divided into three groups (30 fish per group). One group was injected intramuscularly with 100 μL PBS containing 20 μg pcIL-2 (200 μg/mL), the other group with 100 μL PBS containing 20 μg pcN3 (200 μg/mL) as control, and the last group with the 100 μL PBS as the blank control. The injection dose and concentrations were performed as our previously work [32]. At 24, 48, 72 and 120 h post treatment, the spleens from three fish in each group were collected for total RNA extraction. The expression levels of poIL-2, β-defensin, CD40-1, CD8α, IFN-γ and TNF-α in spleens were detected using qRT-PCR as described above.

2.10. pcIL-2 treatment and challenge

The flounders were randomly divided into two groups (30 fish per group). The fish of two groups were intramuscularly injected with 100 μL PBS containing 20 μg pcIL-2 and pcN3, respectively. At 72 h post injection, the fish injected with pcIL-2 and pcN3 were both challenged with 200 μL of live HIRRV ($1 \times 10^{7.5}$ $TCID_{50}$/mL). Mortality was daily recorded for 15 days.

2.11. Statistical analysis

The fold change for the relative gene expression was determined by the $2^{-\triangle\triangle Ct}$ method [25]. All the data was expressed as mean ± SD. Statistical comparisons between groups were made using one-way ANOVA with Duncan's multiple range tests. Statistical analysis was performed using IBM SPSS Statistics 19. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Cloning and sequence analysis of poIL-2

The full-length cDNA sequences of poIL-2 (GenBank accession nr. KY307833) was 989 bp with an ORF of 420 bp, including an N-terminal signal peptide of 20 aa and a 5′ UTR of 67 bp and a 3′ UTR of 499 bp with three mRNA instability motifs (ATTTA), and containing a putative atypical polyadenylation signal sequence (AATTAAA) located upstream of the poly (A) tail. The theoretical molecular weight of the mature peptide of poIL-2 is about 15.69 kDa, and the isoelectric point is 4.42. A typical signal peptide of the first 20 aa residues in the deduced aa sequences of poIL-2, a complement control protein domain located from 22 to 75 aa and a typical Pfam: IL-15 domain located from 28 to 132 aa

