Fish & Shellfish Immunology 51 (2016) 70–76

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

A transmembrane C-type lectin receptor mediates LECT2 effects on head kidney-derived monocytes/macrophages in a teleost, *Plecoglossus altivelis*

Hai-Ling Ma [a], Yu-Hong Shi [a], Xue-Heng Zhang [a], Ming-Yun Li [a], Jiong Chen [a, b, *]

[a] Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Ningbo University, Ningbo 315211, China
[b] Collaborative Innovation Center for Zhejiang Marine High-efficiency and Healthy Aquaculture, Ningbo University, Ningbo 315211, China

ARTICLE INFO

Article history:
Received 1 December 2015
Received in revised form
6 February 2016
Accepted 8 February 2016
Available online 12 February 2016

Keywords:
Antibody neutralization
Bacterial killing
Chemotaxis
C-type lectin receptor
Cytokine expression
LECT2
Monocyte/macrophage
Phagocytosis

ABSTRACT

Leukocyte cell–derived chemotaxin 2 (LECT2) is a multifunctional cytokine involved in many diseases in which immune dysfunction is present. Ayu LECT2 (PaLECT2), which interacts with a C-type lectin receptor (PaCLR), was shown to activate ayu head kidney-derived monocytes/macrophages (MO/MΦ) to improve the outcomes of fish upon bacterial infections. However, it is not known if PaCLR mediates PaLECT2 effects on ayu MO/MΦ. In this study, we determined the role of PaCLR in signal transduction of PaLECT2 on ayu MO/MΦ. We expressed the PaCLR ectodomain in *Escherichia coli* and produced a refolded recombinant protein (rPaCLR) that was then used to produce the anti-PaCLR IgG (anti-PaCLR) for neutralization. Addition of the refolded PaLECT2 mature peptide (rPaLECT2m) to ayu MO/MΦ cultures, increased cytokine expression, induced chemotaxis, and enhanced phagocytosis and bactericidal activity of these cells were observed. When we added anti-PaCLR to block the ectodomain of PaCLR, these effects were significantly inhibited. Based on our previous works and the data presented here, we conclude that PaCLR mediates the immunomodulatory effects of PaLECT2 on ayu MO/MΦ, thus defining a mechanism by which LECT2 protects fish against pathogens.

1. Introduction

Leukocyte cell-derived chemotaxin-2 (LECT2) was first isolated from culture fluid of the human (*Homo sapiens*) T cell line, SKW-3, and was identified as a cytokine with neutrophil chemotactic activity [1]. Current evidences suggest that LECT2 is a multifunctional protein involved in many diseases where immune dysfunction is present, such as sepsis, cancer, diabetes and severe liver injury [2–6]. In addition to mammals, a large number of LECT2 genes had also been characterized in teleosts such as rainbow trout (*Oncorhynchus mykiss*) [7], ayu (*Plecoglossus altivelis*) [8], longtooth grouper (*Epinephelus bruneus*) [9], grass carp (*Ctenopharyngodon idella*) [10], and Asian seabass (*Lates calcarifer*) [11]. Fish LECT2 expression increased dramatically after infection and LECT2 gene polymorphisms were shown to be significantly associated with bacterial disease resistance [11–13], while LECT2 expression in mammals always decreased upon bacterial infection [3,14]. Recently, we found that ayu LECT2 (PaLECT2) interacted with a C-type lectin receptor (PaCLR), and that PaCLR was mainly expressed in the head kidney and leukocytes [8]. Addition of recombinant ayu LECT2 mature peptide (rPaLECT2m) to ayu head kidney-derived monocytes/macrophages (MO/MΦ) induced chemotaxis, increased phagocytosis, increased bactericidal activity, and altered expression of several different genes mainly involved in actin cytoskeleton, pattern recognition receptors, and cytokines [15,16]. In a recent *in vivo* study, we found that intraperitoneal injection of LECT2 could improve the outcomes of *Vibrio anguillarum*-infected ayu through the mediation of MO/MΦ [5], similar to that reported in mice [3]. These studies revealed that, in fish, LECT2 enhanced the MO/MΦ functions to combat bacterial infections. However, no studies have examined if PaCLR is the receptor for PaLECT2 in ayu MO/MΦ.

