Fish & Shellfish Immunology 34 (2013) 909–919

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Trunk kidney of grass carp (*Ctenopharyngodon idella*) mediates immune responses against GCRV and viral/bacterial PAMPs *in vivo* and *in vitro*

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ARTICLE INFO

Article history:
Received 27 September 2012
Received in revised form
2 January 2013
Accepted 4 January 2013
Available online 16 January 2013

Keywords:
Grass carp (*Ctenopharyngodon idella*)
Trunk kidney
Immune response
Grass carp reovirus
PAMPs

ABSTRACT

Trunk kidney is a vital organ for excretion in teleosts. There have been sporadic reports of processing pathogens for the immune function in trunk kidney. However, molecular processes of pathogen recognition receptors (PRRs) responding to virus and viral/bacterial pathogen-associated molecular patterns (PAMPs) are poorly elucidated in trunk kidney. In the present study, we investigated transcriptional profiles of twelve representative immune-related genes (TLRs (TLR3, TLR7 and TLR22); RLRs (RIG-I, MDA5 and LGP2); NLRs (NOD1 and NOD2); adapter molecules (MyD88 and IPS-1); effector molecule type I interferon (IFN-I) and immunoglobulin M (IgM)) in trunk kidney tissue of grass carp (*Ctenopharyngodon idella*) (designated as Ci) injection of grass carp reovirus (GCRV) utilizing quantitative real-time RT-PCR (qRT-PCR). Furthermore, mRNA expression patterns of these genes (IgM excepted) were examined post GCRV infection and polyinosine-polycytidylic acid (poly(I:C)), lipopolysaccharide (LPS) or peptidoglycan (PGN) stimulation in primary trunk kidney cells of grass carp. The relative values of CiTLR3, CiTLR22 and CiMyD88 were increased post GCRV challenge and viral/bacterial PAMPs stimulation. The mRNA transcriptions of CiTLR7 were obviously activated with GCRV challenge. Remarkably, the mRNA expressions of CiRIG-I, CiMDA5, CiLGP2 and CiIPS-1 were largely up-regulated with GCRV challenge and viral/bacterial PAMPs stimulation. Interestingly, the expression tendencies of CiNOD1 and CiNOD2 were differential not only in GCRV challenge and poly(I:C) stimulation, but also in LPS and PGN stimulation. It was demonstrated that CiIFN-I induced powerful anti-viral and anti-bacterial effects in trunk kidney. In addition, the expression of CiIgM was induced at 72 h post GCRV injection *in vivo*. Collectively, these results suggest that trunk kidney of grass carp serves as an important immune organ, and plays crucial roles in triggering anti-viral and anti-bacterial immune responses both *in vivo* and *in vitro*.

1. Introduction

The innate immune system serves as the first line of protection against invading microbial pathogens through a number of germ line-encoded pattern recognition receptors (PRRs) [1]. PRRs recognize conserved pathogen-associated molecular patterns (PAMPs) to trigger the innate immune response and subsequently adaptive immunity. Upon virus invasion and PAMPs stimulation, PRRs initiate signaling pathways that lead to the induction of inflammatory cytokines and type I interferon (IFN-I). Currently, three major classes of PRRs have been identified, including toll-like receptors (TLRs), retinoic acid inducible gene I-like receptors (RLRs) and nucleotide oligomerization domain-like receptors (NLRs) [2].