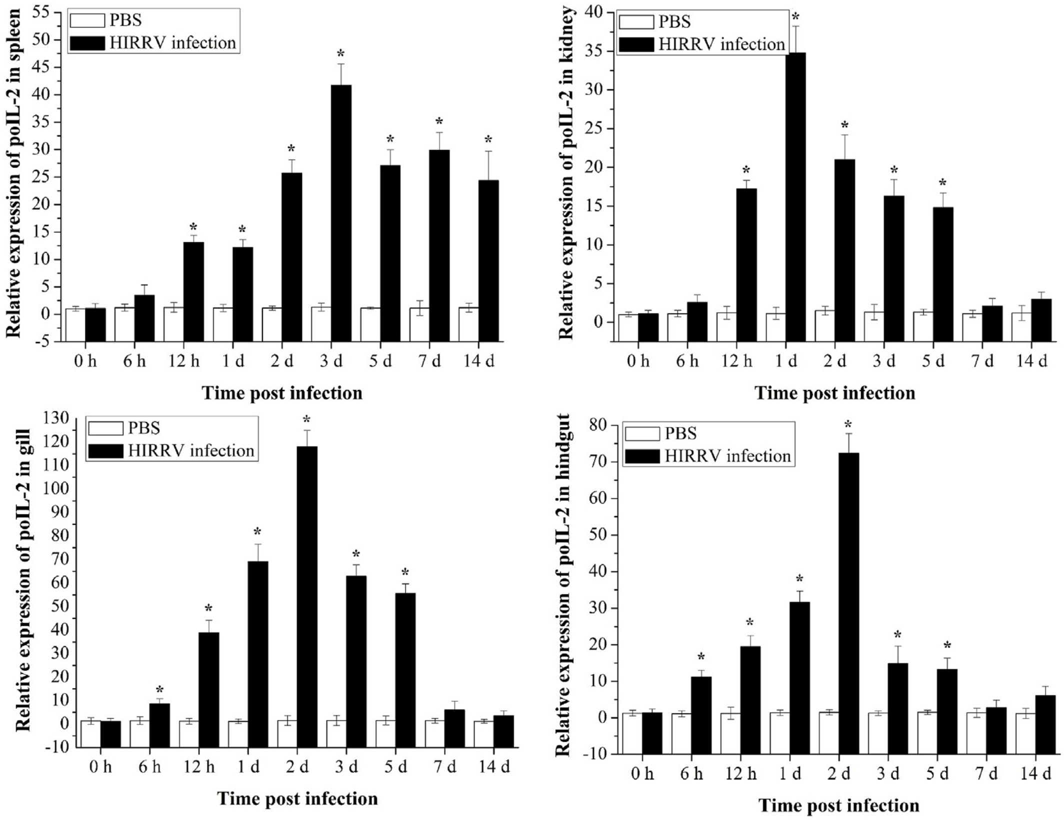

Fig. 7. The expression profiles of poIL-2 gene in spleen, kidney, hindgut and gill following infections with HIRRV. All data were normalized to EF-1α and data are expressed as relative to the control. Error bars indicate standard deviation of the mean. Asterisks indicate significant difference compared with control groups ($p < 0.05$).

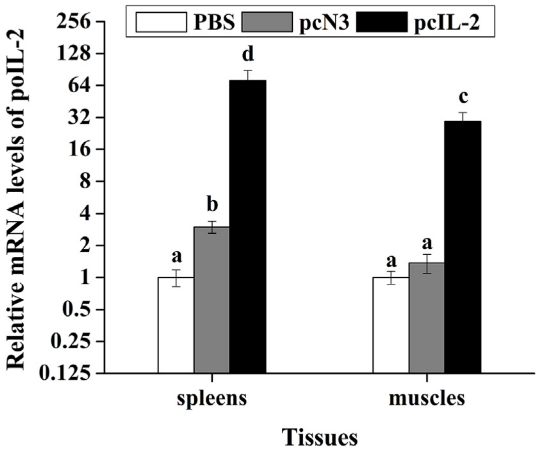

Fig. 8. Detection of poIL-2 transcript levels in the spleens and muscles of flounder at 5 d post intramuscular injection by qRT-PCR. All data were normalized to 18S rRNA and data are expressed as relative to the control. Error bars indicate standard deviation of the mean. Asterisks indicate significant difference compared with control groups ($p < 0.05$).

were identified in poIL-2 by the SMART program (Fig. 1).

3.2. Sequence homology and phylogenetic relationships of poIL-2

Blast homology analysis showed that the aa sequences identity between poIL-2 and mammalian IL-2 was fairly low, ranging from 0.9% to 22.5%, with 20.9% identity to cow IL-2 and 22.5% identity to human IL-2. The poIL-2 shared 28%–53% aa sequences identity with IL-2s of other fish species, with lowest identity to carp (*Cyprinus carpio carpio*) IL-2 and highest identity to turbot (*Scophthalmus maximus*) IL-2 (Fig. 2). Moreover, multiple alignment of the aa sequences revealed that poIL-2 not only own the classical IL-2 family signatures of "C-X(3)-EL-X(2)-(T/V)-(V/M/L)-(K/T/R)-X-EC" and "DS-X-(F/L)Y(A/T/S)P", but also possessed seven conserved cysteines aligned well with that of the fish species IL-2s (Fig. 2). Furthermore, IL-2, IL-4, IL-6 and IL-15 of teleost fish were used for the phylogenetic tree construction, and the results showed that the tree was mainly separated into two main clusters, corresponding to IL-2/IL-15 and IL-4/IL-6, and poIL-2 located in the IL-2/IL-15 clade and grouped closely with fugu (*Takifugu rubripes*), pufferfish (*Tetraodon nigroviridis*), sea bass (*Dicentrarchus labrax*) and large yellow croaker (*Larimichthys crocea*) (Fig. 3). In addition, only three α helices were identified in poIL-2 molecule using the YASPIN secondary structure prediction method (Fig. S1).

