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Immunological characteristics of Interleukin-2 receptor subunit beta (IL-2Rβ) in flounder (*Paralichtlys olivaceus*): Implication for IL-2R function

Xiujuan Zhou[a], Jing Xing[a,b,*], Xiaoqian Tang[a,b], Xiuzhen Sheng[a], Wenbin Zhan[a,b]

[a] Laboratory of Pathology and Immunology of Aquatic Animals, KLMME, Ocean University of China, 5 Yushan Road, Qingdao, 266003, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266071, China

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ABSTRACT

Interleukin-2 receptor subunit beta of flounder (*Paralichthys olivace*, fIL-2Rβ) was annotated on the NCBI, its gene was cloned and characterized functionally in this study. And then the amino acids sequences and tertiary structure of fIL-2Rβ were analyzed, respectively. RT-PCR and ImageJ analyzed showed that fIL-2Rβ mRNA were expressed in the gill, spleen, kidney, intestines, liver, blood, muscle and skin, which showed high signals in spleen and blood. And then the recombinant protein of fIL-2Rβ extracellular region and its polyclonal antibodies were produced, native fIL-2Rβ molecules in flounder peripheral blood leukocytes (PBLs) were identified at 60.7 kDa by Mass spectrometry, which were in accordance with the molecular mass of full fIL-2Rβ protein calculated on the predicted protein sequence. Then the IL-2Rβ+ cell in T/B lymphocytes were characterized by Flow cytometry and indirect immunofluorescence assay, respectively. The results showed that the percentages of IL-2Rβ+ leukocytes, IL-2Rβ+/CD4+, IL-2Rβ+/IgM+ lymphocytes were 18.4 ± 2.7%, 4.5 ± 0.8%, 4.3% ± 0.5 in PBLs, and were 13.6 ± 0.9%, 4.6 ± 1.1%, 6.1% ± 0.4 in spleen, similarly, the percentages of IL-2Rβ+ leukocytes, IL-2Rβ+/CD4+, IL-2Rβ+/IgM+ lymphocytes were 9.4 ± 0.3%, 4.0 ± 0.5%, 5.7 ± 0.1% in head kidney, respectively. After KLH injection, compared with control group, the gene expression of IL-2, IL-2Rβ, CD3, TCR, CD79b and IgM in spleen of flounder were up-regulated, respectively ($p < 0.05$). And the FCM results showed that the percentages of IL-2Rβ+ leukocytes in PBLs were significantly increased post Keyhole limpet hemocyanin (KLH) injection, which peaked 23.9 ± 0.9% at 9th day ($p < 0.05$). To our knowledge, those results first reported that the characteristics of IL-2R and IL-2R + molecules were expressed on both B and T lymphocytes in fish. At the same time, this study lays a foundation for further exploring the interaction between IL-2 and IL-2R to promote cell proliferation and carrying out biological functions.

1. Introduction

Interleukin-2 receptor (IL-2R), as the specific receptor of interleukin-2(IL-2), perform their biological functions by binding with IL-2 to transmit signals into cells insides, which induced the downstream signal expression and participated in immune response [1,2]. The reaction of IL-2 and IL-2R have a wide function on the immunomodulation. The characteristics of IL-2R and the reaction of IL-2 with IL-2R in immunoregulation have been clarified in mammals, while were barely in the bony fish. Therefore, analyze the characteristics of interleukin-2 receptors (IL-2R) is essential for further study in the IL-2R biological functions in teleost.

In mammals, IL-2R consists of three subunits: the IL-2R alpha, IL-2R beta, and IL-2R gamma chains [3]. Mammalian IL-2 functionally signals via either high or intermediate-affinity IL-2 receptors. The three receptor chains are located on different CHs of IL-2 and differentially expressed and modulated [4]. IL-2R associate on the cell surface to produce a high-affinity receptor complex capable of binding to IL-2 [5]. IL-2 binds with IL-2R to move inward rapidly, which triggers a series of intracellular signal transduction processes, and induced cell growth, proliferation and other activities. IL-2R gamma chain is also subunit of the receptors for IL-4, IL-7, and IL-9, IL-12, IL-15 [6]. At the moment, the intracellular region of IL-2R alpha is too short to function as a signal transducer [7]. The longest IL-2R beta (IL-2Rβ) in the intracellular region has many binding sites for protein phosphorylation [8]. Therefore, IL-2Rβ plays an indispensable role in signal transduction due to its sequence and structural characteristics [9]. IL-2R mediates the multipotency of IL-2 on lymphocytes including T cells, B cells and NK cells.

* Corresponding author. College of Fisheries, Ocean University of China, 5 Yushan Road, Qingdao, 266003, China.
E-mail address: xingjing@ouc.edu.cn (J. Xing).

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Table 1
Primers.

Name	Primer Sequence	Application
fIL-2Rβ-F	5′-CCGGAATTCCACAAAGAGTCACAAACTCGAGG-3′	Extracellular domain cloning
fIL2Rβ-R	5′-ACGCGTCGACTTATGGTGTCGGTTGCAGAAGC-3′	
βactin-F	5′-GTCCCTGTATGCCTCTGGTC-3′	Internal control
βactin-R	5′-TGTCACGCACGATTTCCCTC-3′	
18SRNA-F	5′-GGTCGTGATGCCCTTAGATGTC-3′	
18SRNA-R	5′-AGTGGGGTTCAGCGGGTTAC-3′	
CD3-F	5′-ATCGCCATGACGCTCCTC-3′	Q-PCR
CD3-R	5′-AAGCCTTTGGTTTGATCGTG-3′	
IL-2-F	5′-AGAGGATGCCAGTATCGGTT- 3′	
IL-2-R	5′-CAACGACCTCCGCAGAATGT-3′	
IL-2Rβ-F	5′-TGTAGGCTGTAGTCTCGTCTTCG-3′	
IL-2Rβ-R	5′-CCTATCGCTGGGCTTGACC-3′	

The combination of IL-2 and IL-2R complex can cause intracellular signal events and ultimately lead to cell proliferation [10]. Study shown that NK lymphocytes from human IL-2Rβ transgenic mice are able to proliferate rapidly and develop potent LAK cytotoxicity in *vitro* [11]. In addition, dysregulated expression of the IL-2Rβ abrogates development of NK cells [12]. What's more important is that T cell proliferation and development of IFNc-producing effector $CD8^+$ T cells depend upon IL-2Rβ [13].

The T/B lymphocytes antibodies of flounder were produced in our previous studies. And studies showed that IL-2 can be used as an adjuvant to improve the efficacy of candidate vaccines. To date, the gene of IL-2Rβ in teleost were annotated on NCBI in a few species of fish, such as *Scophthalms maximus*, *Lates calcarifer*, *Seriola dumerili* etc. However, its immunological characteristics have not been reported in fish. In order to analyzed immunological characteristics of IL-2R, this study have identified the IL-2Rβ gene, and its gene expression profile was analyzed in flounder. And then the native flounder IL-2Rβ (fIL-2Rβ) protein have been identified by polyclonal antibody against recombinant fIL-2Rβ. The IL-2Rβ + cell in T/B lymphocytes were characterized by Flow cytometry and indirect immunofluorescence assay, respectively. Cloning and characterizations analyses of IL-2Rβ may allow to better understand the involvement and mechanisms of IL-2 in regulating the immune response in flounder and perhaps other teleost.

2. Materials and methods

2.1. Animals

Flounder (*Paralichthys olivaceus*, 35 ± 5 g) were obtained from a fish farm (Rizhao, Shandong, China) and acclimated in the wet lab with continuous aerated seawater and fed with commercial pellets at a daily ration of 0.7% of their body weight. All of the fish were held in the wet lab for two weeks before the experiments for acclimatization and evaluation of overall fish health. Only health fish, as determined by general appearance and level of activity, were used in the following experiments. One part was used for mRNA and leukocytes preparation, the others were used for injection experiments.

Healthy New Zealand white rabbits weighing of 2.5 kg and BALB/c mice aging of six-week were purchased from Qingdao Animal Experimental Center (Shandong, China), and then were used for antibodies production.

This study was carried out strictly in line with the procedures 81 in the Guide for the Use of Experimental Animals of the Ocean University of China in agreement with the International Guiding Principles for Biomedical Research Involving Animals (EU84 2010/63). All efforts were dedicated to minimize suffering.

