Fish & Shellfish Immunology 55 (2016) 281–292

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Identification and functional characterization of multiple interleukin 12 in amberjack (*Seriola dumerili*)

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ARTICLE INFO

Article history:
Received 15 April 2016
Received in revised form
20 May 2016
Accepted 22 May 2016
Available online 27 May 2016

Keywords:
IL-12
IFNgamma
Cell-mediated immunity
Amberjack
Yellowtail

ABSTRACT

Interleukin (IL) -12 is a heterodimeric cytokine mainly produced by monocytes, macrophages, and dendritic cells in mammals. IL-12p70 composed of IL-12p35 and IL-12p40, is known to play a crucial role in promoting cell-mediated immunity (CMI) through Th1 differentiation and IFN-γ production. Although two types of *IL-12p35 (p35a, p35b)* and three types of *IL-12p40 (p40a, p40b* and *p40c)* have been identified in several fish species, the knowledge on functional characteristics of teleost IL-12 is still limited. In the present study, we cloned two types of *IL-12p35* and three types of *IL-12p40* genes in amberjack and yellowtail, and analyzed their expressions in response to stimulation with *Nocardia seriolae* in amberjack. As a result, four types of *IL-12 (IL-12p35a, p35b, p40a and p40b)* and *IFN-γ* mRNA were increased by live-*N. seriolae* stimulation but not by formalin-killed *N. seriolae*, suggesting that four types of *IL-12* (*p35, p35b, p40a and p40c)* participate in promoting CMI. Subsequently, we produced six types of recombinant IL-12p70 (rIL12p70) protein in insect cells. Head kidney leukocytes were cultured with formalin-killed *N. seriolae* and six types of rIL-12p70 to elucidate the role of amberjack IL-12p70 in induction of CMI. After stimulation, *IFN-γ* expression was elevated whereas *IL-10* expression was suppressed in Head kidney leukocytes stimulated with four types of rIL-12 (p40a/p35a, p40c/p35a, p40a/p35b, p40a/p35b). On the other hand, two types of rIL-12 (p40b/p35a, p40b/p35b) only elicited down regulation of *IL-10* expression. These results indicate that all amberjack IL-12p70 isoforms are involved in Th1 -differentiation and promotion of CMI with different manners. Fish IL-12 has a potential for the promising vaccine adjuvant.

1. Introduction

Interleukin-12 (IL-12) is a heterodimeric cytokine that shows a key roles on activation of T-cells and natural killer cells in mammals [1,2]. IL-12, consisting of an alpha chain (p35) and beta chain (p40), acts as heterodimeric IL-12p70 which is mainly expressed in antigen presenting cells (APCs), such as monocytes, macrophages, and dendritic cells [3–5]. Production of IL-12p35 is induced by bond of Major histocompatibility complex (MHC) class II on the surface of APCs and T-cell receptor, whereas IL-12p40 is produced by bonding CD40 on the surface of APCs to the CD40-ligand expressed in T-cells [6,7]. IL-12p70 secreted by APCs, promote induction of cell-mediated immunity (CMI) through the Th1 differentiation by binding to IL-12Rβ1 and IL-12Rβ2 heterodimer on Th0 cells [8,9]. The activated Th1 cells lead to the priming of macrophages and cytotoxic T-cells via production of Th1-type cytokines, such as Interferon (IFN)-γ, Tumor necrosis factor (TNF)-α and IL-2 [8,9]. Th1 cells also inhibit the humoral immune response with limiting Th2 differentiation from Th0 cells by production of IFN-γ. On the other hand, IL-10, produced by Th2 cells controls an induction to humoral immunity by suppressing IFN-γ production in Th1 cells [10–14].

To date, teleost *IL-12* genes have been identified in European seabass, common carp, fugu, Atlantic halibut, Atlantic salmon, rainbow trout, orange spotted grouper, and rock sea bream [15–22], showing that teleost possess two types of *IL-12p35* (*p35a, p35b*) and three types of *IL-12p40* (*p40a, p40b, p40c*) genes, which are through to be derived by genome duplication event prior to teleostean split [23]. Although effects of IL-12 on induction of Th1-

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http://dx.doi.org/10.1016/j.fsi.2016.05.025
1050-4648/

cytokines (IFN-γ and TNF-α) gene expression was reported in several fish species as in mammals [15–22], the knowledge of functional differences among multiple IL-12p70 isoforms is limited in fish.

In the present study, we have isolated five types of *IL-12* gene *(IL-12p35a, p35b, p40a, p40b* and *p40c)* from amberjack (*Seriola dumerili*) and yellowtail (*Seriola quinqueradiata*). These two species are among the most popular marine fish for aquaculture in Japan and widely consumed all over the world. Nocardiosis caused by Gram-positive intercellular bacterium, *Nocardia seriolae*, cause serious economic losses in amberjack and yellowtail aquaculture production [24–26]. Therefore, an effective vaccine is urgently needed for the control of Nocardiosis. Recently, we demonstrated that cell-mediated immunity (CMI) has a crucial role in protection from infection of intercellular bacterium in ginbuna crucian carp *Carassius auratus langsdorfii* [27–29]. In addition, innate immunity-related cytokine genes were increased in *N. seriolae*-infected Japanese flounder [30]. Furthermore, Nayak et al. [31] reported that $CD8^+$ T cells and IgM^+ cells were increased at the early times sensitization with live and inactivated *N. seriolae*, while $CD4^+$ T cells were increased only by live *N. seriolae* infection in ginbuna crucian carp. Moreover, administration of recombinant IFN-γ improved survival rate after infection with *N. seriolae* [31]. These data suggest that induction of CMI is an important in the protection from *N. seriolae* infection in fish. In this study, gene expression levels of *IL-12p35* and *IL-12p40* in response to live or inactivated *N. seriolae* were analyzed in amberjack. In addition, bioactivity of amberjack recombinant IL-12p70 was evaluated to clarify the contribution of IL-12 in induction of CMI.