3.3. Tissue distribution of IL-2 transcript in healthy flounder

The expressions of poIL-2 in the tissues of healthy flounder, including PBLs, gill, skin, spleen, head kidney, trunk kidney, liver, foregut, midgut, hindgut, muscle, stomach and heart were determined by qRT-PCR. Results showed that IL-2 mRNA transcript was detected in all of the examined tissues, with highest expression observed in PBLs, spleen and hindgut, the moderate expression in gill, trunk kidney and stomach, and the weakest expression in other tissues of flounder (Fig. 4).

3.4. Expression patterns of poIL-2 in PBLs after different stimulations

To investigate the expression patterns of poIL-2 in cultured PBLs *in vitro*, PBLs of flounder were isolated and primary cultured, and then stimulated with Poly (I: C), LPS, PHA or Con-A, respectively. Results

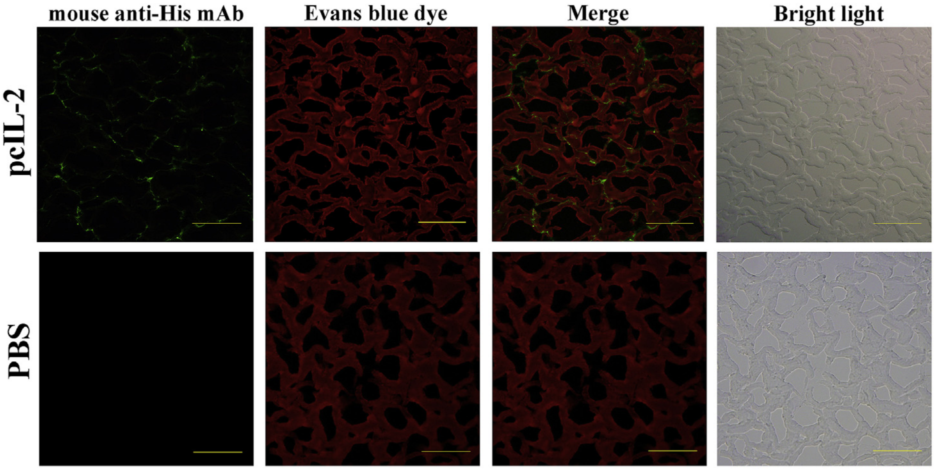

Fig. 9. Detection of His/Myc-tagged poIL-2 proteins in muscle of flounder at 5 d post intramuscular injection by IIFA. The green fluorescence represents the expression of His/Myc-tagged poIL-2, and the muscle tissues were counterstained in red by Evans blue dye. The third panel in row presents the image of the first two panels with digital overlays to visualize the co-localization. The fourth panel in row presents the bright light of muscle tissues. Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

showed that the mRNA levels of poIL-2 in PBLs were significantly increased post stimulation with these four stimulants compared with the control group ($p < 0.05$). Among them, the PHA and LPS induced much higher mRNA levels of poIL-2 compared with Con-A and Poly (I:C), the highest mRNA level occurred at 12 h post stimulation with PHA (81.2-fold), while the peak appeared at 6 h post LPS stimulation (74.1-fold) (Fig. 5).

3.5. Expression patterns of poIL-2 after E. tarda and HIRRV infection

qRT-PCR was used to examine the patterns of poIL-2 expression in spleen, kidney, gill and hindgut of flounder in response to *E. tarda* and HIRRV infection. Results showed that the expressions of poIL-2 in the four tissues of *E. tarda* infected flounder were significantly up-regulated post challenge, and reached their first peaks at 6 h post infection, then underwent a decline. However, the poIL-2 expression level increased again and reached their second peaks at day 3 or 5 post challenge (Fig. 6). Post challenge with HIRRV, significantly up-regulated expressions of poIL-2 in these four tissues was also observed, the peak expression levels of poIL-2 occurred at day 1–3, which was much delayed compared with those of *E. tarda* infection group, and there was no second expression peak observed in HIRRV infection group (Fig. 7).