Ayu is an economically important fish in East Asia and bacterial and viral infections hinder ayu culture development [17]. It is therefore important to study modulation of the ayu immune response against pathogens. In this study, we overexpressed PaCLR in *Escherichia coli* and developed an antibody against PaCLR. Using

* Corresponding author. Laboratory of Biochemistry and Molecular Biology, School of Marine Sciences, Ningbo University, Ningbo 315211, China.
E-mail address: jchen1975@163.com (J. Chen).

http://dx.doi.org/10.1016/j.fsi.2016.02.013
1050-4648/

antibody neutralization, we determined if PaCLR mediates the immunomodulatory effects of PaLECT2 on ayu MO/MΦ.

2. Materials and methods

2.1. Fish management

Healthy fish, weighing 40–50 g each, were purchased from a commercial farm in Ninghai County, China and kept in 100 L tank at 20–22 °C with regular feeding as previously described [5]. The fish were acclimatized to laboratory conditions for two weeks before experiments were conducted. All experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Ethics Committee of Ningbo University.

2.2. Production of refolded recombinant PaLECT2 and PaCLR

Recombinant PaLECT2 mature peptide (rPaLECT2m) containing no endotoxin was produced as previously reported [15]. Primers CLR-F: 5′- CCATATGTGCGTTGATGGTTGGATCCA -3′ and CLR-R: 5′-GGAATTCTCAGTTGAGCATGATTGCGT -3′ were designed to amplify the sequence that encodes the extracellular region of PaCLR (EMBL accession number: FN396582). Amplicons with the expected size were digested by *Nde* I and *Eco*R I (underlined in the primer CLR-F and CLR-R, respectively), and subsequently inserted into the pET28a(+) vector, resulting in the construction of the recombinant plasmid pET28a-PaCLR. Then, the related target protein was expressed in *E. coli* BL21 (DE3). Recombination PaCLR (rPaCLR) was expressed as an inclusion body, and then purified as previously reported [15]. In brief, solubilized rPaCLR was purified eluted with buffer B (0.1 M Tris-HCl, 0.5 M NaCl, 500 mM Imidazole and 8 M urea, pH 7.5) increasing from 0 to 100% on an HisTrap™ FF (GE Healthcare, Shanghai, China). The peak fractions were dialyzed with solubilization buffer (50 mM KH_2PO_4, 1 mM EDTA, and 300 mM KCl, containing 8 M urea and 0.2 M DTT, pH 8.0), and subsequently concentrated 10 times using a 10,000 NMWL spin filter (Millipore, Shanghai, China). Refolding of solubilized rPaCLR was performed using urea gradient (8 to 2 M) size-exclusion chromatography on an XK 16/100 column packed with Superdex 75 gel media (GE Healthcare). Peak fractions were pooled, concentrated using a 10,000 NMWL spin filter and desalted on a 5-ml Bio-Gel P-6 desalting column (Bio-Rad, Shanghai, China). All procedures were carried out at 4 °C.

2.3. rPaCLR antibody preparation

Antibody preparation was performed as previously described [18]. Briefly, rPaCLR, emulsified with Freund's incomplete adjuvant, was used to immunize mice by intraperitoneal injection once every seven days for a total of four injections. Control mice were injected with complete Freund's adjuvant. Blood was collected and sera were separated. Anti-PaCLR IgG (anti-PaCLR) and control isotype IgG (isoIgG) were purified using protein G chromatography media (Bio-Rad). Specificity of anti-PaCLR was tested by Western blot analysis, and visualized using an enhanced chemiluminescence (ECL) kit (Advansta, Menlo Park, USA).