2.2. mRNA and leukocytes preparation

For mRNA preparation, the gill, spleen, kidney, intestines, liver, blood, muscle and skin were collected aseptically from six flounder, respectively. The total RNA was extracted by TRIzol method according to the manufacturer's instructions. The quantity and concentration of the RNA were detected by NanoDrop ND-8000 spectrophotometer (Thermo Scientific, USA), and the integrity of RNA was evaluated on a 1.5% agarose gel. The cDNA was synthesized by using Reverse Transcriptase M-MLV kit (TaKaRa, China) according to manufacturer's instructions. And then the cDNAs of muscle, kidney, spleen, gill, heart, skin, PBL, liver, stomach and intestine were used as templates in RT-PCR and fIL-2Rβ gene cloned.

For leukocytes of peripheral blood, head kidney and spleen preparation, the isolated protocol according to a method described previously [14]. In brief, the peripheral blood was drawn from the caudal vein and diluted in solution (65% RPMI-1640 containing 20 IU ml^{-1} heparin, 0.1% w/v NaN_3 and 1% w/v BSA). The head kidney and spleen were extirpated, and cell suspensions were prepared by squeezing the tissue with 65% RPMI-1640 solution through a nylon gauze filter. Then the peripheral blood and cell suspensions were centrifuged and the supernatants were laid over a 1.020–1.070 g/cm^3 discontinuous Percoll density gradient. After centrifuged, the leukocytes layers from the Percoll interface were collected. The leukocytes of peripheral blood, head kidney and spleen were used for Flow cytometry, and the peripheral blood leukocytes (PBLs) also were used in indirect immunofluorescence assay and Western blotting.

2.3. RT-PCR

The cDNAs of gill, spleen, kidney, intestines, liver, blood, muscle and skin collected from heath flounder were used as templates in RT-PCR under the following PCR conditions in RT-PCR under the following PCR conditions: pre-denaturation at 95 °C for 5 min, followed by 35 cycle of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30s and a final extension of 72 °C for 10 min. The β-actin gene was used as an internal control. The PCR products for flounder interleukin-2 receptor subunit beta (fIL-2Rβ, primers in Table 1) were electrophoresed on 1.5% agarose gels. And the mRNA expression of fIL-2Rβ have been analyzed and quantitated by ImageJ analysis software.

2.4. Analysis of flounder interleukin-2 receptor beta sequence

To analysis fIL-2Rβ gene, the sequence of fIL-2Rβ (accession No. XP_019962172.1) was retrieved from the NCBI database. Then, the basic information of the IL-2Rβ were analyzed using DNA star and the online SMART tool (http://smart.embl-heidelberg.de/). The amino acid sequence was assessed using Expert Protein Analysis System (http://www.expasy.org/) [15]. Its signal peptide was predicted using SignalP v4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Its phosphorylation site was analyzed using Netphos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/). The potential open reading frame (ORF) was analyzed using ORF Finder program (http://www.ncbi.nlm.nih.

gov/gorf/gorf.html). To analysis the amino acids of fIL-2Rβ, a multiple amino acid sequence alignment was conducted in ClustalX software. MEGA 5.0 software was used to construct and analyze a phylogenetic tree using the neighbor-joining method with 1000 bootstrap trials [16]. Moreover, a three-dimensional model of fIL-2Rβ was created using the template model of human IL-2Rβ in the Swiss-Model Repository Server (http://swissmodel.expasy.org/).

2.5. Production of flounder interleukin-2 receptor beta recombinant protein and its antibody

mRNA of spleen as template, the extracellular region of fIL-2Rβ excluding its signal peptide was amplified with the forward primers (fIL-2Rβ-F, Table 1) containing a SalI site and a reverse primer (fIL-2Rβ-R, Table 1) containing an EcoRI site. Following digestion with SalI and EcoRI, the purified PCR fragment was inserted into the pET28a (+) expression vector (Merck millipore, USA). The constructed recombinant plasmid pET28a (+)-IL-2Rβ was then transformed into *E. coli* complete cells (Merck millipore), and then induced during exponential growth with isopropylb-D-thiogalactopyranoside (IPTG) for 6 h at 30 °C prior to harvest. The recombinant proteins were affinity-purified using His Trap™ HP Ni-Agarose (GE healthcare, China) according to the manufacturer's instructions. The concentrations of purified proteins were determined by the Bradford method.

The polyclonal antibody against recombinant protein fIL-2Rβ (rfIL-2Rβ) were produced as previously described [17]. The male New Zealand white rabbits and BALB/c mice were immunized four times at biweekly intervals with 100 and 20 mg purified rfIL-2Rβ protein in CFA, respectively. After four boosters, the serum samples were collected and purified by protein G agarose affinity chromatography (Thermo Scientific, USA), and then anti-flounder rfIL-2Rβ polyclonal antibodies (Abs) were obtained. At the moment, the mouse anti-flounder rfIL-2Rβ polyclonal antibodies were produced in the similarly. The Abs titer was tested by ELISA, and the specificity of the Abs were analyzed using Western blotting and Mass spectrometry.

2.6. Western blotting and mass spectrometry

The PBLs lysates and purified rfIL-2Rβ were applied on SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, Germany). And then the membrane was blocked with PBS containing 4% BSA for 1 h at 37 °C, and incubated with Abs for 1.5 h at 37 °C, then washed three times with PBST. Antibody binding was detected with goat-anti-rabbit IgG-alkaline phosphatase conjugate (diluted 1:4000 in PBS) (Merck Millipore) for 1 h at 37 °C, and washed three times with PBST. Finally, the bands were stained with freshly prepared substrate solution (100 mM NaCl, 100 mM Tris and 5 mM MgCl2, pH 9.5) containing NBT (Sigma) and BCIP (Sigma) for 5 min and stopped by washing with distilled water. Serum from no immunized rabbit instead of the primary antibodies reacted with purified recombinant protein of flounder IL-2Rβ was used as control. The immune-reactive proteins in leukocyte lysates were excised from corresponding polyacrylamide gels and analyzed by Mass spectrometry.

2.7. Flow cytometry and indirect immunofluorescence assay

The heath flounder leukocytes isolated from peripheral blood, head kidney and spleen were adjusted to 1×10^7 cells/ml with PBS. For one color flow cytometry, the leukocytes were incubated with Abs for 1.5 h at 37 °C, subsequently samples were washed three times with PBS containing 5% (v/v) Newborn Calf Serum, then incubated goat-anti-rabbit Ig-Alexa Fluor® 647 (1:1000, Thermo Fisher Scientific, USA) for 1 h in the dark at 37 °C, and then washed again. After that, the cell suspensions were analyzed with an Accuri C6 cytometer (BD Accuri., USA).

For double immunofluorescence staining and two-color flow cytometry, the mixture of two kinds of antibodies (rabbit anti-rfIL-2Rβ polyclonal antibodies have mixed with mouse anti-IgM monoclonal antibodies [18], mouse anti-rfIL-2Rβ polyclonal antibodies have mixed with mouse anti-CD4-1/CD4-2 polyclonal antibodies [19], respectively) were used as primary antibody, and the mixture of FITC-conjugated goat-anti-mouse IgG (1:1000 diluted in PBS, Sigma) and Alexa Fluor 649-conjugated goat anti-rabbit IgG (1:1000 diluted in PBS, Sigma) were used as secondary antibodies. For immunofluorescence observation in microscope, the PBLs were counterstained with DAPI (Bio-Legend) for 10 min at 37 °C in dark. After the last washing, 20 μl PBLs suspension was dripped onto APES coated slides, and the PBLs were settled and fixed onto slides after 2 h, and then observed under a fluorescence microscope (Evos FL Auto2, Thermo, USA). Unimmunized rabbit serum was used as negative controls instead of Abs as primary antibody. The mixture of unimmunized rabbit and mouse serum were used as negative control.

2.8. Injection experiments

The injection experiment was performed as described by Xing et al. [20], but with slight modification. In brief, heath flounder were randomly divided into two group (50 fish in per group). On the day of injection, KLH (Keyhole limpet hemocyanin, cat No. H7017, Sigma, USA) and PBS were emulsified with Freund's complete adjuvant (cat No. F5881, Sigma, USA) at equal volume, respectively, and then were injected to fish intraperitoneally (100 μl per individual), respectively.