To date, more than 13 TLR members have been found in mammals [3]. They are implicated in the detection of a vast range of pathogens including viruses, bacteria, protozoa and fungi [3,4]. Various viruses and bacteria are perceived by intracellular TLRs (TLR3, TLR7/TLR8 and TLR9). Generally, TLR3, TLR7/8 and TLR9 recognize double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and CpG DNA nucleic acids, respectively [4,5]. Unlike TLR3, TLR22 occurs exclusively in aquatic animals (fish and amphibians), and recognizes long-sized dsRNA [6,7]. Upon virus infection or PAMPs stimulation, TLR7/TLR8 and TLR9 recruit the adapter molecule MyD88 (myeloid differentiation factor 88) to elicit immune responses. In contrast, TLR3 and TLR22 rely on TIR domain-containing adapter-inducing IFN-β (TRIF) to mediate downstream signaling pathways. Subsequently, MyD88 and TRIF

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http://dx.doi.org/10.1016/j.fsi.2013.01.003

lead to the activation of nuclear factor-κB (NF-κB) and interferon regulatory factors-3/7 (IRF-3/7). Ultimately, IFN-I and inflammatory cytokines are induced to exert immune effects [3].

RLRs compose of three members: RIG-I (retinoic acid inducible gene I), MDA5 (melanoma differentiation associated gene-5) and LGP2 (laboratory of genetics and physiology 2), and they play a crucial role in modulating antiviral innate immunity [8,9]. RIG-I and MDA5 contain two N-terminal caspase recruitment domains (CARDs), a DExD/H box RNA helicase domain and a C-terminal repressor domain (RD), whereas LGP2 lacks CARDs. The CARDs mediate downstream signaling cascades and induce the activation of interferon-β promoter stimulator 1 (IPS-1; also known as MAVS, CARDIF or VISA) [10]. IPS-1 functions as an adapter molecule, coupling with RIG-I/MDA5 to activate the kinases of TBK1 (TANK-binding kinase 1) and IKK-ε (inhibitor of nuclear factor Iκ kinase-ε), subsequently, they activate a set of transcription factors, including IRF-3/7 and NF-κB that induce productions of IFN-I and ISGs (IFN-stimulated genes) [8]. The third member LGP2 is identified as a negative or positive regulator of signaling transduction in RLR pathways [11,12].

Consisting of more than 20 members, NLRs represent a large class of intracellular sensors that can detect microbial pathogens and endogenous danger signals in mammals [13]. NLRs can be grouped into molecules that contain either a CARD or a Pyrin motif, CARD-containing NOD1 and NOD2 can indirectly interact with IPS-1 leading to the activation of NF-κB and IRF-3. Subsequently, they induce the cytokines and IFN-I productions [13].

Like mammals, fish possess a large number of TLRs, RLRs and NLRs, which have been researched [14–19]. It is evidenced that fish have similar mechanisms to trigger the immune response against pathogens matching with mammals [20,21].

IFN-Is can elicit a rapid and potent immune response controlling viruses and bacteria infections. In particular, they are considered as a bridge of the innate and adaptive immunity in mammals [22]. Recently, significant progress has been made in understanding the fish and amphibian IFN systems [21,23–25].

Compared with the innate immunity, the adaptive immunity depends mainly on antigen receptors to generate highly specific immune responses in living organisms [26]. Immunoglobulins (Igs) bind antigens with high specificity, and they play an essential role in adaptive immunity [27]. IgM is well conserved in both mammals and teleosts, particularly, fish can produce IgM as a primary antibody response post pathogenic infections [28].

Grass carp (*Ctenopharyngodon idella*) is the momentous economic species for aquaculture in China. However, it is susceptible to several epidemics, especially the hemorrhage disease, caused by grass carp reovirus (GCRV), a dsRNA virus [29]. Poly(I:C) (polyinosine–polycytidylic acid) is a model for dsRNA analog inducing the production of IFN-I in many organisms [30]. LPS (lipopolysaccharide) is an outer-membrane element of gram-negative bacteria, and PGN (peptidoglycan) is a major component of gram-positive bacterial cell walls [31]. Since eukaryotic organisms do not contain LPS and PGN in their cellular structures, they are ideal targets for detecting bacterial infections.

