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RESEARCH ARTICLE

Characterization of a nucleotide-oligomerization domain (NOD) like receptor C5 (NLRC5) subfamily member from black rockfish (*Sebastes schlegelii*), portraying its transcriptional responses against immune stimulants

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Abstract Nucleotide-oligomerization domain like receptors (NLRs) are recently identified group of pattern recognition receptors which involve in sensing broad range of pathogen associated molecular patterns or damage associated molecular patterns to trigger corresponding immune responses in host cells. In this study, we identified and characterized a NLRC5 family member from a previously established black rockfish cDNA database, designating as '*RfNLRC5*'. The complete open reading frame of *RfNLRC5* consists of 5808 bp which encodes for a protein of 1936 amino acids with the predicted molecular mass of 213 kDa. Intriguingly, RfNLRC5 harbored only two typical domain signatures of NLR superfamily, namely NACHT domain and LRRs. However, it was phylogenetically closely related to the telostan counterparts. As expected, RfNLRC5 shared significant sequence compatibility with its teleostan counterparts, eminently with that of large yellow croaker. As detected by our qPCR assay, RfNLRC5 was universally distributed in tissues examined, albeit with different levels. Therein, more pronounced expression levels were detected in blood cells and spleen tissues. After treating the naïve fish with immune stimulants; lipopolysaccharides and Polyinosinic:polycytidylic acid (poly I:C), *RfNLRC5* mRNA expression in blood cells and spleen tissues was found to modulate significantly with notable inductive responses. Collectively, our results in this study hint a potential role of *RfNLRC5* in host innate immune responses against bacterial or viral infections.

Keywords NLRC5 · Black rockfish · Tissue specific mRNA expression · Immune-stimulation · Transcriptional modulation

Introduction

Innate arm of the host immunity is less specific but rapidly responsive system against foreign invaders, including pathogenic organisms. Pathogen recognition receptors (PRRs) plays a key role in this first line host defense system through recognition of evolutionary conserved molecular motifs of invading pathogens, designated as pathogen associated molecular patterns (PAMPs). This recognition leads to trigger different immune signaling pathways or shape-up the appropriate inflammatory reactions for an immediate response (Takeuchi and Akira 2010). These responses in-turn activate the adaptive immune system, generally through activation of dendritic cells. PRRs are usually cytosolic, secreted or show transmembrane localization. For instance, toll like receptors are most extensively studied group of PRRs which are localized on cell surface and endosome membranes to sense extracellular pathogens or phagocytosed pathogens (Kawai and Akira 2010). On the other hand RIG-1 like receptors (RLRs), AIM2 like receptors and nucleotide binding oligomerization domain—containing receptors (NOD like receptors (NLRs) are exclusively located in the cytosol and nucleus (Takeuchi and Akira 2010; Liu et al. 2013),

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whereas peptidoglycan recognition proteins are either cytosolic, secreted or localized on the cell membrane.

NLRs are recently identified group of PRRs which are consisted of mainly three types of domains; namely, N-terminal effector domain, central nucleotide binding domain (NBD or NATCH domain) and C-terminal leucine-rich repeats (LRRs). LRRs usually sense pathogens by binding to selective ligands, whereas NATCH domain involves in oligomerization and auto-activation of the molecule. N terminal effector domain is important in forming protein–protein interactions, signal transduction and triggering the respective immune signaling cascades (Rosenstiel et al. 2008). As N terminal effector domains, NLRs harbor different domains, including caspase recruitment domain (CARD), a baculovirus inhibitor of apoptosis protein repeat (BIR) and pyrin domain (PYD). Therefore, based on the n-terminal domain NLRs are divided into four main subfamilies, namely NLRA, NLRB, NLRC and NLRP. However, members of the NLR family which do not show significant homology to the N-terminal domain of the aforementioned subfamily members are categorized under NLRX subfamily (Fritz et al. 2006; Proell et al. 2008; Ting et al. 2008; Opitz et al. 2009). NLRC family is mainly consisted of several members including NLRC1 (NOD1), NLRC2 (NOD2), NLRC3, NLRC4 and NLRC5.