2.8.1. Realtime quantitative PCR

For Realtime quantitative PCR (Q-PCR), the spleen was randomly collected from three fish in each group at 0 h, 6 h, 12 h, 24 h, 36 h, 48 h, 72 h and 96 h after injection. Additionally, total RNA extraction followed the Trizol method. Then, cDNA was synthesized using the PrimeScript™ reagent Kit with gDNA Eraser (TaKaRa, China) according to the manufacturer's instructions, and then the IL-2, IL-2Rβ, CD3, TCR, CD79b and IgM gene expression were quantified using Q-PCR. The cDNA from spleen was used as templates, and Q-PCR was carried out using SYBR Green I Master (Roche, Switzerland) in a LightCycler® 480 II Real Time System (Roche, USA). The specific primers are shown in Table 1, which were identified by sequencing of all the products. The cDNA concentrations were adjusted to 100 ng/ml by NanoDrop 8000. The thermal cycling profile consisted of an initial denaturation at 95 °C for 30 s, followed by 45 cycles of denaturation at 95 °C for 5 s and extension at 60 °C for 30 s. An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 °C to 95 °C [21]. The expression level of genes in blank control individuals was defined as 1. Each assay was performed in triplicate using the 18S gene as an internal control. All data were analyzed using the $2^{-\triangle\triangle Ct}$ method.

2.8.2. Flow cytometry

For detected the variation of IL-2Rβ+ cell by FCM, the PBLs were collected from three fish in each group at 1d, 3d, 5d, 7d, 9d, 11d post injection. The PBLs were incubated with Abs for 1.5h at 37 °C, subsequently samples were washed three times with PBS containing 5% (v/v) Newborn Calf Serum, then incubated goat-anti-rabbit Ig-Alexa Fluor® 647 (1:1000, Thermo Fisher Scientific, USA) for 1h in the dark at 37 °C, and then washed again. After that, the IL-2Rβ+ cell was analyzed with an Accuri C6 cytometer (BD Accuri., USA).

2.9. Statistics

The data were presented as the mean ± SD. Statistical analysis was performed using the software SPSS 19.0. The statistical analysis used One-way analysis of variance (ANOVA) and Duncan's multiple comparisons using SPSS19.0 software. The level of significance was defined as $p < 0.05$.

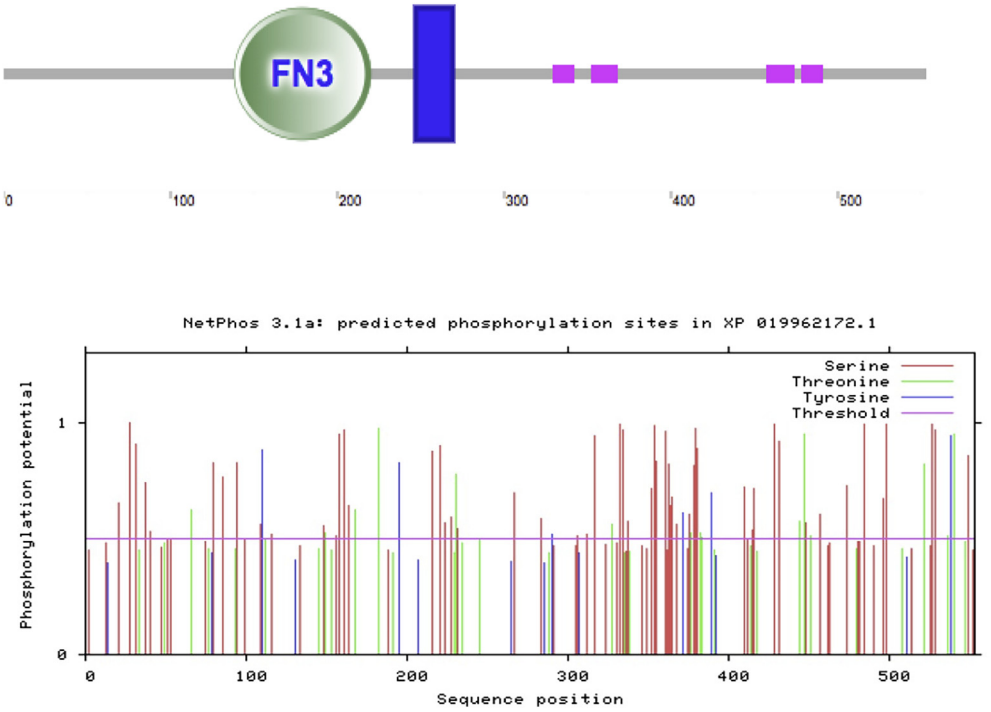

Fig. 1. Conserved domains and predicted phosphorylation sites in the protein sequence of flounder IL-2Rβ. Conserved domain: FN3 domain (aa138-aa220).

3. Results

3.1. Characteristics of fIL-2Rβ sequence

In this study, the basic sequence information and structural features of the fIL-2Rβ protein have been analyzed by on-line biological analysis software. FIL-2Rβ is a membrane-bound protein that consists of a 27-aa signal peptide, 119-aa extracellular region (aa28-aa246), a single 23-aa transmembrane domain (aa247-aa269), and a 223-aa cytoplasmic region. And its extracellular region contains an FN3 domain (aa138-aa220), which belongs to the FN3 superfamily and included the evolutionarily conserved FN3 domain. Netphos 3.1 Server analyzed results showed that FIL-2Rβ contains 76 phosphorylation sites which play an important role in cell signal transduction (Fig. 1).

Multiple alignment of the deduced fIL-2Rβ amino acid sequence with other known IL-2Rβ teleost proteins was performed using the ClustalX program. Different amino acids are labeled in different letters, and conserved cysteine residues (C) are labeled in deep red. Conserved tryptophan residues (W), which play a key role in the three-dimensional conformation of proteins, are marked in arrowheads (Fig. 2). In addition, fIL-2Rβ shares a relatively high identity of 28.0%–65.0% with known members of the teleost fish. The highest identity was the 65.0% with *Scophthal musmaximus*, and the lowest identity was 28.0% with *Lepisosteus oculatus*. Besides, the phylogenetic tree revealed that the fIL-2Rβ cluster was most closely related to *S. musmaximus* in terms of its genetic evolutionary position (Fig. 3). The accession number used in Figs. 2 and 3 were listed in Supplement Table 1 and Supplement Table 2.

To imitate the tertiary structure of fIL-2Rβ, with human IL-2Rβ as the template, the tertiary structure of the fIL-2Rβ was modeled. The results show that the fIL-2Rβ has an overall tertiary structure similar to that of human IL-2Rβ (Fig. 4). In the IL-2/IL-2 receptor system of human, IL-2Rβ, as a specific receptor of IL-2, its extracellular region was interacted with helices A and C of IL-2 [5].

3.2. Tissue distribution of fIL-2Rβ mRNA

RT-PCR results showed that fIL-2Rβ mRNA expressed in gill, spleen, kidney, intestines, liver, blood, muscle and skin in healthy flounder (Fig. 5A). In addition, the mRNA expression of fIL-2Rβ have been analyzed and quantitated by ImageJ analysis software in Fig. 5B. The results showed that high signals were observed in blood and spleen, and weak expression was detected in the gill, kidney, liver, muscle and skin.

3.3. Identification of native fIL-2Rβ protein

According to fIL-2Rβ sequence published in NCBI, the rfIL-2Rβ was successfully expressed, compared with the non-induced bacteria (Fig. 6A, lane1), SDS-PAGE results revealed that the purified proteins presented distinct bands at 27 kDa (Fig. 6A, lane3), it was in accordance with the predicted molecular masses. And then rabbit-anti-rfIL-2Rβ antibody was successfully produced. The specificity of rabbit-anti-rfIL-2Rβ were analyzed by Western blotting, and the results showed that it could specifically recognized rfIL-2Rβ (Fig. 6B, lane5). Moreover, the antibody could specifically recognize about 60.7 kDa bands in leukocytes lysates of flounder peripheral blood, which were in accordance with the molecular mass of full fIL-2Rβ protein calculated on the predicted protein sequence. It was no positive result in negative control (Fig. 6B, lane4). The 60.7 kDa protein was confirmed to be the native fIL-2Rβ by Mass spectrometry analysis (Fig. 7). The results indicated the Abs specifically recognized native fIL-2Rβ molecules and could be used in following experiments.

