Fish & Shellfish Immunology 50 (2016) 79–90

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Identification and characterization of a novel NOD-like receptor family CARD domain containing 3 gene in response to extracellular ATP stimulation and its role in regulating LPS-induced innate immune response in Japanese flounder (*Paralichthys olivaceus*) head kidney macrophages

Shuo Li [a, 1], Xiaoli Chen [a], Gaixiang Hao [a], Xuyun Geng [b], Wenbin Zhan [c], Jinsheng Sun [a, *]

[a] Tianjin Key Laboratory of Animal and Plant Resistance, College of Life Sciences, Tianjin Normal University, 393 West Binshui Road, Xiqing District, Tianjin 300387, China
[b] Tianjin Center for Control and Prevention of Aquatic Animal Infectious Disease, 442 South Jiefang Road, Hexi District, Tianjin 300221, China
[c] Laboratory of Pathology and Immunology of Aquatic Animals, LMMEC, Ocean University of China, Qingdao 266003, China

ARTICLE INFO

Article history:
Received 6 December 2015
Received in revised form
20 January 2016
Accepted 22 January 2016
Available online 25 January 2016

Keywords:
NLR
NLRC3
Extracellular ATP
Lipopolysaccharide-induced innate immunity
Paralichthys olivaceus

ABSTRACT

Nucleotide oligomerization domain (NOD)-like receptor (NLR) family with a caspase activation and recruitment domain (CARD) containing 3 (NLRC3) protein is an important cytosolic pattern recognition receptor that negatively regulates innate immune response in mammals. Hitherto, the immunological significance of NLRC3 protein in fish remains largely uncharacterized. Here we identified and characterized a novel *NLRC3* gene (named *poNLRC3*) implicated in regulation of fish innate immunity from Japanese flounder *Paralichthys olivaceus*. The poNLRC3 protein is a cytoplasmic protein with an undefined N-terminal domain, a NACHT domain, a fish-specific NACHT associated domain, six LRR motifs, and a C-terminal fish-specific PYR/SPYR (B30.2) domain but only shares less than 40% sequence identities with the known Japanese flounder NLRC proteins. *poNLRC3* gene is ubiquitously expressed in all tested tissues and is dominantly expressed in the Japanese flounder head kidney macrophages (HKMs). We for the first time showed that *poNLRC3* expression was significantly modulated by the stimulation of extracellular ATP, an important danger/damage-associated molecular pattern in activating innate immunity in *P. olivaceus*. Importantly, we revealed that poNLRC3 plays an important role in positively regulating ATP-induced *IL-1beta* and *IL-6* gene expression, suggesting the involvement of poNLRC3 in extracellular ATP-mediated immune signaling. In addition, we showed that *poNLRC3* mRNA expression was up-regulated in response to LPS and *Edwardsiella tarda* immune challenges. Finally, we showed that down-regulating the endogenous *poNLRC3* expression with small interfering RNA significantly reduced LPS-induced proinflammatory cytokine gene expression in the Japanese flounder HKM cells. Altogether, we have identified a novel inducible fish NLR member, *poNLRC3*, which is involved in extracellular ATP-mediated immune signaling and may positively regulate the LPS-induced innate immune response in the Japanese flounder HKM cells.

1. Introduction

The nucleotide-binding oligomerization domain receptors, also known as NOD-like receptors (NLRs), are intracellular sensor proteins that recognize pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs) and mediate the innate immune response to a wide range of pathogens, tissue damage and other cellular stresses [1,2]. NLR proteins have a complex domain architecture with variable N-terminal effector domains, a central nucleotide binding and oligomerization (NACHT) domain that shares homology with the AAA+ superfamily of ATPases, and a variable C-terminal leucine-rich repeats (LRRs)

* Corresponding author.
E-mail addresses: shuo76@yahoo.com (S. Li), jssun1965@aliyun.com (J. Sun).
[1] S. Li is a senior author.

http://dx.doi.org/10.1016/j.fsi.2016.01.029
1050-4648/

domain involved in ligand recognition [3]. Ligand binding leads to oligomerization and activation of NLRs and the activated NLRs then recruit other proteins through homotypic interactions with their N-terminal domains, typically either a pyrin or a CARD domain, and activate NF-κB through the NODosome pathway or caspase-1 through the inflammasome mechanism [4].

Based on their evolutionary and domain structure, NLRs can be divided into NLRA, NLRB, NLRP and NLRC subfamilies [5]. NLRC subfamily is further classified as five subgroups: NLRC1 (also referred as NOD1), NLRC2 (formerly known as NOD2), NLRC3 (also referred as NOD4), C2TA (MHC class II transactivator protein, also referred as CIITA) and NLRC5 [6]. NLRC subfamily members usually have one or two N-terminal CARD domains with the exception of NLRC3 and NLRC5 proteins, in recruiting caspase-1 or kinases and activation downstream signaling. NLRC3, initially termed as *CATERPILLER* gene, or *CLR16.2*, was firstly identified from human T lymphocytes and is predominantly expressed in human immune tissues and cells [7]. Further studies indicated that NLRC3 is conserved in vertebrates and possesses the characteristic NOD and leucine-rich repeat configuration but with an undefined CARD domain [5]. In contrast to most NLRs which activate innate immunity, mouse NLRC3 inhibits Toll-like receptor signaling *via* modification of the signaling adaptor TRAF6 and transcription factor NF-κB [8]. Recent studies also indicated that mammalian NLRC3 negatively regulates STING-dependent innate immune activation in response to cytosolic DNA, cyclic di-GMP, and DNA viruses and reduces IFN and other cytokine production [9].