NLRs can sense broad range of PAMPs. NLRC1 and NLRC2 are known to recognize peptidoglycan (PGN) derived distinct structural motifs. For instance, NLRC1 sense g-D-glutamyl-meso-diaminopimelic acid in PGN of all Gram negative and some Gram positive bacteria (Chamaillard et al. 2003; Girardin et al. 2003a). On the other hand, NLRC2 was reported to recognize muramyl dipeptide (MDP) in PGN of both Gram positive and negative bacteria (Girardin et al. 2003b). Upon the PAMP recognition, NLRs trigger different immune signaling cascades which can activate NF-κB or mitogen-activated protein kinases (MAPKs) to regulate the expression of different transcriptional factors such as, AP1 and induce the expression of proinflammatory cytokines and chemokines like IL-1β, IL-6, IL-8, TNF-α and IFN-γ (Inohara et al. 2000; Hasegawa et al. 2008; Monie et al. 2009; Rosenzweig et al. 2009; Chen et al. 2010).

To date, several fish counterparts of NLRs were identified from teleostan species. Mammalian NLR homologues were initially identified from zebrafish and categorized into three separate groups of NLR super family, as NLR A, B and C. NLRA showed analogy to mammalian NOD whereas NLRB resembled mammalian NALP. Interestingly, NLRC was unique to fish. Five NLR counterparts (NOD1, NOD2, NLRC3, NLRC5 and NLRX1) were reported from catfish (Sha et al. 2009) and two (NOD1 and 2) were reported from grass carp (Chen et al. 2010). Moreover, two splice variants of NOD2 was identified and characterized from rainbow trout (Chang et al. 2011) whereas fish specific NLRC was also reported from olive flounder (Unajak et al. 2011). In addition, NOD1 counterpart was recently characterized from rohu (Swain et al. 2012).

Black rockfish (*Sebastes schlegelii*) is one of the highly demanded maricultured finfish delicacies in Northeastern Asia, especially in Korea. However, due to the intensive culturing conditions in farms pathogenic infections in these fish was found to increase drastically over the time, severely affecting the crop quality and yield. Therefore, development of a proper disease management system in black rockfish mariculture farming is becoming a necessity to increase the resistance of these creatures to infections. Thus, the investigation of innate immune mechanisms in this fish on a molecular level, and the identification of ways to increase resistance to infection using modern molecular techniques is one of the productive ways to face the pathogenic threat, successfully. In this study, we sought to identify a homologue of NLRC5 from black rockfish and molecularly characterized while deciphering its temporal transcriptional modulation in response to exposure to some chemical derivatives of pathogens.

Materials and methods

Identification and in silico characterization

A contig sequence of our previously established black rockfish cDNA database (Maestroni and Conti 1989) showing homology to known NLRC5 counterparts of other organisms were identified using Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and designated as *RfNLRC5*. Subsequently, the complete coding sequence of *RfNLRC5* was identified and corresponding amino acid sequence was derived using DNAsist 2.2 software. Expected domain conservation in RfNLRC5 was affirmed by ExPASy Prosite database (http://prosite.expasy.org) comparing with that of several vertebrate counterparts and some of the physicochemical properties were determined using ExPASy ProtParam tool (http://web.expasy.org/protparam). The homology of RfNLRC5 was ascertained using pairwise and multiple sequence alignment platforms using Matgat software (Campanella et al. 2003) and ClustalW2 server (http://www.Ebi.ac.uk/Tools/clustalw2), respectively. The phylogenetic relationship of RfNLRC5 with some of its vertebrate counterparts was analyzed by Molecular Evolutionary Genetics Analysis (version 5.0) software (MEGA 5.0) (Tamura et al. 2011) using neighbor-joining platform, with the support of 1000 bootstrap replicates.