2.4. Cell preparation

Ayu head kidney-derived MO/MΦ were isolated as previously described [5]. Briefly, ayu head kidney was washed in RPMI 1640 medium (Invitrogen, Shanghai, China) supplemented with 2% fetal bovine serum (FBS) (Invitrogen), streptomycin (100 μg/ml), penicillin (100 U/ml), and heparin (20 U/ml). The cells were separated by using Ficoll-Hypaque PREMIUM (1.077 g/ml) (GE Healthcare, New Jersey, USA) in combination with centrifugation. Then the cells were seeded in 35-mm dishes at a density of 2×10^7/ml. Non-adherent cells were washed off, and the attached cells were incubated in the complete medium (RPMI 1640, 5% ayu serum, 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin) at 24 °C, with 5% CO_2.

2.5. Cytokine expression analysis

Differences in mRNA expression for tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), interleukin 10 (IL-10) and granulocyte-colony stimulating factor (G-CSF) genes in ayu MO/MΦ were determined using real-time quantitative PCR (qPCR), as previously described[18]. Briefly, ayu MO/MΦ were pre-incubated with 200 μg/ml anti-PaCLR or isoIgG for 40 min, followed by rPaLECT2m (2.5 μg/ml) treatment for 3.5 h. Cells were collected for the exaction of total RNAs. Primers for TNFα, IL-1β, and IL-10 amplification were the same as those previously reported [5]. The primers GCSF-F: 5′- GACCTACTGCTGGCTGCTTC -3′ and GCSF-R: 5′-ATTGGCCACTGACTTTCAGC -3′ were newly designed to amplify a 172 base pair fragment from ayu G-CSF sequence (JP740394). The qPCR protocol used was: 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, in a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, USA) using SYBR® Premix Ex Taq™ (Perfect Real-Time) (TaKaRa). The mRNA expression of cytokines was normalized against that of β-actin, and the quantitative differences in expression between different samples were calculated using the $2^{-\Delta\Delta CT}$ method [19].

2.6. Immunofluorescence assay

Immunofluorescence assay was performed as previously reported [18]. In brief, head kidney-derived MO/MΦ were purified as described in Section 2.4. The cells were fixed in 4% paraformaldehyde for 30 min, then air-dried, followed by soaking in PBS. Cells were blocked with 5% BSA in PBS before incubation with polyclonal mouse antiserum to PaCLR (1:50 dilution) at 4 °C overnight. Cells were visualized with a laser confocal microscope IX81-FV1000 (Olympus, Tokyo, Japan) using DyLight™ 488-TFP ester-conjugated goat-anti mouse IgG (1:200 in PBS). DAPI (1:1000 in PBS) was used to dyeing the nucleus of cells.

2.7. Chemotaxis assay

Ayu MO/MΦ were pre-incubated with 200 μg/ml anti-PaCLR or isoIgG for 40 min. A modified Boyden's chamber method [20] using a Chemotaxicell (Corning, shanghai, China) with a 5 μm pore-size polyvinylpyrrolidone-free polycarbonate membrane were carried out to assess the chemotactic activity of rPaLECT2m on ayu MO/MΦ. rPaLECT2m or BSA was diluted to 0, 0.1, 1, 10, and 100 μg/ml in complete medium. The lower chamber containing rPaLECT2m or BSA was covered with a 5-μm pore nitrocellulose filter. The cells were plated in the upper chamber, and the chambers were incubated for 1.5 h at 24 °C. Cells that migrated into the lower chamber were counted using light microscopy (Nikon, Tokyo, Japan). Each migration assay was performed in triplicate.

2.8. Phagocytosis assay

During logarithmic growth, *E. coli* DH5α were collected and labeled with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, USA) according to the manufacturer's protocol, and FITC-labeled bacteria were hereafter designated as *E. coli*-FITC. Ayu MO/MΦ were pre-incubated with 200 μg/ml anti-PaCLR or isoIgG for 40 min. Then 2.5 μg/ml of rPaLECT2m was added and incubated for