Up to date, several NLRs have been identified in teleost, including zebrafish [10], goldfish [11], rainbow trout [12], orange-spotted grouper [13], Japanese flounder [14,15], and channel catfish [16,17]. However, the immune relevance of NLRs in teleost is till limited. Specially, much less is known about the regulatory function of NLRC3 in fish innate immunity. Currently, the only available literature reported that zebrafish NLRC3-like receptor can bind the inflammasome component apoptosis-associated speck-like protein and is an essential negative regulator of macrophage activation and inflammation in a pyrin and NACHT domains-dependent manner [18].

In the present study, we have identified and characterized a novel *NLRC3* ortholog (termed *poNLRC3*) that was up-regulated by LPS and bacterial challenges from Japanese flounder *Paralichthys olivaceus*. We showed that *poNLRC3* positively regulates the LPS-induced proinflammatory cytokine expression in *P. olivaceus* head kidney macrophages. In addition, we revealed that *poNLRC3* expression was significantly modulated by extracellular ATP stimulation and down-regulation of *poNLRC3* expression significantly reduced ATP-induced *IL-1beta* and *IL-6* gene expression in the HKM cells. These findings for the first time addressed the involvement of NLRC3 in the extracellular ATP-mediated innate immune signaling in teleost and suggested that *poNLRC3* may perform an important role in regulating fish innate immune response.

2. Material and methods

2.1. Fish and maintenance

Japanese flounder *P. olivaceus* were obtained from a local fish farm in Dagang, Tianjin, China and maintained in an aerated recirculating seawater system in the laboratory at 21 °C for two weeks before experimentations. Fish were handled and clinically examined as described in the previous study [19]. Only healthy animals without any pathological signs were selected for experiments.

2.2. RNA isolation, cDNA preparation and gene cloning

Total RNA from *P. olivaceus* tissues was purified by TRIzol reagent (Invitrogen) and treated with DNase I (amplification grade, Invitrogen) before reverse transcription according to the manufacturer's recommendations. RNA from cells was isolated with the RNeasy plus mini kit (Qiagen). RNA quality was examined by a 1.2% formaldehyde-denatured agarose gel stained with ethidium bromide. The purified RNA was quantified by measuring OD_{260} with a NanoDrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific). Aliquots of total RNA (2 μg) were transcribed into cDNAs using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

RT-PCR was performed to amplify the internal sequence of *poNLRC3* gene using a degenerate primer pair (F1/R1, Table 1) and the cDNA templates synthesized from Japanese flounder hepatopancreas tissue. The obtained mid-part sequence was used to design gene-specific primers to amplify the 5′- and 3′-untranslated regions of the Japanese flounder *poNLRC3* cDNA. To obtain the 3′-terminal end of *poNLRC3* cDNA sequence, a nested PCR was performed with a gene-specific forward primer F2 and a universal reverse adaptor primer AP (Table 1). The 5′-terminal end of *poNLRC3* cDNA sequence was amplified using a SMARTer™ RACE amplification kit (Clontech) by two rounds of nested-PCR amplification: the first round of PCR reaction was performed with a forward primer UPM (a mixture of primers UPM-L and UPM-S, Table 1) and a reverse gene specific primer GSP1 (Table 1); the PCR products were then diluted 100 times and a nested amplification with primers NUP/GSP1 (Table 1) was performed in a MyCycler™ gradient thermocycler (Bio-Rad). Gene-specific primer pair F3 and R2 (Table 1) corresponding to the 5′- and 3′-untranslated regions of *poNLRC3* mRNA, respectively, were designed, and RT-PCR was carried out to obtain the complete sequence of the Japanese flounder *poNLRC3* cDNA.

2.3. Bioinformatics analysis

Nucleotide sequence identities were determined by blast against GenBank database using BlastX algorithm at the National

Table 1
Sequence of primers used in this study.

Primer name	Sequence (5′→3′)
F1	CAGAARYTCAYTCTDGACTGG
R1	GACTCTAGAGCCGAGATCAGA
F2	GACCCAAGCCAAGACACT
AP	GCCACGCGTCGACTAGTAC
GSP1	GTGACTAAATCTCCTGCTGAAATATTCG
UMP-L	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UMP-S	CTAATACGACTCACTATAGGGC
NUP	AAGCAGTGGTATCAACGCAGAGT
F3	TGTCAGGACGGATTCTGGAA
R2	ACTTTGCACACGATCGAAGT
FLAG-f	CGGGGTACC AAGAGGAAGAGGAATGAAGA
FLAG-r	TCCCCCGGG CTAACAAAGCAATGCAGAAG
poNLRC3-f	AGCAGCCGTATCTCCATCACC
poNLRC3-r	AAGCATCCACATCCCCGC
IL-1beta-f	CCTGTCGTTCTGGGCATCAA
IL-1beta-r	CACCCCGCTGTCCTGCTT
IL-6-f	CAGCTGCTGCAAGACATGGA
IL-6-r	GATGTTGTGCGCCGTCATC
TNF-alpha-f	CCGACTGGATGTGTAAGGTG
TNF-alpha-r	GTTGTGGGGTTCTGTTTTCTC
IFN-gamma-f	TGTCAGGTCAGAGGATCACACAT
IFN-gamma-r	GCAGGAGGTTCTGGATGGTTT
beta-actin-f	AGGTTCCGTTGTCCCG
beta-actin-r	TGGTTCCTCCAGATAGCAC