Animal rearing and tissue collection

Pre acclimatized healthy fish to the laboratory conditions were obtained from one of the aquariums in Marine Science Institute of Jeju National University, Jeju Self Governing province, Republic of Korea and maintained in 400 L laboratory aquarium tanks filled with aerated seawater at 22 ± 1 °C. Five healthy fish with average body weight of 200 g were sacrificed for the tissue collection. Before scarification, ~1 mL of blood was collected from each fish using sterile syringes coated with 0.2 % heparin sodium salt (USB, USA) and the peripheral blood cells were separated by immediate centrifugation at 3000×g for 10 min at 4 °C. Other tissues including head kidney, spleen, liver, gill, intestine, kidney, brain, muscle, skin and heart were excised and snap-frozen in liquid nitrogen and stored at −80 °C.

Pathogen derived chemical treatment

Transcriptional modulation of *RfNLRC5* upon immune stimulation in healthy rockfish with average body weight of 200 g was determined in a time course immune stimulation experiment. LPS (1.25 mg/mL, *E. coli* 055:B5; Sigma) and Poly I:C (1.5 μg/μL; Sigma) was used as immune stimulants after resuspending or dissolving in phosphate buffered saline (PBS). Fish were intraperitoneally injected with each stimulant in a total volume of 200 μL. For the injection control group, fish were injected exclusively with 200 μL PBS. The blood cells and spleen tissues were sampled from five individuals of each group at 3, 6, 12, 24, 48 and 72 h post-injection, as described above, respectively.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from a pool of tissue samples (~40 mg from each fish) of five individual fish (both un-injected and injected fish) using QIAzol® (Qiagen) following the vendor's protocol. Tissue samples collected from healthy as well as immune challenged fish were further purified using RNeasy MiniElute cleanup Kit (Qiagen). RNA quality was examined by 1.5 % agarose gel electrophoresis and the concentration was determined at 260 nm in μDrop Plate (Thermo Scientific). First strand cDNA was synthesized in a 20 μL reaction mixture containing 2.5 μg of RNA with PrimeScriptTM II 1st strand cDNA Synthesis Kit (TaKaRa). The synthesized cDNA was diluted 40-fold in nuclease free water and stored in a freezer at −80 °C until use.

Transcriptional profiling by qPCR

The basal mRNA expression level of *RfNLR5* in aforementioned collected tissues of healthy fish and its modulation in blood cells and spleen tissues of immune stimulated animals was determined by qPCR technique using previously prepared diluted cDNA samples as templates. PCR was performed using the Dice™ Real time system thermal cycler (TP800; TaKaRa, Japan) in a 10 μL reaction volume containing 3 μL of diluted cDNA from each tissue, 5 μL of 2 × TaKaRa ExTaq™ SYBR premix, 0.4 μL of each primer (RfNLRC5-qF and RfNLRC5-qR; Table 1) and 1.2 μL of ddH_2O as per the essential MIQE guidelines (Bustin et al. 2009). PCR cyclic conditions were as follows: 95 °C for 10 s; 35 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s; and a final cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Each assay was conducted in triplicates to increase the credibility of the results. The baseline was set automatically by the Dice™ Real Time System software (version 2.00). RfCasp10 relative mRNA expression was determined using the Livak ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen 2001) using black rockfish elongation factor-1-alpha (*RfEF1A*) gene as the internal reference (GenBank ID: KF430623; (Liman et al. 2013). The primers used for the internal reference are listed in Table 1. The data are presented as the mean ± standard deviation (SD) of the relative mRNA expression in triplicated experiments. In immune stimulation experiments, the expression levels of *RfNLRC5* mRNA were calculated relative to that of *RfEF1A*. In addition, the expression fold values were further normalized to the corresponding PBS-injected controls at each time point. The relative expression level in the un-injected control at the 0 h time point was used as the baseline reference. To determine statistical significance ($P < 0.05$) between the experimental and un-injected control groups, two-tailed unpaired student's *t* test was carried out.

