Developmental and Comparative Immunology 76 (2017) 238–246

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

NOD1 is the innate immune receptor for iE-DAP and can activate NF-κB pathway in teleost fish

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ARTICLE INFO

Article history:
Received 28 March 2017
Received in revised form
23 June 2017
Accepted 23 June 2017
Available online 24 June 2017

Keywords:
NOD1
iE-DAP
RIP2
NF-κB
Signaling pathway
Teleost fish

ABSTRACT

The innate immune system is the first line for organisms defense against microbial infection, and NOD-like receptors (NLRs) protein family is an important member of innate immunity effector molecules. It has been proved that NLRs are located in the endochylema and can senses of microbial products. NOD1 is one of the representatives of this family, it has been proved that in mammals, NOD1 can distinguish a specific muropeptide (G-D-glutamyl-*meso*-diaminopimelic acid, iE-DAP) which was derived from bacterial peptidoglycans. However, the NOD-mediated intracellular recognition of microorganisms remains largely uncharacterized in teleost fishes. In this study, we use miiuy croaker (*Miichthys miiuy*) as a model to determine NOD1 can response to the infection of Gram-negative bacteria and it is the receptor that can recognize of iE-DAP by LRRs domain, it can activate the NF-κB signaling pathway through recruit RIP2 to induce inflammatory response in teleost fishes. Results showed that NOD1 can recognize the components of Gram-negative bacteria and activate inflammatory response to resistance of bacterial infection. Our study can improve the knowledge on immune system of fishes and provide a theoretical basis for the study of prevention and treatment of fish diseases.

1. Introduction

The immune system can be activated by various receptors that produced by different innate immune cells, such as macrophages, NK cells, neutrophils, and dendritic cells. These receptors can identify a variety of pathogenic microorganisms or their products (Moreira and Zamboni, 2012). The immune system was includes innate and acquired systems. And the former is the first line of host cells to resistance against pathogen invasion (Miller, 1979), it can be activated through recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) which are present on the cell surface or in the cytosolic compartment (Akira and Takeda, 2004). These PRR families include TLRs, RIG-like receptors (RLRs), C-type lectin receptors (CLRs), and NOD-like receptors (NLRs). All PRRs have been extensively explored and are known to play an important role in the pathogen identification and immune response activation (Kawai and Akira, 2009; Schroder and Tschopp, 2010; Bonardi et al., 2012). NLRs are a family of intracellular receptors that do not contain signal peptide and transmembrane domain and is related to both resistance to pathogenic microorganism infection and cellular damage (Shiau et al., 2013; Wilmanski et al., 2008).

NLRs are composed of three domains, a C-terminal ligand binding domain, a centrally positioned NACHT domain, and a N-terminally located effector binding domain (Wilmanski et al., 2008; Inohara et al., 2005). According to the results of the evolutionary and domain structure analyses, NLRs can be divided into four subfamilies, namely, NLRA, NLRB, NLRP, and NLRC subfamilies (Schroder and Tschopp, 2010). Approximately 22 members of the NLR family are present in humans, and more than 30 are present in mice (Ting et al., 2008), recent research found that NLR are also present in invertebrates and lower vertebrates. Such as more than 200 NLR genes are present in purple sea urchins (Hibino et al., 2006), approximately 34 NLR genes in lampreys (Smith et al., 2013), and total of 50 NLR genes in the miiuy croaker (Li et al., 2016), NOD1 is one of them. In miiuy croaker, NOD1 is very conservative compared to other species, it widely distributed in multiple tissues, and can response to infection of Gram-negative bacteria.

NOD1 is a member of the NLRC subfamily and encoded by *CARD4* genes, it was composed of several domains, and each domain has different functions. Past studies showed that it was

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http://dx.doi.org/10.1016/j.dci.2017.06.012
0145-305X/

widely found in various tissues of mammals (Tohno et al., 2008) and fish (Rajendran et al., 2012). In mammals, it can specifically identify G-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) moieties which was derived from Gram-negative bacteria. In addition, it can recruits downstream interacting protein RIP2 through CARD-CARD interaction to induce the activation of NF-κB signaling pathway (Girardin et al., 2003; Chamaillard et al., 2003; Inohara et al., 2001; Xie and Belosevic, 2015). In the process of fish farming, Gram-negative bacteria are the main pathogenic microbes that can causes various fish diseases, *Vibrio anguillarum* is one of them. Thus, studying the pattern recognition receptors that can identify Gram-negative bacterial component is important. Owing to the large differences between fish and mammals in types and quantities of PRRs, the PRRs which can distinguish Gram-negative bacterial component in mammals may not play the same role in fishes (Sepulcre et al., 2009). Currently, because of the limitation in research method, we know very little about the receptors that can respond to the infection of Gram-negative bacteria in teleost fishes. NOD1 is an intracellular receptor that was both coexist in fishes and mammals, it have highly similar between them (Swain et al., 2013), previous studies have shown that the expression of NOD1 was rapidly increased in the tissues of goldfish which were infected by *Aeromonas salmonicida* or *Mycobacterium marinum* (Xie et al., 2013). In this paper, we choose miiuy croaker (*Miichthys miiuy*) as a model species to study the function of NOD1 due to it was possess good immunological research background (Che et al., 2014; Chu et al., 2015; Xu et al., 2016b). We use this species to determine NOD1 can recognize Gram-negative bacterial components and activate NF-κB signaling pathway by recruiting RIP2 to induce immune response in teleost fishes. This study can provide a theoretical basis for the future study of fish immune systems and introduce a new method for the treatment of fish diseases.