3.4. Identification of IL-2Rβ+ leukocytes, IL-2Rβ+/CD4+, IL-2Rβ+/IgM + lymphocytes

IL-2Rβ+ leukocytes, IL-2Rβ+/CD4+, IL-2Rβ+/IgM+ lymphocytes were detected by Flow cytometry in PBLs, spleen, head kidney (Fig. 8). The results showed that the percentages of IL-2Rβ+ leukocytes, IL-2Rβ+/CD4+, IL-2Rβ+/IgM+ lymphocytes were 18.4 ± 1.7%, 4.5 ± 0.8%, 4.3% ± 0.5 in PBLs, respectively. The percentages of IL-2Rβ+ leukocytes, IL-2Rβ+/CD4+, IL-2Rβ+/IgM

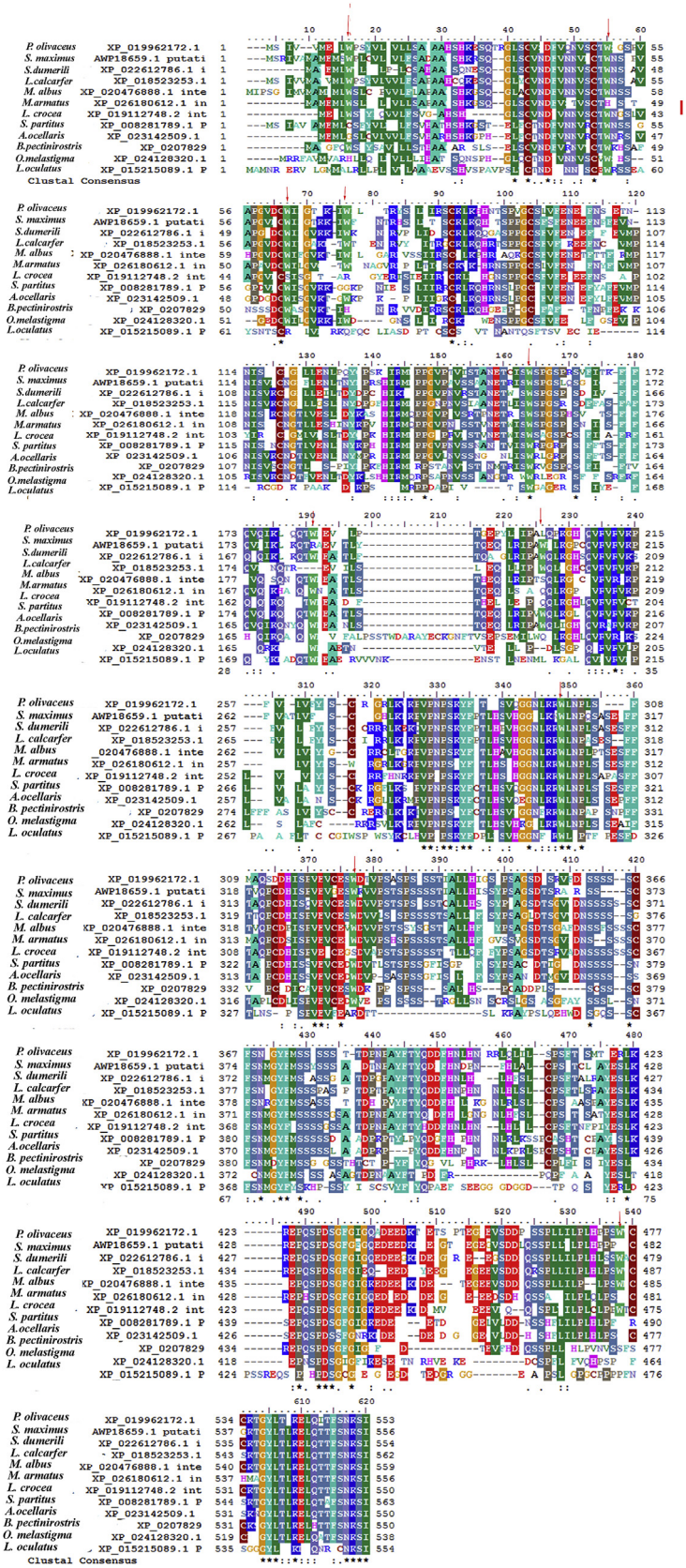

Fig. 2. Alignment of translated IL-2Rβ in different fish species. The accession number for each sequence was listed in the supplement Table 1.

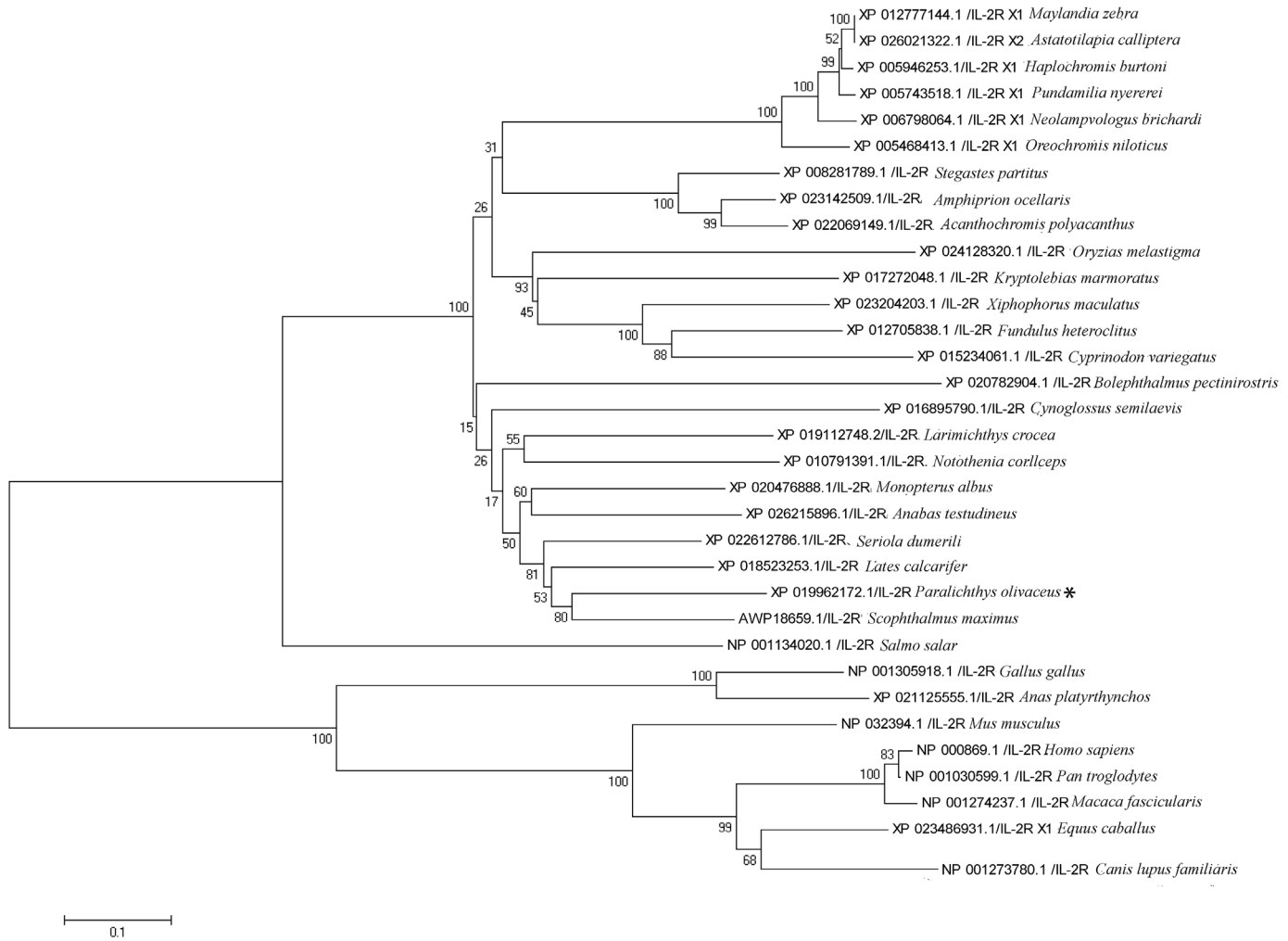

Fig. 3. Phylogenetic tree analysis of IL-2Rβ from flounder and other species. The tree was constructed by the "neighbor-joining" method using MEGA5.0 software. Node values represented the percent of bootstrap confidence derived from 1000 replicates. The accession number for each sequence was listed in the supplement Table 2.