Results and discussion

The complete open reading frame (ORF) of *RfNLRC5* was 5808 bp in length which encodes for a protein of 1936 amino acids with 213 kDa molecular mass and ~6.0 isoelectric point. Sequence details on RfNLRC5 were deposited in NCBI- GenBank database, under the accession number KT004545. According to the predicted domain architecture, RfNLRC5 was consisted of N terminal NACHT domain (residues 230–405) and fourteen C-terminal LRRs (residues 841–868, 869–896, 982–1009, 1092–1119, 1120–1147, 1204–1228, 1310–1334, 1335–1357, 1540–1567, 1621–1648, 1737–1764, 1765–1789, 1793–1820 and 1875–1902) agreeing with typical domain arrangement of NLR superfamily members. However, there was no N-terminal effecter domain found like in mammalian counterparts, although which is not unlike with the previously characterized catfish NLRC5 counterpart

Table 1 Oligomers used in the study

Name	Purpose	Sequence (5′ → 3′)
RfNLRC5-qF	qPCR of *RfNLRC5*	ACACCACGCTACACCTGTCTTTGA
RfNLRC5-qR	qPCR of *RfNLRC5*	TGCACAGTCCACGAGGCCTATTT
RfEFA-F	qPCR for black rockfish EF1A	AACCTGACCACTGAGGTGAAGTCTG
RfEFA-R	qPCR for black rockfish EF1A	TCCTTGACGGACACGTTCTTGATGTT

Table 2 Number of LRRs in the C termini of different vertebrate NLRC5 counterparts as predicted by SMART online server

Species name	Number of LRRs
Black rockfish (*Sebastes schlegelii*)	14
Large yellow croaker (*Larimichthys crocea*)	10
Catfish (*Ictalurus punctatus*)	13
Chickens (*Gallus gallus*)	22
Human (*Homo sapiens*)	22

(Liman et al. 2013). According to the domain structure comparison, RfNLRC5 harbors the maximum (14) number of LRRs among the deferent teleostan NLRC5 counterparts, considered (Table 2). However, compared to teleostan ones, chicken and human NLRC5 consist higher number of LRR motifs. Greater number of LRRs is one of the important factors which contribute to the increased surface area of LRR containing proteins, in turn increasing the efficacy of PAMP recognition (Bell et al. 2003). Thus, according to our comparison we can suggest that non-mammalian NLRC5 counterparts are more efficient in pathogen recognition than teleostans.

As expected, according to our pairwise sequence alignment study, RfNLRC5 shared relatively higher sequence compatibility with its teleostan counterparts, in which sharing eminent identity (60.5 %) and similarity (70.7 %) with large yellow croaker NLRC5 (Table 3). The notably high sequence compatibility of RfNLRC5 showed with NLRC5 counterpart, compared to the other NLRC counterparts (NLRC1, 2 and 3) of the same species affirms that RfNLRC5 is more likely a member of NLRC5 family. This notion was further validated by our phylogenetic reconstruction, in which teleostan NLRC5 were clustered together, closely and independently (Fig. 1). Moreover, therein different NLR family members were separately clustered as expected, where RfNLRC5 was clustered with the relevant counterpart of large yellow croaker. Within the NLRC5 main cluster, non-teleostan NLRC5 similitudes were distinctly clustered, where mammalian (bovine and human) and avian (chicken) counterparts were diverged from a common ancestral origin. Intriguingly, fish specific olive flounder NODC was clustered with rat NOD2, hinting on its homology with the mammalian NOD2 s.

