Fish and Shellfish Immunology 81 (2018) 309–317

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Molecular cloning and bioactivity of an IL-2 homologue in large yellow croaker (*Larimichthys crocea*)

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ARTICLE INFO

Keywords:
Large yellow croaker (*Larimichthys crocea*)
Interleukin-2
Expression
Immune response
T cell development and differentiation

ABSTRACT

Interleukin-2 (IL-2), an important immunomodulatory cytokine, plays a crucial role in promoting the proliferation, activation and differentiation of T cells. Here, the cDNA of an IL-2 homologue (*Lc*IL-2) in large yellow croaker (*Larimichthys crocea*) was cloned by RACE-PCR techniques. The open reading frame (ORF) of *Lc*IL-2 gene is 426 bp long and encoded a precursor protein of 141 amino acids (aa), with a 20-aa signal peptide and a 121-aa mature peptide containing two putative N-glycosylation sites at Asn^{77} and Asn^{101}. The *Lc*IL-2 is preferentially expressed in lymphocytes-rich tissues, such as spleen and blood, and is increased in head kidney and spleen upon inactivated trivalent bacterial vaccine or poly(I:C) stimulation. *Lc*IL-2 expression could also be detected in primary head kidney leukocytes (PKL), primary head kidney macrophages (PKM) and primary head kidney granulocytes (PKG), with the highest level in PKL. In addition, the expression level of *Lc*IL-2 in PKL was slightly induced by LPS or poly(I:C), while markedly induced by PHA or Con-A. The recombinant *Lc*IL-2 protein produced in *Pichia pastoris* could increase the expression of genes involved in Th1 (IL-2, IFN-γ and T-bet) and Th2 (IL-4/13A, IL-4/13B and GATA3) development and differentiation, and of the IL-2 downstream transcription factor STAT5B gene, but inhibit the expression of genes related to Th17 (IL-17A/F2 and IL-17A/F3) development and differentiation. Taken together, our results indicated that *Lc*IL-2 possesses similar structural and functional characteristics to other vertebrate IL-2s, and may play a role in T cell development and differentiation.

1. Introduction

Interleukin-2 (IL-2), an important immunomodulatory cytokine, is also known as a T cell growth factor (TCGF) and was first discovered as a growth-promoter of bone marrow-derived T lymphocytes [1,2]. IL-2 is mainly derived from activated $CD4^+$ T cells [3]. Upon antigen stimulation, all or most T cells produce IL-2 immediately, subsequently, $CD4^+$ T cells differentiate into T helper cell and other T cell subsets [4,5]. Within different T helper cell subsets, Th1 mainly produces IL-2 in large amounts [4]. Certain antigen-presenting cells, such as B cells and dendritic cells (DC), could also produce IL-2 in a small amount [6–8]. Besides these, activated natural killer (NK) cells, and NKT cells also produce the IL-2 [9–11].

IL-2 is a pleiotropic cytokine. The most important function of IL-2 is that it can act as a potent T cell growth factor for both $CD4^+$ and $CD8^+$ T cells [3]. IL-2 could induce the expression of STAT5 (Signal transducer and activator of transcription 5)-dependent IL-12Rβ2 (interleukin 12 receptor, beta 2 subunit) and T-bet (T-box transcription factor TBX21), thus promoting $CD4^+$ T cells to differentiate into Th1 cells that preferentially produce interferon-γ (IFN-γ) [12]. Additionally, it could promote the expression of IL-4 and IL-4 receptor to mediate the Th2 development [13,14]. IL-2 negatively regulated the expression of IL-6Rα and IL-6ST (IL-6 signal transducer) to inhibit the differentiation of Th17 cells, which produce IL-17A, IL-17F and IL-22 [15,16]. Other than these, IL-2 is essential for the development of regulatory T (Treg) cells [17]. It also induces lymphokine-activated killer activity and the cytolytic activity of NK cells [18]. Meanwhile, IL-2 can augment immunoglobulin production of B cells [6], and enhance the activities to induce target cell death in $CD8^+$ T cells [19].

Up to now, only two teleost IL-2 homologues were identified in pufferfish (*Takifugu rubripes*) and rainbow trout (*Oncorhynchus mykiss*) [20,21]. Both of them share same genomic synteny as human IL-2.