+ lymphocytes were 13.6 ± 0.9%, 4.6 ± 1.1%, 6.1% ± 0.4 in spleen, respectively. In addition, the percentages of IL-2Rβ+ leukocytes, IL-2Rβ+/CD4+, IL-2Rβ+/IgM+ lymphocytes were 9.4 ± 0.3%, 4.0 ± 0.5%, 5.7 ± 0.1% in head kidney, respectively. The percentages of IL-2Rβ+ leukocytes in PBLs was higher than that of in spleen and head kidney.

3.5. Double immunofluorescence staining for observing IL-2Rβ+/CD4+, IL-2Rβ+/IgM+ lymphocytes

Fluorescence microscope observation showed that there exists single positive signal of IL-2Rβ, CD4 and IgM leukocytes. In addition, double positive signals, as both diffuse green fluorescent and red fluorescent signals, IL-2Rβ+/CD4+ and IL-2Rβ+/IgM+ were also observed, respectively. The results show that a considerable number of lymphocytes in PBLs exhibited double immunofluorescence staining. White arrows showed that the co-localization of IL-2Rβ and CD4+T

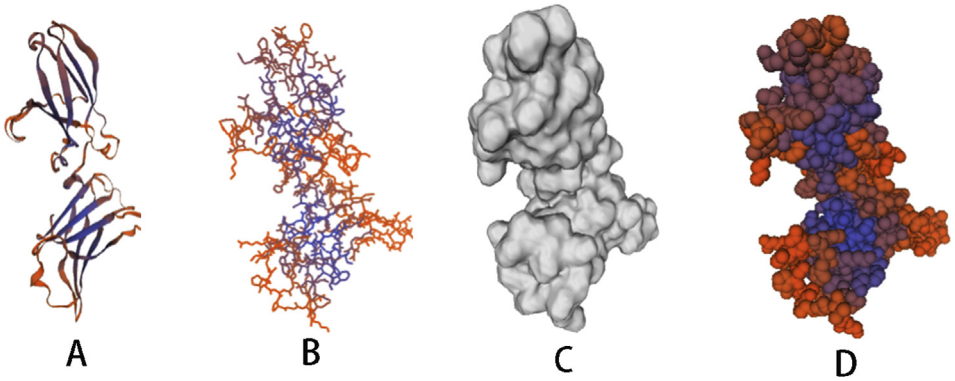

Fig. 4. Tertiary structure model of flounder IL-2Rβ molecule predicted by the program Swiss Model Repository Server. Ã- D refer to catoon, lines, surface, spacefill, respectively.

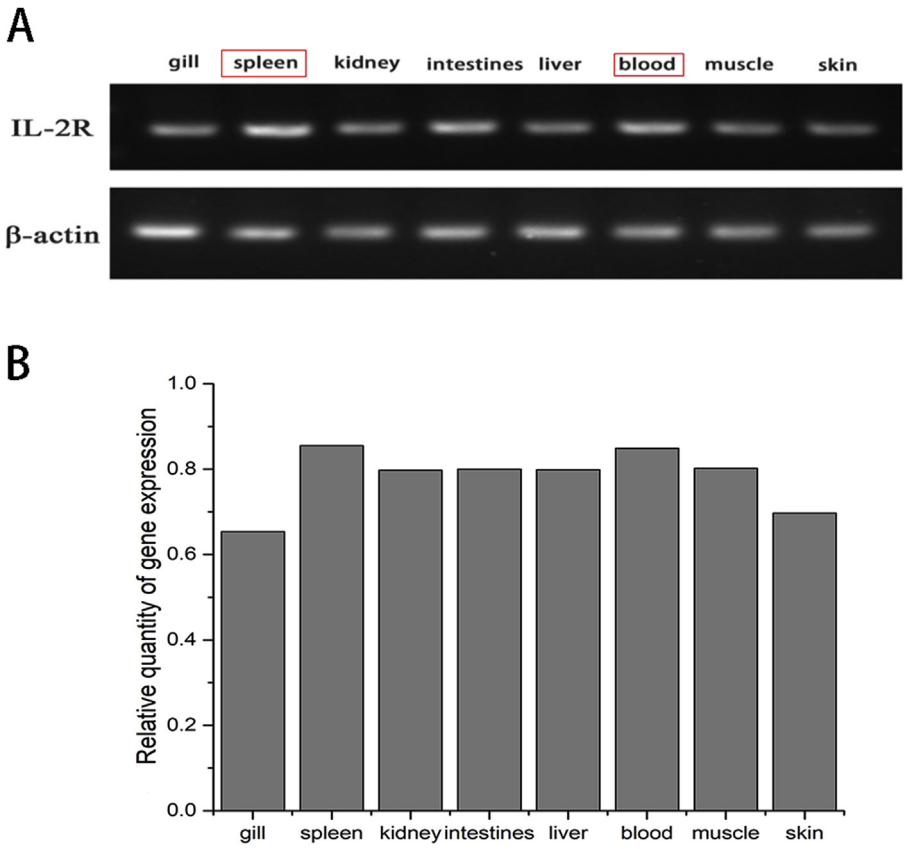

Fig. 5. RT-PCR and ImageJ results of flounder IL-2Rβ in flounder tissues. A: Lane(L) 1. gill; L2. spleen; L3. kidney; L4. intestines; L5. liver; L6. blood; L7. muscle; L8. skin. B: The relative quantity of IL-2Rβ gene expression in different tissues of flounder analyzed by ImageJ.

lymphocytes indicated that flounder IL-2R molecule was expressed on CD4 + T lymphocytes (Fig. 9B). Meanwhile, the co-localization of IL-2R and IgM explained that flounder IL-2R molecule was expressed on IgM + B lymphocytes (Fig. 9D). By contrast, isotype controls utilizing rabbit IgG and mouse IgG as nonrelated primary Abs displayed no fluorescent signals (Fig. 9A and C).

3.6. The variation of IL-2Rβ+ leukocytes after KLH injection

In KLH infection group, the percentages of IL-2Rβ+ leukocytes showed an increase on the 5th day (ANOVA, $p > 0.05$) compared with the control group, and reached the peak at 9th day with 23.9 ± 0.9%, which showed a significant increase compared with control group (ANOVA, $p < 0.05$, Fig. 10A). Additionally, the representative

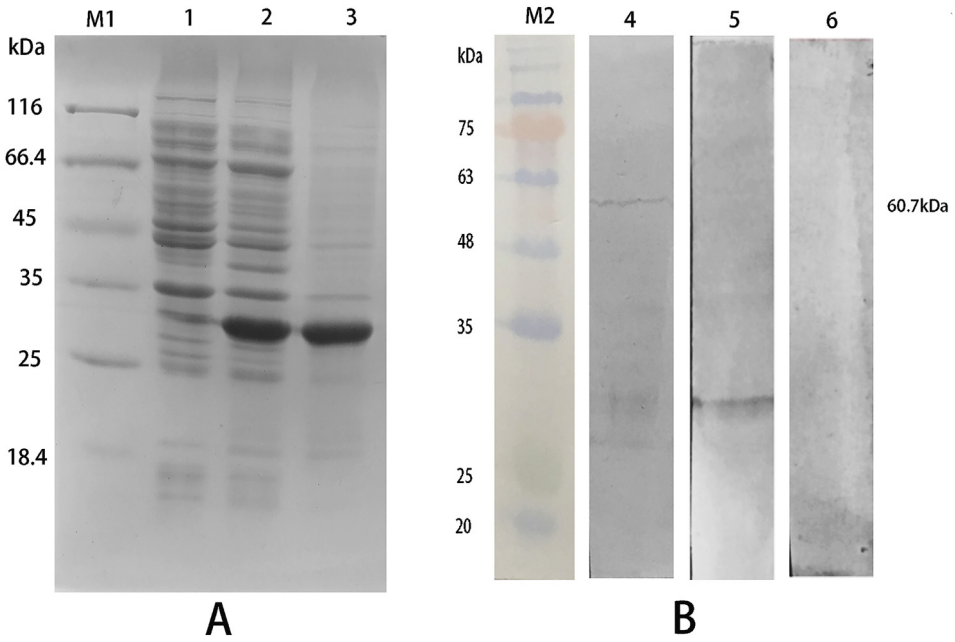

Fig. 6. SDS-PAGE results of flounder IL-2Rβ recombinant proteins and western blotting results of antibodies (Abs) to recombinant proteins and leukocytes lysates. A. Lane M1: molecular mass marker; Lane 1: transformed *E. coli* without IPTG induction; Lane2: transformed *E. coli* induced with IPTG; Lane 3: purified recombinant protein of flounder IL-2Rβ. B. Western blotting results of antibodies (Abs) to recombinant protein and leukocytes lysates. Lane M2, marker; Lane 4, rabbit-anti-rIL-2Rβ (1:1000) reacted with peripheral blood leukocytes; Lane 5, rabbit-anti-rIL-2Rβ (1:3000) reacted with purified recombinant protein of flounder IL-2Rβ; Lane 6, serum from no immunized rabbit instead of the primary antibodies reacted with purified recombinant protein of flounder IL-2Rβ was used as control.