As detected by the qPCR assay, *RfNLRC5* expression was detected in every tissue considered in the study, however with different magnitudes (Fig. 2). Pronounced *RfNLRC5* mRNA expression was detected in blood cells, followed by spleen. However, liver, intestine, muscle and skin tissues exhibited relatively very low mRNA expression. Blood consists pool of cells including immune relevant cells such as macrophages like phagocytes, serving as the circulatory medium in animals. Hence, it is not unlike to observe prominent level of PRR expression including NLRs in blood cells to recognize potential pathogens which can enter into blood stream and trigger the relevant immune signaling pathways. On the other hand, fish spleen also harbors ellipsoids enriched with immune cells like macrophages which involves in phagocytosis (Uribe et al. 2011). Moreover, it also comprises blood cells including immune relevant cells such as phagocytes, cytotoxic cells and dendritic cells. Thus, tissues in spleen may also express notably higher amount of NLRs to detect different PAMPs of invaded pathogens. Similar to our observation, catfish NLRC5 was found to express ubiquitously in all tissues examined with very low expression in liver, muscle and ovary tissues (Sha et al. 2009). On the other hand, flounder NLRC expression was universally distributed in tissues examined; but more abundant in brain, gill, kidney and peripheral blood leukocytes (PBLs) (Unajak et al. 2011).

In order to decipher the expressional modulation of *RfNLRC5* upon pathogen stress, healthy rockfish were stimulated using LPS or poly I:C as pathogen derived molecular motifs having bacterial or viral origin, respectively. According to our qPCR assay, after LPS treatment, *RfNLRC5* mRNA expression was significantly elevated from its basal level at early [at 6 h post stimulation (p.s.)] as well as late phase (at 24 h p.s. and 48 h p.s.) of the experiment in blood cells with an early (3 h p.s.) down regulation) (Fig. 3A). On the other hand, its transcription was initially up-regulated (6 h and 12 h p.s.) and later (48 h and 72 h p.s) downregulated in spleen against LPS treatment (Fig. 3A). The overall inductive transcriptional response elicited upon LPS treatment suggests that RfNLRC5 may have a role in sensing and triggering immune responses against bacterial, especially Gram negative bacterial pathogens; as NLRs are known to detect inflammatory stimuli to mediate the formation of inflammasomes and stimulate the secretion of cytokines and

Table 3 Percentage similarity and identity values of RfNLRC5 with different NLRC5 homologes

No.	Species name	Protein	NCBI GeneBank accession number	Amino acids	Identity (%)	Similarity (%)
1	*Larimichthys crocea* (large yellow croaker)	NLRC5	KKF09127	1672	60.5	70.7
2	*Ictalurus punctatus* (catfish)	NLRC5	NP001186995	1726	37.8	58.7
3	*Homo sapiens* (human)	NLRC5	NP115582	1866	28.1	48.6
4	*Gallus gallus* (chicken)	NLRC5	AEY11256	1862	27.7	51.2
5	*Bos taurus* (cattle)	NLRC5	XP001250847	1868	27.4	48.1
6	*Homo sapiens* (human)	NLRC3	NP849172	1065	17.9	29.4
7	*Homo sapiens* (human)	NLRC2 (NOD2)	NP071445	1040	17.8	28
8	*Mus musculus* (mouse)	NLRC3	NP001074749	1102	17.6	30.4
9	*Bos taurus* (cattle)	NLRC3	XP584462	1065	17.5	29.8
10	*Rattus norvegicus* (rat)	NLRC2 (NOD2)	NP001099642	932	16.9	26.5
11	*Paralichthys olivaceus* (olive flounder)	NLRC	ADX66441	1175	16.9	26.5
12	*Danio rerio* (zebrafish)	NLRC3	XP001920433	940	15.4	26.6
13	Homo sapiens (human)	NLRC1 (NOD1)	NP006083	953	15.3	26.3
14	*Ictalurus punctatus* (catfish)	NLRC1 (NOD1)	ACM45224	946	15	25.6