2. Material and methods

2.1. Preparation of tissue and macrophage

To obtain the infected liver tissues, in brief, healthy fish were divided into control group and experiment group, then the individuals in experiment group were intraperitoneal injection of 1 ml suspension of *V. anguillarum* (1.5×10^8 CFU/ml), and at 24 h after injection, dissect the individual, and the tissues from three individuals were removed to −80 °C. The additional details were as described in previous studies (Li et al., 2015).

In order to isolate the macrophages, the head kidney tissues were collected from three healthy miiuy croakers, and then chopped these tissues to perform sterile filtration using 100 μm pore size of the cell filter in L-15 medium that contained streptomycin (100 μg/ml), penicillin (100 IU/ml), 2% FBS and heparin (20 U/ml). The cell suspension were added into 51% Percoll (Pharmacia, USA) separating medium and then centrifuged for 40 min at 400 g, 4 °C. Next, remove the supernatant and obtain the cells from interface and cleaned two times with L-15 medium. Afterward, the cells were seeded in 6 well plate in L-15 medium at a density of 4×10^7 per well and cultured in the incubator at 26 °C with 4% CO_2. On the second day, the cell pellet was replaced with fresh complete L-15 medium that contained 20% FBS. For the PGN and iE-DAP treatment, the cells were challenged with 3 μg/ml PGN (69554, Sigma) and 10 μg/ml iE-DAP (tlrl-dap, InvivoGen), respectively, and collected the cells at 3 h, 6 h, 12 h, 24 h and 48 h for RNA extraction. The cells with no treatment was considered as the control, and each experiment had three biological replicates.

2.2. Real-time quantitative PCR analysis

Total RNA that extracted from macrophages and liver using TRIzol reagent (Invitrogen) was subjected to reverse transcription reaction using the FastQuant RT Kit (Tiangen) which can eliminate genomic contamination. Using specific primers to check the expression of miiuy croaker NOD1, NF-κB, IL-6 and TNFα, β-actin as the internal control. The individual that has been injected with physiological saline and the macrophages that have not been challenged with stimulant were as the mock control. SYBR® Premix Ex Taq™ (Takara) and 7300 real-time PCR system (Applied Biosystems, USA) were used to perform real-time quantitative PCR, and the reaction mixture includes 6 μl ddH_2O, 10 μl SYBR Premix (2 ×), 0.4 μl ROX Dey (50 ×), 0.8 μl of each primer (10 μM) and 2 μl cDNA template. The amplification cycling conditions were 95 °C for 30s and followed by 40 cycles 95 °C for 5s and 60 °C for 34 s. After each analysis, the dissociation curve was conducted to determine target specificity. For each experimental sample, the triplicate experiment were performed. All of primers were displayed in supplemental Table 1.

2.3. Plasmid constructions

The gene of miiuy croaker NOD1 was amplified using specific primers that possess the HA tag and the restriction enzyme sites of *Kpn I* and *Xba I*, then the PCR product was enzyme at 37 °C for 10 min (Takara) and inserted into pCDNA3.1 vector. The PCR product of miiuy croaker RIP2 was digestion and cloned into the pCDNA3.1-flag vector between *BamH* I and *Xho* I sites. The recombinant plasmids were validated by double enzyme digestion and sequencing. The mutants plasmids that named as NOD1-ΔCARD, NOD1-ΔLRR1, NOD1-ΔLRR2-4, NOD1-ΔLRR5-7 and NOD1-ΔLRR were generated through PCR on the basis of NOD1 recombinant plasmid by using specific primers. The oligonucleotide of NOD1-shRNA was designed by using the RNAi Target Sequence Selector website (Clontech), the sequence of three NOD1 shRNAs and control shRNAs were displayed in supplemental Table 1. After the annealing reaction, the sequences were inserted into the pSIRENRetroQ-ZsGreen1 vector between *BamH* I and *EcoR* I sites (Clontech). All of the plasmids were extracted by Endotoxin-Free Plasmid DNA Miniprep Kit (Tiangen), all of primers were displayed in supplemental Table 1.

2.4. Cell culture, transfection and luciferase reporter assays

HEK293 is an easy operating tool cell line was purchased from the ATCC (American type culture collection). The cells were cultured in DMEM high glucose medium that contained 100 μg/ml streptomycin, 2 mM L-glutamine, 10% FBS (Gibco) and 100 units/ml penicillin, under humidified conditions at 37 °C, 5% CO_2. Before experiment, HEK293 cells were inoculated to 24-well culture plate at a suitable density, when the cell density reached about 80% of the culture plate, transfect plasmids into cells. Firstly, mixed the plasmids with the transfection reagent in Opti-MEM®I Reduced Serum Medium (Gibco), and then the mixture was added to the cell culture plate. After 6 h incubation, replace to fresh medium. To verify the role of NOD1, the NOD1 expression plasmid was transfected into cells together with NF-κB, ISRE, IFNα and IFNβ luciferase reporter gene plasmids, respectively, and the control group were transfected with the equal amount of empty plasmids and luciferase reporter gene plasmids. To detect the recognition between ligands and NOD1, after transfection of NOD1 expression plasmid and NF-κB reporter gene plasmid, the cells were transfected with equivalent amounts (1 ug) of LTA, PGN, poly(I:C), iE-DAP and Zymoscan A, respectively, then collected the cells to detect the luciferase activity.