A

MSHIVVMELLWPSYVLIVLLSANAAHSHK**ESQTR**GLSCVSDFVQNVSCTWSGSPVAPGVDCWIHGTRK**IWILENSTR**YSRLMIRSCKL
KHHNTSPVGCSLVFENEDFNSYETNNISLECDGKLLENLPQYQPSKNIKMNPPGVPTVISTANETCISWSPGSPRSVFITKFNFQVQIKLK
QQTWK**EVQSLPTGEPYLKIPALQPK**GHYQVRVRVKPSDRPNSHWSNWSPTTSWITFQEQNWLLQPTPLILLGMMLPVGFLVILVFYL
SCVRKGRLKVKPVPNPSKYFHTIYSVQGGNLKKWLNPLSSYFMAQSDDHISPVEVCESWDTVPSASPSTSSTIALLHIGSFPSAGSDSSK
VFDDSSSSSCFSNIGYFMSSTSSSITTDPNPAYFTYQDDFHNLHNNRRLQLILSPSFTSSMTHERLKREPQSPDSGFGIGQADEEDKTEETS
MPTEGDEVSDDPKSSPLLILPLHPPSWVCPTSSALSPPQLPSLNQIWSDSQEEEEEEDVTAAYGNSEAWPVAGTMCRSSSMPAEPCKTG
YLTIKELQITFSNKSI

B

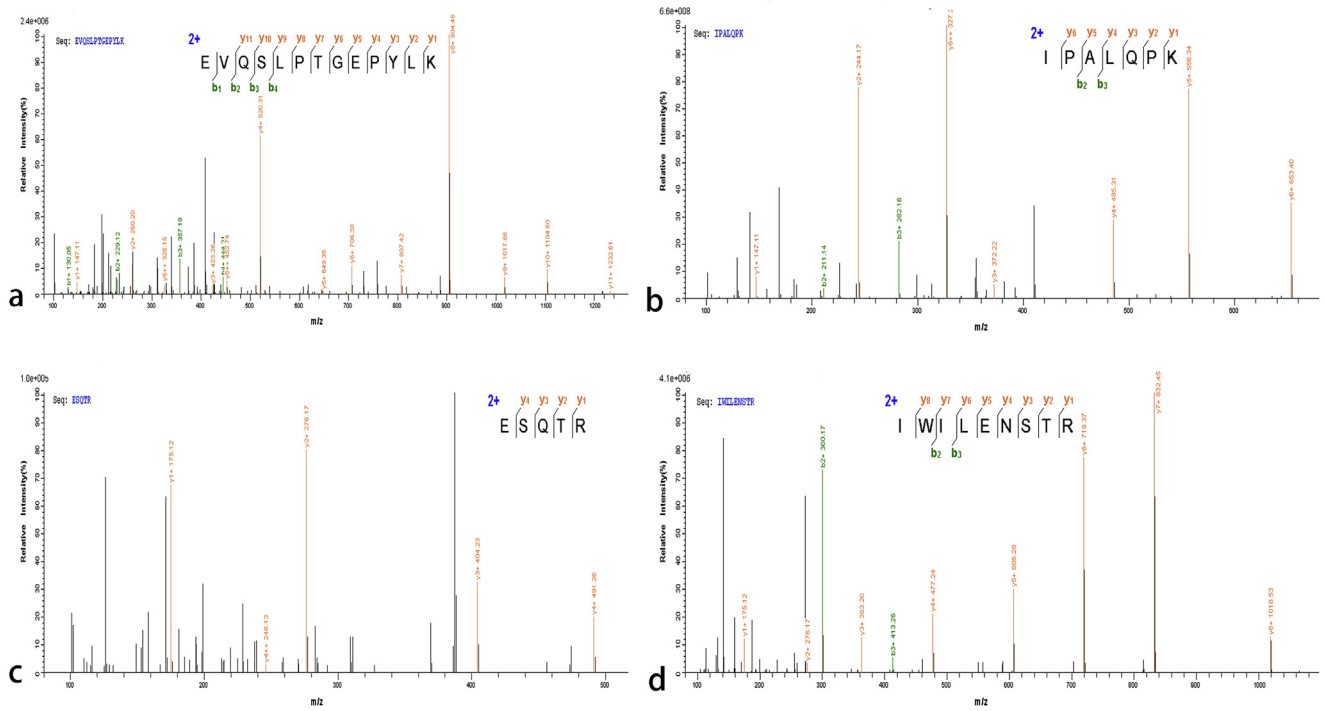

Fig. 7. Identification of the 60.7 kDa protein recognized by rabbit anti- flounder IL-2Rβ polyclonal antibodies in PBLs using mass spectrometry. (A) The four peptides (a, b, c, d) that most matched the flounder IL-2Rβ sequence were underlined. (B)The LC–MS/MS results of the four peptides.

fluorescence histograms of IL-2Rβ+ leukocytes at the peak time of control and KLH group after infection were shown in Fig. 10B1 and B2.

3.7. Expression of related-gene after KLH injection

Expression levels of related-gene after KLH injection were summarized in Fig. 11. The Q-PCR analysis showed that IL-2, IL-2Rβ, CD3, TCR, CD79b and IgM genes in the spleen of fish were up-regulated after KLH injection compared with control group. (ANOVA, $p < 0.05$). The CD3 gene was significantly upregulated and reached the peak at 36 h, then declined and reached the second peak at 96 h. Similarly, IL-2R and IL-2 genes were peaked at 24 h and 72 h after injection, respectively. And compared with the control groups the mRNA levels of TCR gene was significantly increase and reached their maximum at 12 h after injection. Similarly, the expression peak time of CD79b and IgM genes appeared at 48 h post injection, then declined to normal levels (ANOVA, $p < 0.05$).

4. Discussion

In this study, IL-2Rβ of flounder have been characterized. Expression in mRNA, protein level and its characterizations of IL-2Rβ were analyzed, respectively. And as we know, it's for the first time to analyzed immunological characteristics of IL-2Rβ in the teleost. Protein sequences analysis showed that fIL-2Rβ were contained a 246-aa extracellular domain and a 223-aa cytoplasmic region. There are 76 phosphorylation sites in the long intracellular domain, which play an important role in signal transduction [22]. At moment, four important pathways of IL-2: Janus kinase (JAK)–signal transducer, activator of transcription (STAT), phosphoinositide 3-kinase (PI3K)–AKT and mitogen-activated protein kinase (MAPK) pathways were related to protein phosphorylation [23,24]. The gene of flounder IL-2 have been cloned in our previous study (accession number: KY307833.1), prediction of phosphorylation sites of IL-2Rβ provides a reference for further study of IL-2-IL-2R signaling pathway in our lab. Phylogenetic analysis using the neighbor-joining method showed that fIL-2Rβ clustered into a major clade within the fish IL-2Rβ subgroup, yet distinct from that formed by mammalian IL-2Rβ. The highest identity was the 65.0% with turbot (*Scophthal musmaximus*). The reason for this is that the flounder and turbot have similar taxonomic status, which may have conservative gene evolutionary processes.