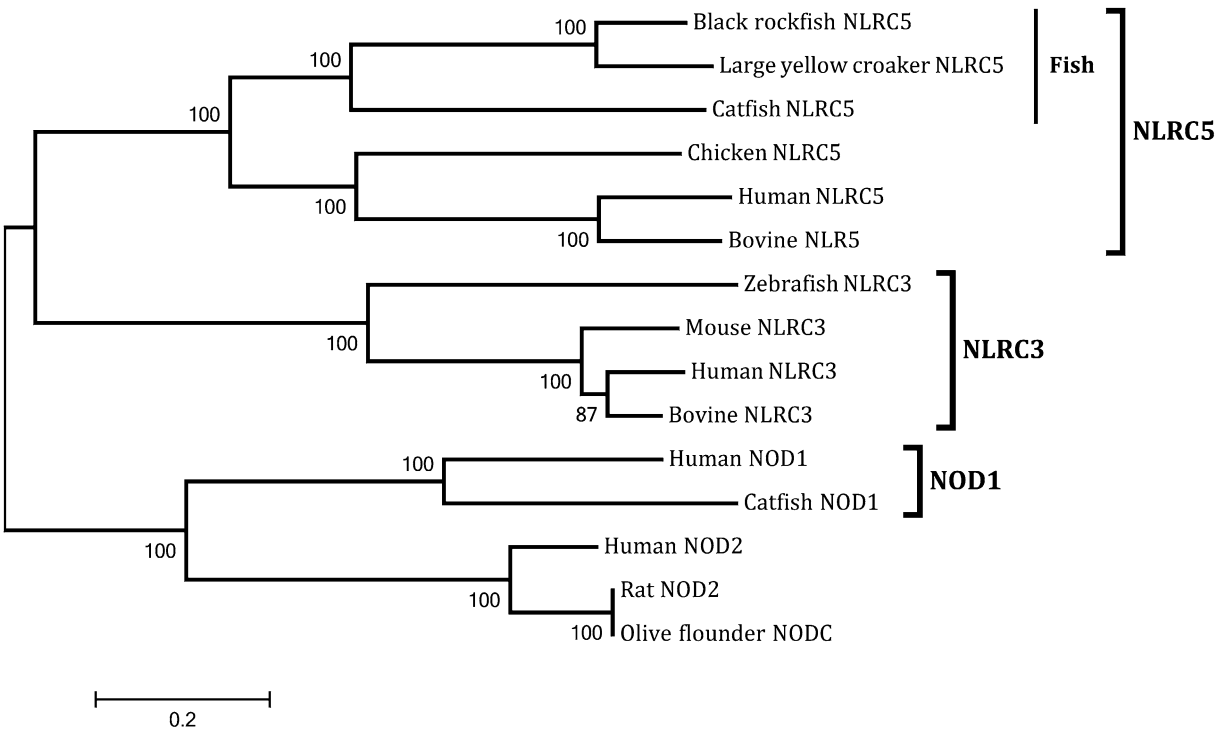

Fig. 1 Phylogenetic reconstruction of RfNLRC5 generated based on ClustalW multiple sequence alignment with different vertebrates and invertebrates under the neighbor-joining platform using MEGA version 5.0. Bootstrap supporting values are denoted at the tree branches and NCBI-GenBank accession numbers of used NLR homologues are mentioned in Table 3