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https://doi.org/10.1016/j.fsi.2018.07.040
Received 13 June 2018; Received in revised form 12 July 2018; Accepted 17 July 2018
Available online 18 July 2018

Furthermore, recombinant rainbow trout IL-2 produced in *Escherichia coli* had a role in inducing the expression of STAT5, Blimp-1 (B lymphocyte-induced maturation protein), as well as of IFN-γ, gIP-10 (interferon gamma-induced protein 10) and IL-2 in *in vitro* cultured primary head kidney leucocytes [20]. In this study, an IL-2 homologue (*Lc*IL-2) was cloned and characterized in large yellow croaker. Its expression patterns in normal or immune-stimulated tissues and primary immune-related cells were investigated. Recombinant *Lc*IL-2 protein (r*Lc*IL-2) produced in *Pichia pastoris* could induce the expression of genes involved in Th1 and Th2 development and differentiation, and of transcription factor STAT5B gene, but inhibit the expression of genes involved in Th17 development and differentiation *in vivo*. These results therefore indicated that *Lc*IL-2 homologue may be involved in T cell development and differentiation.

2. Materials and methods

2.1. Cloning and bioinformatics analysis of LcIL-2 cDNA

A partial cDNA fragment encoding large yellow croaker IL-2 homologue was predicted from the large yellow croaker genome data (**JRPU00000000**) [22]. To obtain the full-length cDNA of *Lc*IL-2, 5′- and 3′- RACE PCR were performed using 5′-Full RACE Kits with TAP and 3′-Full RACE Core Sets (TaKaRa, China) according to the manufacturer's instructions. The resulting PCR products were cloned into a pMD18-T simple vector (TaKaRa, China), sequenced and assembled to obtain the full-length *Lc*IL-2 cDNA sequence. To confirm the integrity of the cDNA sequence, PCR was performed and the resulted PCR products were sequenced. Primers used in these experiments were listed in Supplementary Table 1.

Sequence similarity analysis was performed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Signal peptide prediction was conducted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Putative glycosylation sites were predicted by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Multiple sequence alignment was performed with Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic maximum likelihood tree was constructed by PhyML software using JTT (Jones, Taylor, and Thornton) amino acid substitution model with aLRT test parameter [23]. The genomic DNA organization of IL-2 genes was analyzed by Splign program (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi?textpage=online&level=form) through aligning the *Lc*IL-2 cDNA sequence with its genomic DNA sequence. Gene synteny at the *IL-2* loci in large yellow croaker and other species was analyzed using the NCBI database.

2.2. Fish and challenge experiments

Large yellow croakers (weight: 102 ± 15.7 g; length: 21 ± 1.7 cm) were purchased from a mariculture farm in Ningde, Fujian, China. Various tissue samples including spleen, kidney, intestine, gill, heart, liver, skin, stomach, blood and brain were collected from at least six healthy fish. For challenge experiments, fish were maintained with a flow-through seawater supply at 25 °C. After acclimating for 7 days, healthy fish were used for the challenge experiments. Two groups of 40 fish each were intraperitoneally injected with inactivated trivalent bacterial vaccine consisting of 1.0×10^9 colony-forming units/mL of *Vibrio alginolyticus, Vibrio parahaemolyticus* and *Aeromonas hydrophila* each at a dose of 0.1 mL/100 g fish or poly(I:C) at a dose of 0.25 mg/100 g fish, respectively. The trivalent bacterial vaccine was prepared as described previously [24], and was used to elicit both innate and acquired immune processes in fish [25]. The head kidney and spleen were collected from six fish in both groups at different time points post-injection (0, 6, 12, 24, 48 and 72 h). Another group of 40 fish was injected with PBS at a dose of 0.1 mL/100 g fish as control. All samples were frozen immediately in liquid nitrogen and stored at −80 °C until RNA extraction.