The RT-PCR and ImageJ analysis showed that fIL-2Rβ were expressed in all tissues examined, with the high levels in the spleen and blood, which were important organs in lymphocytes function. This result is consistent with study for salmon IL-1 [25]. In humans and other mammals, the adaptive immune system is composed of lymphocytes subsets and cytokines that consist of a precise regulatory network for the development of cellular or humoral immunity [26]. The production of IL-2 in mammals is tightly regulated and largely restricted to activated $CD4^+$ T cells, natural killer (NK) cells, as well as dendritic cells (DCs) on stimulation with microbial compounds [27,28], which were agreement with the results of FCM and indirect immunofluorescence assay in this study. The FCM and indirect immunofluorescence assay results have detected the IL-2Rβ+/CD4+, IL-2Rβ+/

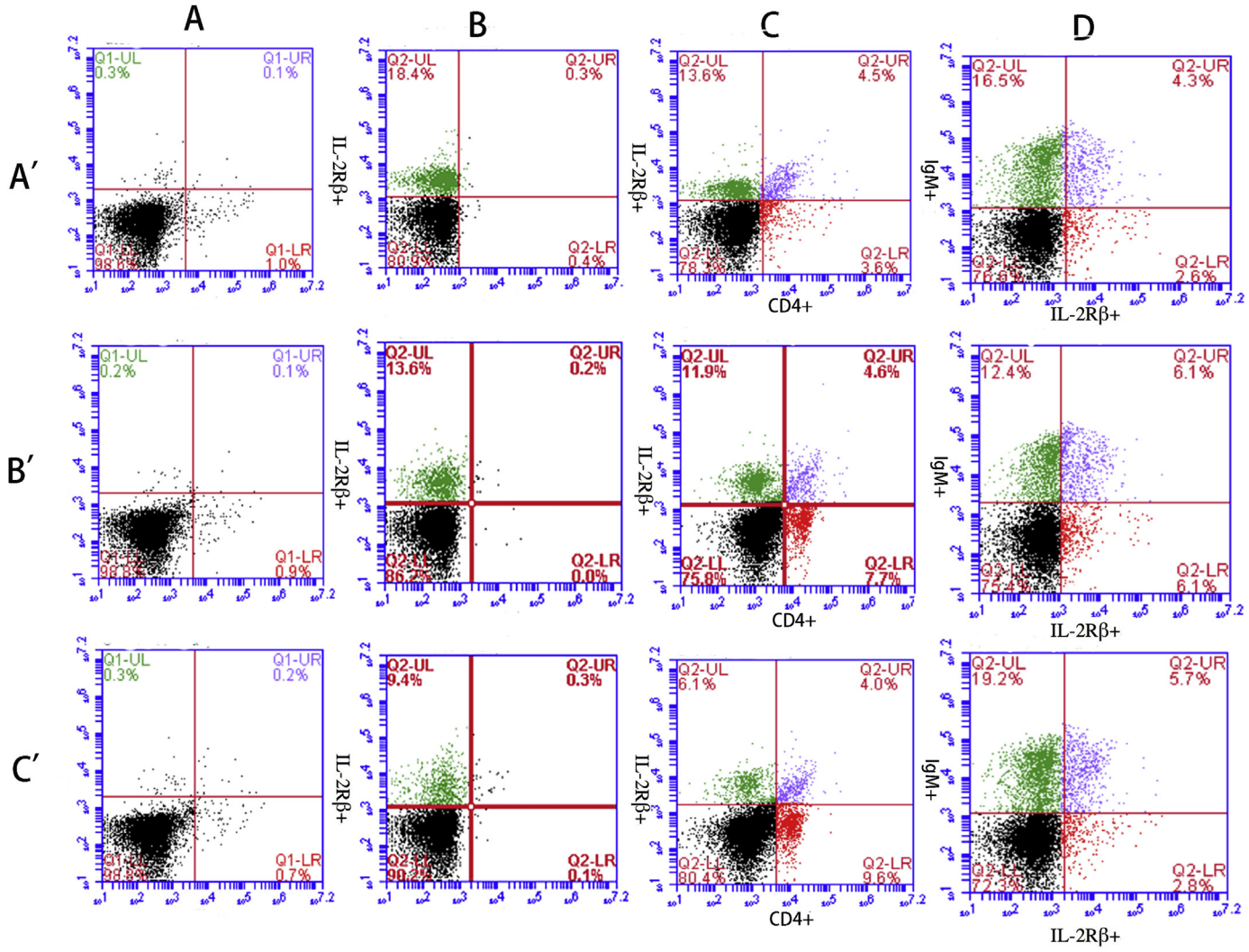

Fig. 8. FCM detection of IL-2Rβ + cells, $CD4^+$ IL-2Rβ + cells, IgM + IL-2Rβ + cells in leukocytes from flounder peripheral blood, spleen, head kidney. A. The negative controls using non-immunized as primary antibody. B. Single staining of leukocytes cells with anti- IL-2Rβ.C. Double staining of leukocytes cells with rabbit anti-$CD4^+$ and mouse anti- IL-2Rβ Abs. D. Double staining of leukocytes cells with mouse anti-IgM Abs and rabbit anti- IL-2Rβ. A'∼C′, in leukocytes from flounder peripheral blood, spleen, kidney, respectively.

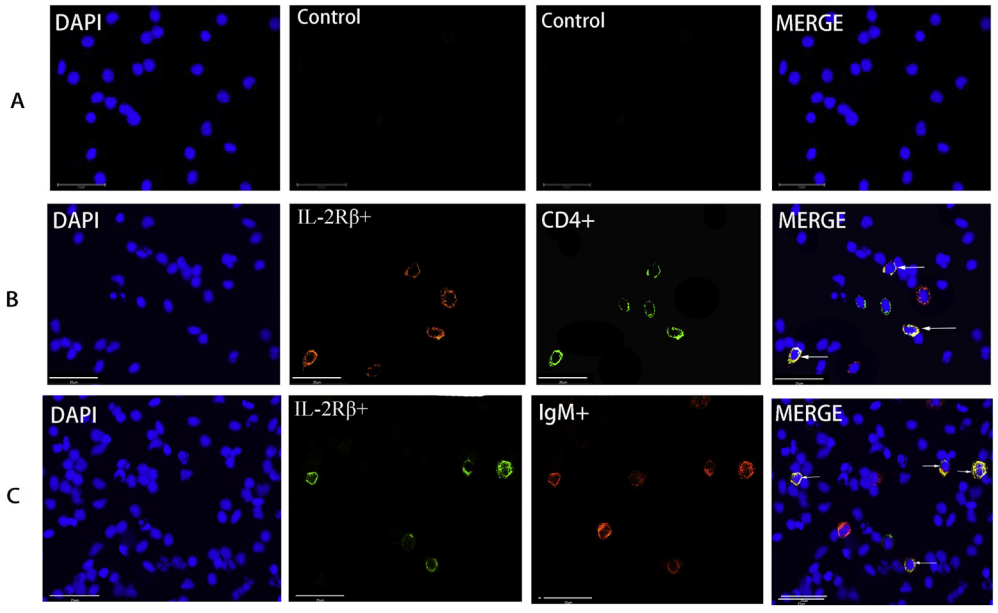

Fig. 9. Double Immunofluorescence staining results of $CD4^+$ IL-2Rβ + cells, IgM + IL-2Rβ + cells lymphocytes. A. Negative controls. B. $CD4^+$ IL-2Rβ + cells. C. IgM + IL-2Rβ + cells lymphocytes. Mouse anti-flounder IgM Mabs, Abs against CD4-1, CD4-2, mouse anti-flounder IL-2Rβ and rabbit anti-flounder CD40 Abs were used in this experiment. Arrows indicate double positive cells. Bar = 25 μm.