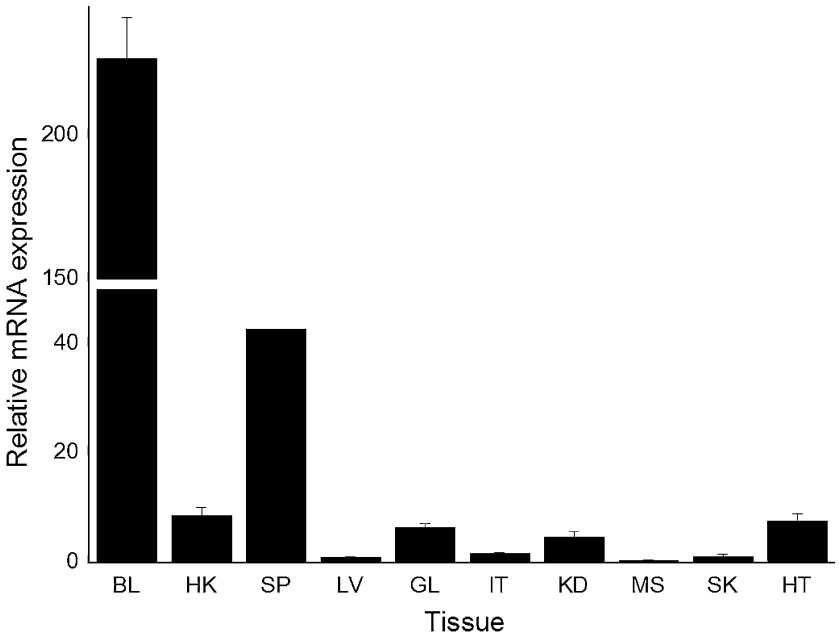

Fig. 2 Tissue-specific distribution of *RfNLRC5* expression in black rockfish measured using quantitative real-time polymerase chain reaction (qPCR). Fold-changes in expression are shown relative to the level of mRNA expression in liver tissue. *BL* blood, *HK* head kidney, *SP* spleen, *LV* liver, *GL* gill, *IT* intestine, *KD* kidney, *MS* muscle, *SK* skin, *HT* heart. *Error bars* represent SD (n = 3)