2.3. Primary immune-related cells

The *in vitro* cultured primary head kidney leukocytes (PKL), primary head kidney macrophages (PKM) and primary head kidney granulocytes (PKG) were prepared as reported previously [26,27]. Briefly, head kidney was removed from individual freshly-killed large yellow croaker under sterile condition and gently pushed through a 70-μm nylon mesh (BD, USA) to get single cell suspension. The effluent was then washed with ice-cold L-15 medium containing 2% FBS, 10 IU/mL heparin, 200 IU/mL penicillin (Gibco, USA) and 200 mg/mL streptomycin (Sigma, USA). To isolate PKL, the single cell suspension was loaded onto freshly prepared

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1       AAACTAGTACATCACCTCTGCTATACAACTTGCACATCACACTGACAAGCCTTCCAGCTGAATATGGAGCACTTT
1                                                                   M  E  H  F
76      ATCAGGATTGCCTTTTGGATTGTTACGCTCTCTGGTTGTCTCCTATCTCTACCTATACCTGAAAATAATATTCCT
5        I  R  I  A  F  W  I  V  T  L  S  G  C  L  L  S  L  P  I  P  E  N  N  I  P
151     TTACTGAGAAAACATGTCAAATGCCCAGCAGAAGCAACATTCTACACCCCATCAAATGTGAATGAGCAATGCCTC
30       L  L  R  K  H  V  K  C  P  A  E  A  T  F  Y  T  P  S  N  V  N  E  Q  C  L
226     ACTGCTGCCCTGGTCTGCTGTGAGAAGGAACTTCAAACAGTCGCAAATGAGTGTAATGACACCCAGAACGGTACA
55       T  A  A  L  V  C  C  E  K  E  L  Q  T  V  A  N  E  C  N  D  T  Q  N  G  T
301     TTCATCCACCTGGCTCAAGAATACTTGACAGAGGTCATAGAAAATTTGGAAAAGAAGAATGGAAATGTATCCATA
80       F  I  H  L  A  Q  E  Y  L  T  E  V  I  E  N  L  E  K  K  N  G  N  V  S  I
376     ACCTCCACAGAATGTGCCTGTGAGAACTGGACTGAAAATCCTTTCGATGTCTTTCTGAACAAAGCGGAATCTGTA
105      T  S  T  E  C  A  C  E  N  W  T  E  N  P  F  D  V  F  L  N  K  A  E  S  V
451     CTTCAGCAAGTATACGCTGCATACTCAACTTCAGCCTAAAAAAGAAAGTAGCTTCAGTCCAACAAAACTGGGGAA
130      L  Q  Q  V  Y  A  A  Y  S  T  S  A  *
526     ACCTTTGTGTTGCAGCTTCCCCATCAGCTGCCCTGAGAAACATTGCAGGAAGTCTAAAGCAGAGCTCATGCGCTG
601     CAGAAAATGACAGCTAAACTTCACTTTGTGTGCGATGACACATCCAACCACTAATTCTGAAGCAAATGGACTTTT
676     CAAGAATATAACCATGTGTTTTAAGGATTTTATGTAATCAAGCAACATCTACACACGTTGAATGACGTGATCTCA
751     GGTATATCCACTTTTAGTGAGGGCTAAGATTCTGAACACTGCTCCGAAAGATTTGTATTTATAACCATGTTATTG
826     CAATATGGACATAAGGCAACTATTTATGCTGAATTTTGTTTATTTTTACTACATACTAATACAATTGTATATCCA
901     AGTATATATATATATTTTAGGATTTCTACAAGAAACTGCTGTGAAGTGAATGTTAAAGTTCTCAAGTTGATTTTG
976     ACCATTGAGAATGTATTTGAAGTAAGCTCCTATATATCTCTAAATTTCTGTTTATACGTAATTTATATATTTATT
1051    TAATATGTATGACATATGTTGGTACTCTAAACTTAAGTTTAATTTAATAAATGTTATCTCTAAGAAAAAAAAAAA
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Fig. 1. Nucleotide and deduced amino acid sequences of *Lc*IL-2.
The predicted signal peptide is underlined (residues 1-20) and two putative N-glycosylation sites are in bold. Stop codon TAA is indicated with an asterisk. A typical polyadenylation signal (AATAAA) is shown in bold and italicized. Four mRNA instability signal (ATTTA) are boxed in the 3′-UTR, the poly(A) tail is italicized and underlined.