3.6. Distribution, transcription and expression of pcIL-2 in flounder tissues

The ORF gene of poIL-2 was inserted into the expression vector pcN3 and resulting in recombinant plasmid pcIL-2. The recombinant plasmids were then verified by digested with *Kpn*Iand *Eco*RI, and the results showed the ORF gene of poIL-2 was successfully inserted into pcN3, and the theoretical molecular mass of digested fragment was in accordance with inserted gene (Fig. S2). The distribution and transcription of pcIL-2 in spleens and kidneys of vaccinated flounder were respectively detected by PCR and RT-PCR. PCR analysis revealed that pcIL-2 was detected in spleens and kidneys of pcIL-2-vaccinated flounder, but not in pcN3- and PBS-vaccinated flounder (Fig. S3A). RT-PCR analysis revealed that poIL-2 mRNA derived from pcIL-2 were also found in pcIL-2-vaccinated flounder, while no mRNA transcripts of poIL-2 derived from pcIL-2 were detected in PBS- and pcN3-vaccinated flounder (Fig. S3B). qRT-PCR was used to detected the expression of poIL-2 derived from pcIL-2 in spleens and muscles (injection site) at 5 d post vaccination, respectively. In muscle tissues, the poIL-2 mRNA in pcIL-2-vaccinated group were significantly up-regulated compared to that in PBS- and pcN3-vaccinated group ($p < 0.05$) (Fig. 8). Meanwhile, IIFA showed that the proteins of His/Myc-tagged pcIL-2 were detected in the muscle tissues of pcIL-2-vaccinated flounder. In contrast, no his-tagged proteins were found in PBS group (Fig. 9).

3.7. Expression of flounder IL-2, β-defensin, CD40-1, CD8α, IFN-γ and TNF-α after pcIL-2 treatment in vivo

To investigate the roles of poIL-2 in the immune regulation, the eukaryotic expression plasmid pcIL-2 was constructed and intramuscularly injected. The expression profiles of poIL-2, *β*-defensin, CD4-1, CD8α, IFN-γ and TNF-α in each experiment group were detected by qRT-PCR and showed in Fig. 10. Results revealed that the expression of all detected genes in spleens of pcIL-2-vaccinated group were significantly higher than those in the PBS- and pcN3-vaccinated group ($p < 0.05$), and mainly displayed the similar trend of increasing firstly, reaching their peaks at 72–120 h post vaccination and then declined.

3.8. Immune enhancement effect of pcIL-2 on flounder challenged with E. tarda and HIRRV

When the flounders pre-treated with pcIL-2 or pcN3 were challenged with live HIRRV, the cumulative mortality rates were recorded and shown in Fig. 11. In the control group, the challenged fish appeared typical hemorrhagic symptom with injection of HIRRV at the 3rd day, and finally the cumulative mortality came up 93.3% at 15th day. On the contrary, flounder pretreated with pcIL-2 exhibited higher resistance against HIRRV infection. The mortalities of pcIL-2-treated flounder challenged with HIRRV were 56.7% with a RPS of 39.2%.

4. Discussion

This study reports the identification and sequencing of IL-2 gene of flounder, and for the first time characterized the function and activity of this molecule in the teleost immune system. The predicted poIL-2 protein was 140 aa, which was a little shorter than that reported in large yellow croaker (142 aa) [22], but larger than that in Japanese pufferfish (127 aa) [21] and in trout (122 aa) [20], and shared approximately 40% similarity with Japanese pufferfish IL-2. Similarly, previous research also showed that IL-2 is a divergent cytokine and even amongst eutherians there is low sequence identity between species [33]. Comparisons across species have suggested a relatively low degree of evolutionary conservation of IL-2 homologues. A complement control protein domain located from 22 to 75 aa was found in poIL-2, suggesting that poIL-2 play a role in the complement immune system [34]. A typical domain named Pfam: IL-15 located from 28 to 132 aa was detected in poIL-2, and the gene ontology function revealed that the domain could regulate the immune response with or without IL-15 [35]. Seven cysteine residues in poIL-2 were found and aligned well with that of the fish species IL-2s in present study. The seven cysteines in fish are suggested to form the inter-chain disulfide bonds and render them more stable in their living water environment [36,37]. Moreover,