The kidney is an important tissue for processing pathogens [32], and many researches focus on head kidney mediating immune responses in teleosts [33–35]. However, the immune mechanism in trunk kidney remains largely unknown. In our previous researches, the up-regulations of some immune-related genes (such as CiMDA5 and CiMyD88) transcripts are obvious post GCRV injection by semi-quantitative reverse transcription-PCR (semi-qRT-PCR) in trunk kidney of grass carp [36,37]. Additionally, Japanese flounder LGP2, IPS-1 and MDA5 are expressed strongly in trunk kidney according to differential tissue expressions [17,18,38]. Therefore, the precise and systematic expression profiles of classical immune-related genes can contribute to clarify immune functions of trunk kidney. Furthermore, better understanding of immune defense mechanisms may be conducive to the development of management strategies for disease control and for comprehensive studies of the immune system in teleosts.

In the present study, we investigated transcriptional profiles of twelve representative immune-related genes injection of GCRV in grass carp trunk kidney *in vivo*. Moreover, we tested corresponding genes (IgM excepted) after GCRV infection and poly(I:C), LPS or PGN stimulation in grass carp trunk kidney *in vitro*.

2. Materials and methods

2.1. Fish treatment and GCRV injection

200 grass carp (20 ± 2 g) were obtained from a fish farm (Shaanxi Province, China), and cultured at 28 °C for two weeks before processing. The fish were randomly and equally distributed into two containers. Animals were fed with baits twice a day at the same conditions. In the injected group, fish were intraperitoneally injected with 100 μl of GCRV (097 strain, 3.63×10^7 $TCID_{50}$/ml) [31], suspended in phosphate buffer solution (PBS) per gram body weight. Similarly, fish were injected with equal volume of PBS in the control group. Four individuals were sacrificed at each time point and their trunk kidneys were harvested at 0, 6, 12, 24, 48 and 72 h.

2.2. Primary cells culture, GCRV infection and viral/bacterial PAMPs stimulation

Four trunk kidney tissues were isolated from freshly killed grass carp by forceps and scissors in sterile laminar flow cabinet. Then, the trunk kidneys were washed with PBS six times (nearly transparent) to remove blood cells and impurities under sterile conditions. After that, the tissue was cut up finely with scissors. Fragments of the tissue (about 1 mm^3) were suspended in PBS and digested with trypsin (0.25%; Sigma, USA) for 20 min at room temperature, then 10% fetal bovine serum (FBS) (Biosource, USA) was added to stop the digestion. Afterward, they were centrifuged at 1500 rpm for 6 min, and the supernatant liquid was removed. After that, the cells were resuspended and transferred in 6-well plates supplemented with Medium 199 (Sigma), 10% FBS, 100 IU/ml of penicillin (Sigma) and 100 μg/ml of streptomycin (Sigma) [29]. Subsequently, they were incubated at 28 °C in a 5% CO_2 humidified atmosphere. Three days later, trunk kidney cells were washed with PBS three times and digested with trypsin for about 3 min at room temperature. After the centrifugation, the supernatant liquid was removed, and the cells were resuspended with the Medium 199 and 10% FBS. Then they were counted using a hemocytometer and cultivated at a final concentration of 2×10^6 cells/ml in 24-well plates. After 24 h incubation, the cells were washed with PBS three times and cultured in Medium 199 without FBS. Soon after, they were challenged with GCRV and stimulated with poly(I:C), LPS or PGN, respectively. The dose of GCRV (3.63×10^7 $TCID_{50}$/ml) was 2 μl/well [31]. The terminal concentration of poly(I:C) was 5 μg/ml. Additionally, the terminal concentration of LPS and PGN was 10 μg/ml. The detailed instructions were provided by our previous study [31]. For time-dependent expression profiles, four parallel cells were harvested at 0, 6, 12, 24, 48 and 72 h with 1000 rpm for 8 min, respectively.

2.3. Sample collection, RNA extraction and cDNA synthesis

All samples (including tissues and cells) were homogenized in 800 μl RNAiso Plus (TaKaRa, Japan) and preserved at −80 °C for RNA