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Characterization and functional assessment of the NLRC3-like molecule of the goldfish (*Carassius auratus* L.)☆

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ABSTRACT

The NLRC3-like (NLRC3L) molecule from the goldfish transcriptome database was identified and characterized. Quantitative gene expression analysis revealed the highest mRNA levels of NLRC3L were in the spleen and intestine, with lower mRNA levels observed in muscle and liver. Goldfish NLRC3L was differentially expressed in goldfish immune cell populations with highest mRNA levels measured in PBLs and macrophages. We generated a recombinant form of the molecule (rgfNLRC3L) and an anti-CT-NLRC3L IgG. Treatment of goldfish primary kidney macrophages *in vitro* with ATP, LPS and heat-killed *Aeromonas salmonicida* up-regulated the NLRC3L mRNA and protein. Confocal microscopy and co-immunoprecipitation assays indicated that goldfish rgfNLRC3L interacted with apoptosis-associated spec-like protein (ASC) in eukaryotic cells, indicating that NLRC3L may participate in the regulation of the inflammasome responses. The dual-luciferase reporter assay showed that NLRC3L over-expression did not cause the activation of NF-κB, but that it cooperated with RIP2 to down-regulate NF-κB activation. Our results indicate that the NLRC3L may function as a regulator of NLR pathways in teleosts.

1. Introduction

The cytosolic receptors called NOD-like receptors (NLRs) recognize intracellular bacteria, fungi and protozoan parasites. The NLRs are evolutionarily highly conserved and are present in animals from sea urchins to mammals (Ting and Davis, 2005; Carneiro and Travassos, 2013; Xie et al., 2013; Zhu et al., 2013). In teleosts, the first reported NLRs were identified in the zebrafish genome (Stein et al., 2007). Three subfamilies of NLRs were present in zebrafish: the first resembled mammalian NODs, the second resembled mammalian NLRPs, and the third was reported to be a unique subfamily of genes having similarities to both mammalian NOD3 and NLRPs (Laing et al., 2008). Subsequently, fish NLRs were reported in grass carp (Chen et al., 2010), rainbow trout (Chang et al., 2011), channel catfish (Rajendran et al., 2012; Sha et al., 2009), rohu (Swain et al., 2012a,b), orange-spotted grouper (Hou et al., 2012), Japanese founder (Park et al., 2012; Unajak et al., 2011), goldfish (Xie et al., 2013), and miiuy croaker (Biswas et al., 2016; Li et al., 2015). The results of these studies indicated the presence of inducible NLRs, and that teleost NLRs shared the conserved structural domains with their mammalian counterparts.

In mammals, NLRC3 contains one N-terminal caspase activation and recruitment domain (CARD), an intermediary nucleotide-binding oligomerization domain, and a C-terminal leucine-rich repeat (LRR). Compared to other NLRs, NLRC3 was relatively less studied and was believed to be the negative regulator of the NF-κB pathway (Schneider et al., 2012). NLRC3 was shown to down-regulate mammalian TLR4-dependent NF-κB activation by interacting with TRAF6 (Fiorentino et al., 2002; Schneider et al., 2012), as well as type I interferon signaling by interacting with the DNA sensor STING (Zhang et al., 2014). In recent studies, the cross-talk between NLRC3 and inflammasome has also been demonstrated to coordinate NLR-mediated inflammatory responses by competing with ASC for pro-caspase-1 binding via CARD-CARD interactions (Eren et al., 2017; Gültekin et al., 2015).

In teleosts, a few studies examined the expression of NLRC3 and NLRC3L in response to bacterial infections. As early as in 2009, Sha and co-workers investigated the expression profiles of NLRC3 in catfish, but no significant changes of the mRNA level of NLRC3 were observed upon challenge with *Edwardsiella ictaluri* (Sha et al., 2009). Later, seabass NLRC3 was characterized and its mRNA levels were shown to be up-regulated following exposure to LPS and both Gram-negative and Gram-positive bacteria (Unajak et al.,

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2011). These authors suggested that teleost NLRC3 may play a role in IL-1β induction after LPS-stimulation (Unajak et al., 2011). In addition, Li and colleagues identified and characterized NLRC3 in Japanese flounder, and found that the NLRC3 expression was up-regulated following stimulation with extracellular ATP (Li et al., 2016b). Similarly, seabass NLRC3 was demonstrated to be a pivotal cytosolic innate immune receptor that recognized a wide array of PAMPs (Paria et al., 2016). More recently, two NLRC3 genes were characterized in mucosal tissues of turbot following bacterial challenge and a NLRC3-like (NLRC3L) gene from blunt snout bream was studied in response to *Aeromonas hydrophila* infection (Zhou et al., 2017).

Previous studies in our lab have demonstrated that goldfish ASC associated with CARD-containing proteins including caspase-1 and receptor-interacting serine/threonine kinase 2 (RIP2) (Xie and Belosevic, 2016). The ASC possesses PYD and CARD domains, and it functions as a central adaptor for the formation of inflammasomes (Chen et al., 2014; Hoss et al., 2017), and regulation of PYD-only proteins (POPs) (Bedoya et al., 2007; Dorfleutner et al., 2007) and CARD-only proteins (COPs) (Druilhe et al., 2001; Lee et al., 2001). Given that most of the NLRs are CARD-containing proteins, it is likely that ASC may also be involved in the regulation of other NLRs. Mammalian ASC has been shown to interact with NLRC3, and this interaction has been shown to interfere with the assembly and activity of inflammasome complexes (Eren et al., 2017; Gültekin et al., 2015). It is not known whether ASC functions in a similar manner in teleosts.