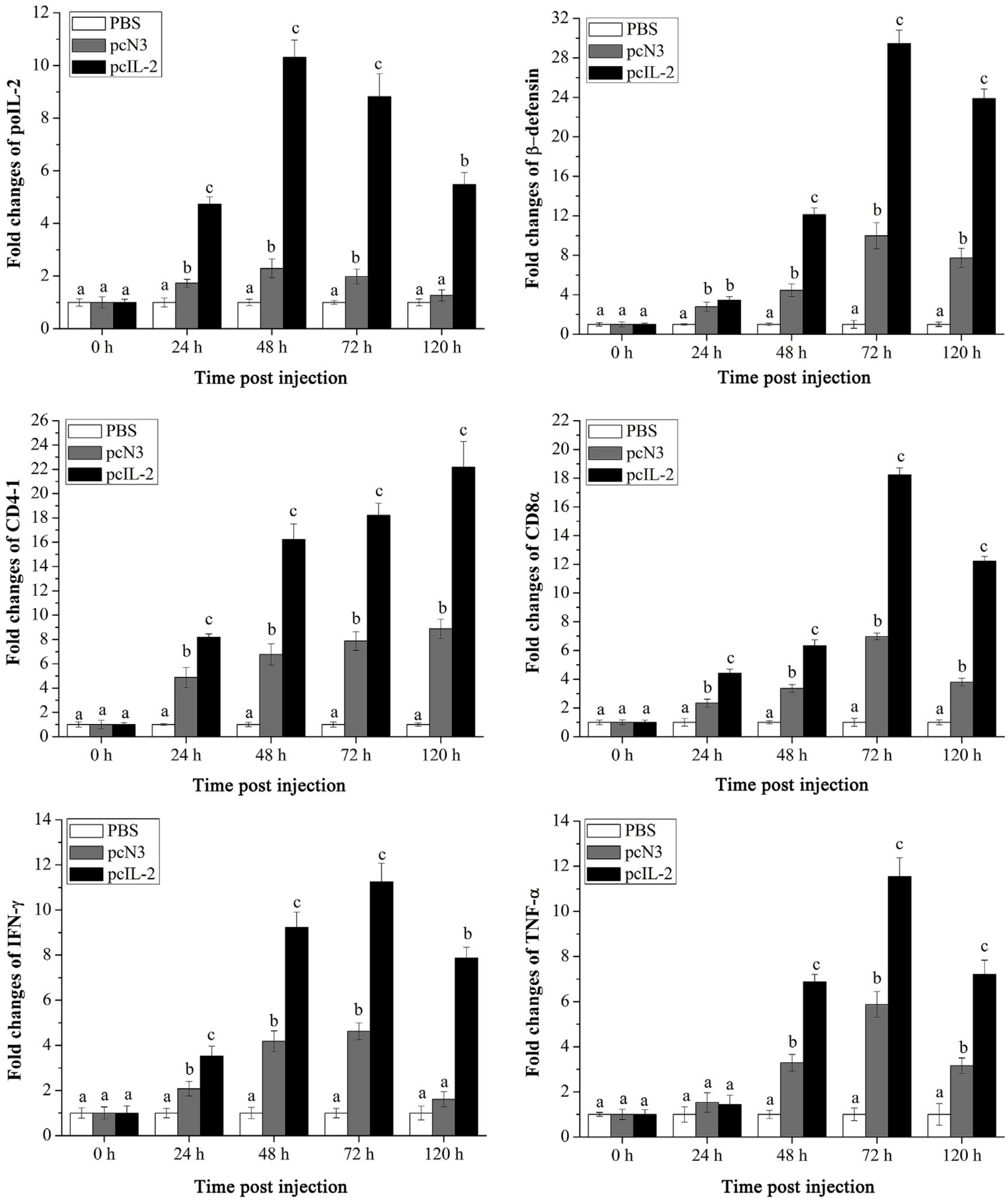

Fig. 10. Expressions of poIL-2, *β*-defensin, CD4-1, CD8α, IFN-γ and TNF-α in spleens of flounder after treatment with pcIL-2 or pcN3. All data were normalized to EF-1α and data are expressed as relative to the control. Error bars indicate standard deviation of the mean. Different letters indicate significant differences between groups ($p < 0.05$).