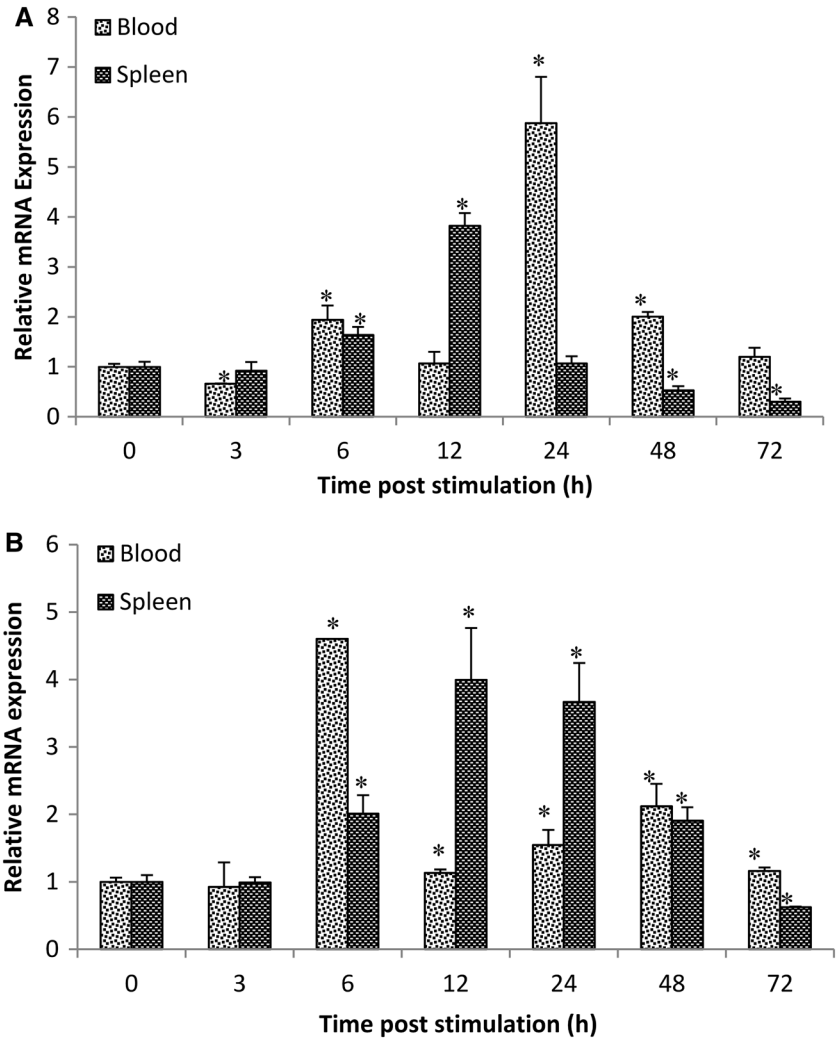

Fig. 3 Temporal modulation of mRNA expression in Blood cells and spleen tissues upon immune stimulation with **A** LPS and **B** Poly I:C as determined by qPCR. The relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method. The black rockfish EF1A gene was used as the internal reference gene and mRNA expression was further normalized to the corresponding PBS-injected controls at each time point. The relative fold-change in expression at 0 h post-injection (Un-injected control) was used as the baseline. *Error bars* represent SD (n = 3); * $P < 0.05$

chemokines including IL-1β and IL-18 (Franchi et al. 2006; Brodsky and Monack. 2009). This suggestion can be further validated by the finding which affirms that fish TLR4 s are unable to recognize LPS (Sepulcre et al. 2009). However, the initial and later downregulations of *RfNLRC5* in blood cells and spleen tissues of black rockfish, respectively may represent a mRNA turnover event under a stressed condition. The difference between overall transcriptional response in blood and spleen tissues may plausibly explain by the difference of their basal mRNA expression levels, respectively. Therein abundant expression of *RfNLRC5* level in blood cells may sufficient enough to sense LPS in early phase of an infection, although significant elevation may need to compensate for that level in spleen tissues, since *RfNLRC5* expression level of which is lesser than that in blood cells. Similarly, following the incubation with LPS, flounder NLRC was also showed time dependent inductive transcriptional responses in kidney leukocytes. However, it was not significantly modulated in flounder PBLs (Unajak et al. 2011).

Following the stimulation with poly I:C, *RfNLRC5* mRNA expression was significantly elevated in blood cells from 6 h p.s. to 72 h p.s. continuously with different fold changes (Fig. 3B). Transcript level was markedly elevated at 6 h p.s. Similarly, in spleen tissues transcription was elevated from 6 h p.s., but until 48 h p.s. continuously, with significant down regulation at 72 h p.s (Fig. 3B). NLR expression was reported to be enhanced by IFN-γ, which is mainly produced against viral infections (Hisamatsu et al. 2003; Iwanaga et al. 2003). Thus, detected transcriptional responses evoked against poly I:C treatment might have mediated by over produced IFN-γ in response to the viral dsRNA analog, poly I:C treatment. These suggestion further hints on the plausible involvement of RfNLRC5 in IFN-γ mediated antiviral immune responses. However, further studies are merited to ascertain this suggestion. Similar to our observation, upon poly I:C or live viral infection expression of NLRs counterparts were reported to be induced in rohu and grass carp (Chen et al. 2010; Swain et al. 2012).

In summary, we have identified a homologue of NLRC5 family members from black rockfish. RfNLRC5 harbored typical domains of NLR superfamily, excluding n-terminal effector domain. Moreover it is phylogenetically related to the teleostan NLRC5 counterparts. *RfNLRC5* was found to express ubiquitously in tissues examined with the eminent basal transcript level in blood. After treating the healthy fish with poly I:C and LPS, *RfNLRC5* mRNA expression was observed to modulate significantly, with inductive responses. Collectively, our findings suggest that RFNLRC5 may play an important role in sensing viral or bacterial pathogens.

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Compliance with ethical standards

Conflict of interest Authors declared no conflict of interest on this manuscript.

Compliance of bioethics Fish care and challenge experiments were reviewed and approved by Animal Care and Use Committee of Jeju National University.

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