2. Materials and methods

2.1. Fish

Artificially hatched amberjack *Seriola dumerili* and yellowtail *Seriola quinqueradiata*, weighting between 120 and 130 g, were maintained in running seawater at a temperature of 25 °C–28 °C, and were fed commercial pellets on a daily basis.

2.2. Bacteria

Nocardia seriolae 024013 strain, which was isolated from yellowtail, was cultured in Brain heart infusion (BHI). The bacteria were incubated for 5 days at 25 °C prior to use.

2.3. cDNA cloning of amberjack IL-12p35 and IL-12p40 genes

Head kidney, spleen, and gill excised from amberjack were stimulated with 50 μg/ml of lipopolysaccharide (*E. coli* O127:B8, Sigma-Aldrich, USA) in 5% FBS containing RPMI-1640 medium at 25 °C for 6 h. After incubation, these tissues were homogenized and total RNA was extracted using RNAiso Plus (Takara, Japan), followed by reverse-transcribed into the first strand cDNA using GeneRacer kit (Thermo Fisher Scientific, USA) according to manufacturer's instruction. Primer sets used for cDNA cloning of *IL-12p35* and *IL12p40* in amberjack and yellowtail are listed in Supplementary Table 1 and Table 2, respectively. Degenerated primers were designed based on conserved sequences of IL-12p35 and IL-12p40 genes from European seabass, Atlantic halibut, orange spotted grouper, fugu and tilapia. Degenerate PCR was conducted to obtain partial sequences of *IL-12p35* and *IL12p40* cDNA with BioTaq DNA polymerase (Bioline, UK). PCR reactions for RACE were performed with KOD–Plus–Neo DNA polymerase (TOYOBO, Japan). The amplified DNA was subcloned into the pGEM®-T Easy vector (Promega, USA) and sequenced using ABI 3500xL Genetic Analyzer (Thermo fisher Scientific).

2.4. Structural and phylogenetic analyses

Putative leader sequence was predicted by using SignalP (http://www.cbs.dtu.dk/services/SignalP/) [32]. Multiplex sequence comparison analysis was conducted by CLUSTAL W program (http://clustalw.ddbj.nig.ac.ip/index.php/) [33]. The phylogenetic tree was constructed by the neighbor-joining method. Possible N-linked glycosylation sites were identified by NetNGlyc 1.0 server (http://www.cds.dtu.sk/services/NetNGlyc/) [34].

2.5. Expression analysis of IL-12 genes in response to Nocardia seriolae

Amberjack were euthanized with an overdose exposure of 2-phenoxyethanol to remove the head kidney. The organ was pressed on cell strainer (CORNING, USA) and suspended with RPMI-1640 containing 5% FBS. The suspension was applied to a Percoll (GE Healthcare, USA) at density gradients of 1.040 and 1.070 g/ml and centrifuged at 1600 rpm for 30 min at 4 °C. The head kidney leukocytes were collected at the interface of 1.040–1.070 g/ml and washed three times with RPMI-1640 containing 5% FBS. The isolated head kidney leukocytes were incubated in 24-well plates (1.5×10^6 cells/well) with stimulants such as 1×10^7 CFU/well live cells or formalin-killed *N. seriolae* for 3, 6, 9 and 12 h at 25 °C. Total RNA was subsequently extracted from the leukocytes and, reverse transcribed into cDNA using ReverTra Ace® (Toyobo). Real-time PCR was performed using GoTaq® qPCR Master Mix (Promega) and Mini opticon™ System (Bio-Rad, USA) with specific primer sets: *IL-12p35a, IL-12p35b, IL-12p40a, IL-12p40b, IL-12p40c, IFN-γ, IL-10* and *EF-1α* (Table 1). Five individual fish were examined and statistical analysis of expression data was performed using one way ANOVA, followed by Tukey's post hoc test ($P < 0.05$ or $P < 0.01$).

2.6. Plasmid construction and transfection

To produce IL-12 heterodimer, ORF of IL-12p35 and IL-12p40 were amplified by PCR using specific primer sets containing *Sph*I or *Kpn*I and *Sac*II recognition sequences and GS linker sequences (GGTGGCGGTGGGTCTGGTGGAGGTGAATCT), respectively (Table 2). PCR products of IL-12p35 and IL-12p40 were fused using overlapping site and cloned into insect cell expression vector pMIB/V5-His B (Thermo Fisher Scientific). High Five cells were transfected with the expression vector and incubated with 20 μg/ml blastcidin S.

2.7. Production and purification of amberjack rIL-12

A cell after incubation, culture media were collected and centrifuged at 1600 rpm for 5 min. The supernatant were loaded on HiTrap HP anion-exchange column (GE Healthcare) and were washed with 20 mM Tris-HCl. Subsequently, rIL-12 were eluted by loading 20 mM Tris-HCl, 1M NaCl. The eluted fraction was loaded into HisTrap Ni-NTA affinity column (GE Healthcare) and recombinant protein was purified with 500 mM imidazole. Purity of rIL-12 was confirmed by SDS-PAGE and Western blotting. Briefly, purified fraction were electrophoresed in 8% SDS-PAGE gel and stained by Coomassie Brilliant Blue. For Western blotting analysis, PVDF membranes with transferred proteins were blocked with 1% skim milk containing PBS. The membrane were incubated with mouse anti-His antibody (1:2000, Wako, Japan) for 1 h at room temperature. After primary antibody reaction, the secondary antibody reaction was conducted with HRP-conjugated anti-mouse IgG + IgM (1:2000, Jackson Immunoresearch, USA) on the same