poIL-2 shares the unique protein fold characteristic of four antiparallel α helices in tertiary structure as many other cytokines [38,39]. Furthermore, the classical IL-2 family signature of “C-X(3)-EL-X(2)-(T/V)-(V/M/L)-(K/T/R)-X-EC” and “DS-X-(F/L)Y(A/T/S)P” were detected in poIL-2 protein, which indicated that poIL-2 is a new member of IL-2 family. In addition, poIL-2 showed high similarity with IL-15 in the aa sequences and protein structures, suggesting that they have a close similarity in biological function. Previous studies have also shown that IL-2 and IL-15 have functional synergy [40,41].

Previous studies have yielded inconsistent results about the expression of IL-2 in healthy fish. In healthy Japanese pufferfish, no constitutive expression of IL-2 was detected in different tissues [21]. However, constitutive expression of IL-2 was observed in all tested tissues of large yellow croaker, with highest levels in spleen and blood [42]. Similarly, in present study, poIL-2 was detected to be constitutively expressed in all the examined tissues, with much higher expression levels in PBL, spleen, hindgut and gill, which indicated that abundant T lymphocytes are present in these four tissues that are predominant systemic and mucosal immune organs and play vital roles in protecting against pathogens invasion [43–45]. Consistent with previous findings in teleost, the highest expression level of poIL-2 in primary cultured PBL could be induced by incubation the mitogen PHA in present work, and a slightly increased expression levels were observed in Poly (I:C) and Con-A treatment groups [20,21,42,46]. These results indicated that the T lymphocytes were effectively activated to transcribe the IL-2 mRNA by mitogens stimulation [47]. To be noted, the microbial-derived molecule, LPS, did not induced a significant increase of IL-2 expression in head kidney cells of Japanese pufferfish at the concentration of 1 μg/mL [21], but significantly higher level of IL-2 mRNA was observed in in head kidney leukocytes of large yellow

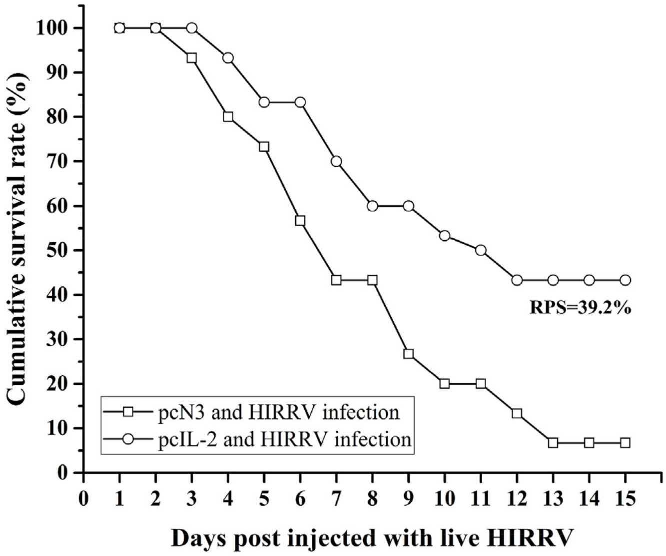

Fig. 11. Accumulative mortalities of flounder pre-treated with pcIL-2 or pcN3 within 15 d post HIRRV challenge.