Studies on most of teleost NLRC3s and their homologues (NLRC3Ls) were primarily based on the examination of gene expression induced by different immune stimuli and/or fish pathogens, there is relatively little published information whether NLRC3 interacts with CARD-domain containing proteins and whether it plays a role in the regulation of the NLRC3 NF-κB pathway in teleosts. In this study, we characterized the NLRC3L of the goldfish and examined its possible involvement in the regulation of NLR pathways.

2. Materials and methods

2.1. Fish

Goldfish (*Carassius auratus* L.) were purchased from Aquatic Imports (Calgary, AB) and kept in the Aquatic Facility of Department of Biological Sciences, University of Alberta. The fish were maintained at 17 °C using a flow-through water system and a simulated natural photoperiod. Fish were fed to satiation daily with trout pellets, and acclimated to this environment for at least three weeks prior to use in experiments. All fish ranged from 10 to 15 cm in length. Prior to handling, fish were sedated using a TMS (tricaine methane sulfonate) solution of 40–50 mg/L in water. The animals in the aquatic facility were maintained according to the guidelines of the Canadian Council of Animal Care (CCAC-Canada).

2.2. Goldfish macrophage cultures

The procedures for the isolation and cultivation of primary kidney macrophages (PKM) and the ingredient of culture medium (NMGFL-15) have been described previously (Barreda and Belosevic, 2001; Neumann et al., 2000). Briefly, complete NMGFL-15 medium used for culturing goldfish macrophages contained 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), 100 μg/mL gentamicin (Gibco), 5% carp serum and 10% newborn calf serum (Gibco). Goldfish macrophage cultures were established by seeding freshly isolated kidney leukocytes from individual fish ($18–20 \times 10^6$ cells/fish) into 75 cm^2 tissue culture flasks containing 15 mL of complete medium and 5 mL of cell-conditioned medium (CCM) from previous cultures. The PKM cultures consisted of heterogeneous populations of cells including early progenitors, monocytes and mature macrophages as determined by flow cytometry, morphology, cytochemistry and function. The 3–4-day cultures contained primarily monocytes, while older cultures (6–8 days of cultivation) were predominately mature macrophages (Barreda and Belosevic, 2001; Neumann et al., 2000).

2.3. Isolation of goldfish splenocytes, neutrophils and peripheral blood leukocytes (PBLs)

Goldfish splenocytes, neutrophils and PBLs were isolated as previously described (Grayfer and Belosevic, 2012; Katzenback and Belosevic, 2009; Xie et al., 2013; Xie and Belosevic, 2016).

2.4. Identification of the goldfish NLRC3L (gfNLRC3L)

Goldfish mRNA NLRC3L transcript (Accession No. KT354221.1) was identified from goldfish spleen mRNA transcriptome database and the full coding sequence of gfNLRC3L was further confirmed by Sanger sequencing (Suppl. Fig. 1).

2.5. Analysis of gfNLRC3L expression in tissues and immune cell populations

Preparation of cDNA of goldfish tissues and immune cell populations and the quantitative real-time PCR (Q-PCR) thermocycling parameters were previously described (Xie et al., 2013). Briefly, goldfish specific NLRC3L primers were designed using Primer express software (Applied Biosystems) and the expression was assessed relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). Tissue and cell populations from four individual goldfish ($n = 4$) were used for the Q-PCR analysis, carried out using 7500 Fast software (Applied Biosystems). Total RNA was extracted using TRIzol (Gibco) and reverse transcribed into cDNA using Superscript III cDNA synthesis kit (Invitrogen) according to manufacturer's directions. Primers were deemed acceptable for use if the experimentally derived R2 values from the cDNA dilution curves were greater than 0.980. Subsequent to primer validation experiments, Q-PCR product was resolved by agarose gel electrophoresis and the resulting product sequenced to confirm primer specificity. The RQ values were normalized against the expression seen in the lowest tissue (muscle) and immune cell population (monocytes).

2.6. Construction of plasmids

Plasmids were constructed as previously described (Xie and Belosevic, 2016). Briefly, PCR fragment encoding the C-terminal region of goldfish NLRC3L (CT-NLRC3L) (851–1025 aa) was amplified with gene-specific primers introduced with *Sac* I and *Not* I at their 5′-end (Table S1). The PCR product was digested by restriction enzyme *Sac* I and *Not* I (Invitrogen), and then ligated to the *Sac* I/*Not* I digested pET-28a (+) vector (Novagen). The full sequence of goldfish NLRC3L was amplified with gene-specific primers designed to meet the requirements of the pcDNA3.1/V5-His TOPO TA expression vector (Invitrogen), pcDNA3.1/NT-GFP-TOPO vector (Invitrogen) and pDsRed-Monomer-C In fusion Ready Vector (Clontech). The resulting PCR products were gel extracted using a QIA quick gel extraction kit (Qiagen), ligated into the above vectors, transformed into competent *Escherichia coli* (One Shot mach1-T1), plated onto LB-kanamycin/ampicillin plates and incubated overnight at 37 °C. Positive clones were identified by colony PCR, grown up in LB-ampicillin/kanamycin and the plasmids containing the insert were isolated using a QIA quick Spin miniprep kit (Qiagen).