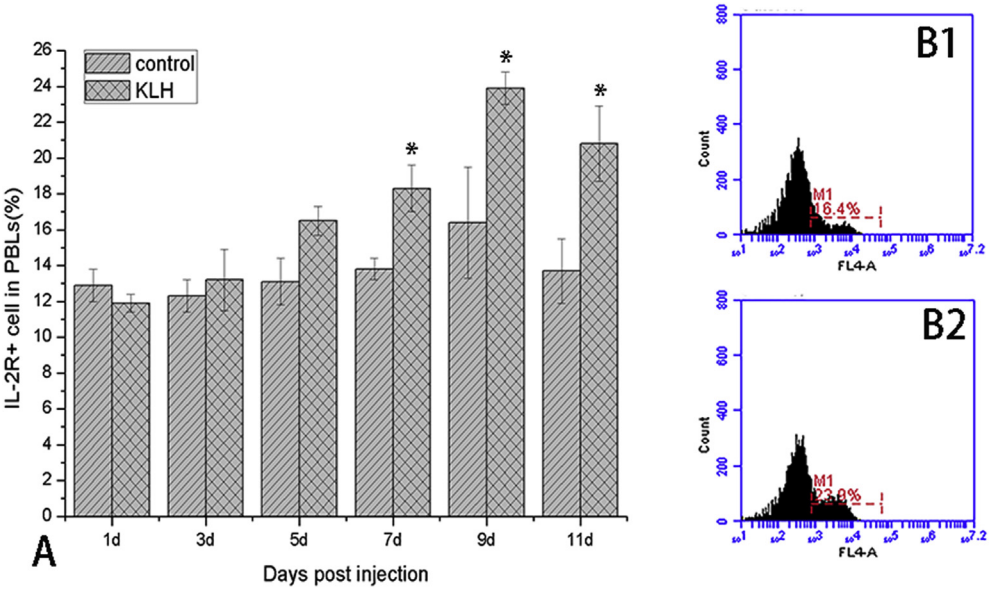

Fig. 10. Percentages of IL-2Rβ+ leukocytes in PBLs of flounder after KLH infection determined by flow cytometry. A. Variations of IL-2Rβ+ leukocytes percentages. "*" represent the statistically significant difference, $p < 0.05$. B. The fluorescent results of IL-2R + leukocytes at maximum. B1: On the 9th day in control group. B2: On the 9th day in KLH group.

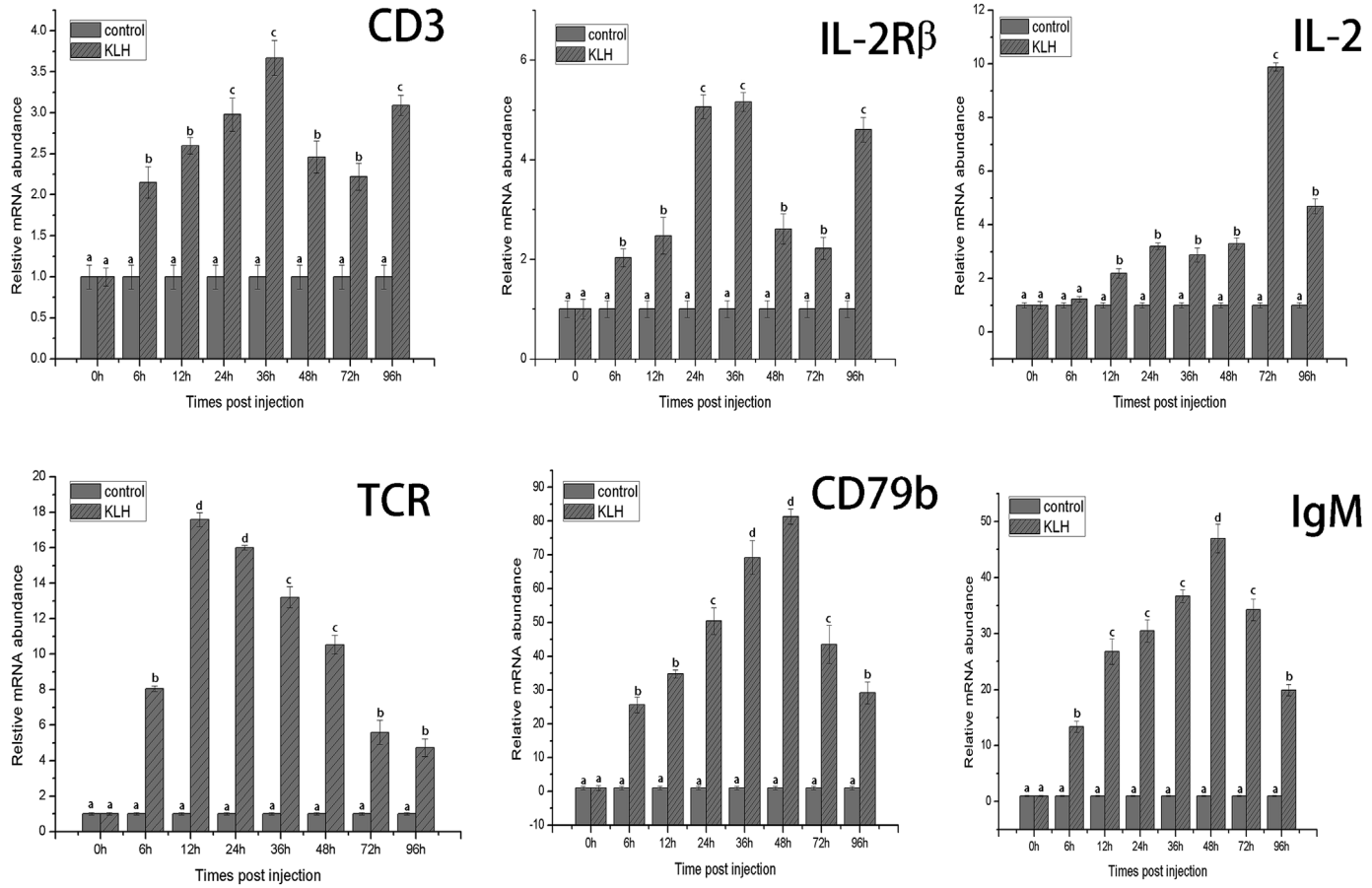

Fig. 11. IL-2, IL-2Rβ, CD3, TCR, CD79b and IgM genes expression in spleen of flounder after injection with KLH. All of the data represent results obtained from at least three independent experiments. Different letters above the bar represent the statistical significance ($p < 0.05$) compared. to each other.

IgM + lymphocytes, respectively. This study firstly reported that the IL-2R molecules were expressed on both B and T lymphocytes in teleost, which illustrated that B and T lymphocytes were the target cell of IL-2–IL-2R–mediated regulatory mechanism.

The FCM results showed that expect being expressed in lymphocytes, IL-2Rβ+ is also expressed in other cells. In the mammals, study showed that, in addition to lymphocytes, IL-2R molecules maybe also distributed on the surface of NK cells, macrophages and DC cells, but now there are lack of antibodies in flounder to label these cells, and no direct results to prove this. This means that IL-2 also plays vital roles in the regulation of other immune cells by reacted with IL-2R [29–31].

For the injection experiment *in vivo*, the percentages of IL-2Rβ+ leukocytes in PBLs were significantly increased post KLH injection compared with control group. And our previous study showed that KLH

can increased the percentages of T/B lymphocytes [20]. KLH as a thymus dependent antigen, B cell differentiation and antibody production require the participation of antigen presenting cells such as macrophages and helper T cells (TH) after KLH injection. It is speculated that the increase of IL-2Rβ+ leukocytes may be related to the variation of T/B lymphocytes, which need to be further explored. The relationship between IL-2R and IL-2 was complicated. Antigen stimulation could induce the secretion of IL-2 and the activation of IL-2 signaling pathway. In this study, the Q-PCR result showed that the expression of IL-2Rβ reached the first peak at 36 h, while the expression of IL-2 reached the peak at 72 h, and then the expression of IL-2Rβ reached the next peak at 96 h. In Flow cytometry and indirect immunofluorescence assay, the results showed that FIL-2Rβ molecule was expressed on both B and T lymphocytes. And the q-PCR showed that the expression of IL-2Rβ were consistent with the expression of CD3 and IgM. In addition, IL-2, as an autocrine cytokine of T cells, its usually upregulated after T cells are activated by antigens. Those results were similar with the study in proliferation of chicken T cells *in vivo* [32]. The interaction between IL-2-IL-2R and lymphocytes needs further study.

Although fIL-2Rβ just one subunit of the fIL-2R, its structural characteristics and expressed on both B and T lymphocytes to illustrated that fIL-2Rβ play the important role in IL-2–IL-2R–pathway. Similar study was published from the IL-4 and IL-4Rα in zebrafish [26]. Analyzed the immunological characteristics of fIL-2Rβ lay a theoretical foundation for IL-2 to exert its immunological function and promote lymphocytes growth, proliferation and differentiation. The progress of IL-2 as vaccine adjuvant to improve immune protection, IL-2–IL-2R–mediated regulatory mechanism would be the further research emphasis.

In conclusion, this study was analyzed the bioinformatic information of fIL-2Rβ amino acids sequence, first reported that the characteristics of IL-2R and IL-2R + molecules were expressed on both B and T lymphocytes in teleost. Those data lay a foundation for further exploring the interaction between IL-2 and IL-2R to promote cell proliferation and the role in immunological function in teleost.

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.2019.07.059.

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