croaker with 50 μg/mL LPS stimulation. We surprisingly found that 500 μg/mL LPS stimulation could induce a comparable expression level of poIL-2 as PHA in PBL. These results suggested that high dose of LPS could stimulate the leukocytes of peripheral blood to express the IL-2. However, the detailed mechanisms need to further investigation. Similar with previous research, our results showed that bacterial infection could significantly induce IL-2 expression [46]. We further found that significantly higher expression of poIL-2 was induced by HIRRV infection. In mammals, the levels of IL-2 were increased remarkably post infections with herpes simplex virus and fatal ebola virus [48,49], which argued that Th1 type response would be dominant in acute virus infection and IL-2 would be upregulated to play an important role in eliminating virus. Interestingly, the expression of poIL-2 exhibited two peaks after *E. tarda* infection, and the first expression peak of poIL-2 occurred at 6 h post infection, which was probably that the pathogen-associated molecular patterns on the surface of *E. tarda* are first recognized by toll-like receptors and then deliver signals to activate immune response [50]. The second expression peak might be due to the colonization and proliferation of *E. tarda* in flounder [51,52]. These results demonstrated that poIL-2 could be significantly upregulated upon bacterial and viral infection, which indicated the potential role of IL-2 in disease resistance in flounder.

In mammals, the functions and activities of IL-2 were well studied. Among its many immune functions, IL-2 is a potent T cell growth factor, which could induce lymphokine-activated killer activity, boost the cytolytic activity of natural killer cells, and augment immunoglobulin production. IL-2 is also known to critically regulate T helper cell differentiation, and has the ability to induce IFN-γ expression [53]. In fish, the bioactivity studies of rIL-2 are still needed to confirm in the piscine immune response. In this study, the constructed eukaryotic expression plasmid encoding poIL-2 was successfully expressed in the tissues of flounders, which then induced upregualtion of several immune related genes. Defensins are important components of antimicrobial peptides in vertebrates, which play crucial roles in combating the invading pathogens [54]. In this study, *β*-defensin significantly up-regulated in spleens after rIL-2 treatment, which indicated that poIL-2 plays an antimicrobial role by enhancing the expression of *β*-defensin. CD4-1 and CD8α were molecular markers of CD4 and CD8 T lymphocytes respectively, which function in antigens presentation and recognition and immune signal transduction after pathogen invasion [55,56]. The up-regulations of CD4-1 and CD8α genes in spleens indicated that the functions of CD4 and CD8 T lymphocytes were activated by pcIL-2. It was reported that exogenous IL-2 can amplify the expression of IFN-γ in mammals [57,58]. Consistently, we observed that the expression of IFN-γ was highly increased in flounder spleen tissues following pcIL-2 treatment, confirming that fish IL-2 could elicit IFN-γ production. Previous results showed that highly expressed IL-1β and TNF-α can induce the expressions of several cytokines and the functions of macrophage [59], which was essential for combating intracellular and extracellular pathogens. TNF-α was markedly induced by pcIL-2 in flounder in this work. This result was supported by the administration of recombinant human IL-2 to mice in peritoneal macrophages and blood monocytes that induced large amounts of TNF-α production [60]. It was also noted that IL-2 was able to directly stimulate TNF production *in vitro* by purified monocytes [61]. In addition, we found that the flounder pre-treated with pcIL-2 showed higher antiviral activity against HIRRV infection, which might due to the strong immune responses activated by pcIL-2. This phenomenon might be related to substantial effects of IL-2 on the cellular immune system. In mammals, IL-2 treatment could activate and expand NK and CD8 + effector to enhance antiviral responses, which was also explored for therapeutic application in different infections [62–64].

In summary, the sequence characteristics and expression profiles of poIL-2 were determined. The poIL-2 has the characteristic structure features present in vertebrate IL-2s, which was highly expressed in PBL, spleen and hindgut. Besides the PHA and Con-A, high dose of LPS could also induce a high expression of poIL-2 in PBL. Intramuscular injection of pcIL-2 could significantly enhance the adaptive and innate immune response, and the flounder pre-treated with pcIL-2 showed an enhanced resistance against HIRRV infection. Our results suggested that the poIL-2 might have adjuvant potential for the fish vaccines against bacterial and virus diseases, which need to be further explored.

Acknowledgements

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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.2019.07.023.

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