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Inflammatory responses in the Japanese pufferfish (*Takifugu rubripes*) head kidney cells stimulated with an inflammasome-inducing agent, nigericin

Soner Bilen [a,1], Gouranga Biswas [b,1], Shohei Otsuyama [c], Tomoya Kono [c], Masahiro Sakai [c], Jun-ichi Hikima [c,*]

[a] Kastamonu University, Faculty of Fisheries, Department of Basic Sciences, Kastamonu 37200, Turkey
[b] Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan
[c] Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan

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ABSTRACT

A cytosolic receptor complex called inflammasome is responsible for mounting inflammatory response by releasing pro-inflammatory cytokines, interleukin (IL)-1β and IL-18. However, inflammatory cascades mediated by the inflammasome are unknown in a lower vertebrate like fish. Therefore, in an *in vitro* experiment, in order to obtain a preliminary information, we conducted transcriptomic analysis of 18 cytokines including pro-inflammatory cytokines in the Japanese pufferfish (*Takifugu rubripes*) head kidney (HK) cells stimulated with an inflammasome-inducing agent, nigericin, and a combination of nigericin and LPS by a multiplex RT-PCR assay (GenomeLab Genetic Analysis System, GeXPS; Beckman Coulter Inc.). Furthermore, expression of IL-1β, IL-6, IL-18, nuclear factor (NF)-κB, nucleotide-binding oligomerization domain 2 (NOD2) and NOD-like receptor X1 (NLRX1) genes was examined in HK cells by a quantitative real-time PCR. Additionally, to confirm functionality of activated inflammatory immunity, we also assessed phagocytic activity, superoxide anion production (NBT assay) and lysozyme activity in the nigericin-stimulated HK cells. An increased gene expression of pro-inflammatory cytokines (IL-1β and IL-18), NF-κB and NOD2 was recorded in nigericin and combined nigericin + LPS- stimulated HK cells. Enhanced cellular (phagocytic activity and NBT assay) and humoral (lysozyme activity) immune parameters in the stimulated cells confirmed induction of inflammatory response. Results suggested probable activation of inflammasome components for processing of the inflammatory cytokines in the Japanese pufferfish.

1. Introduction

The well conserved innate immune system acts as the first line of defense against microbes in all living organisms. The critical role of the innate immune system is initiation of instant response to microbial pathogens, and this is induced by pathogen/pattern recognition receptors (PRRs). PRRs detect molecular patterns of pathogens and induce host innate immune cells towards inactivation of the pathogens (Zhu et al., 2013). Toll-like receptors (TLRs), RIG-I like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are classes of PRRs that have been characterized from teleosts (Laing et al., 2008), rodents, birds (Chen et al., 2009) and humans (Inohara and Núñez, 2003). NLRs are a specialized group of intracellular receptors that represent a key component of the host innate immune system, and in mammals, they are very important on pathogenesis of inflammatory diseases (Chen et al., 2009) with their ability to regulate interleukin (IL)-1β releasing and nuclear factor (NF)-κB signaling. The NLR family contains several proteins and some members (NLRPs or NALPs) form a multi-protein complex called inflammasome by interacting with an inflammatory cysteine protease, caspase-1 with or without involvement of an adaptor protein, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) (Franchi et al., 2012; Martinon et al., 2009; Schroder and Tschopp, 2010). In the inflammasome, caspase-1 is activated through auto-proteolytic maturation to promote cleavage and secretion of biologically active pro-inflammatory cytokines, IL-1β and IL-18 and pyroptotic cell

* Corresponding author. Tel./fax: +81 985 58 7230.
E-mail address: jhikima@cc.miyazaki-u.ac.jp (J.-i. Hikima).
[1] Both the authors contributed equally to this work.

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death (Mariathasan et al., 2006; Martinon et al., 2006; Schroder and Tschopp, 2010). Hence, the inflammasome is a key molecule of inflammatory immune response in vertebrates.

The human and mouse genomes encode 23 and 34 NLRs, respectively (Rathinam et al., 2012). Mammalian NLRs are categorized into five groups, NLRA, NLRB, NLRC, NLRP and NLRX based on their N-terminal effector domains (Hansen et al., 2011). However, in fish, three distinct classes of NLR subfamily (NLRA, B and C) were reported. NLRA resembled mammalian NODs, NLRB to mammalian NLRPs, and NLRC was unique to the teleost fish (Laing et al., 2008). Direct association of fish NLRs in inflammatory immunity is not fully understood compared to the well-evident mammalian reports.

Cytokines are protein mediators produced by immune cells, and are mainly responsible for host innate defense mechanisms. Cytokines include ILs, tumor necrosis factors (TNFs), transforming growth factor (TGF), chemokines and interferons (IFNs) (Savan and Sakai, 2006), are produced by macrophages, lymphocytes, granulocytes, dendritic cells (DCs), mast cells, and epithelial cells, and have proinflammatory, anti-inflammatory and pathogen-killing properties. Cytokines play an important role in the immune system by binding to specific receptors at the cell membrane, setting off a cascade that leads to induction, enhancement or inhibition of a number of cytokine-regulated genes in the nucleus (Mulder et al., 2007).

Nigericin (an NLRP3/NALP3 inflammasome inducer) is a microbial toxin derived from *Streptomyces hygroscopicus*, and acts as a potassium (K^+) ionophore. The NALP inflammasome activation is specific to a low concentration of K^+. Nigericin is a K^+ channel and is recognized to cause a marked efflux of intracellular K^+ (Pétrilli et al., 2007) which is crucial for the activation of caspase-1 (Locovei et al., 2007). The release of IL-1β in response to nigericin has been demonstrated to be NALP3-dependent (Mariathasan et al., 2006). Nigericin requires signaling through pannexin-1 to induce caspase-1 maturation, and consequent IL-1β processing and release (Pelegrin and Surprenant, 2007). Nigericin was used as a stimulator in mouse to understand activation of caspase-1 and pro-inflammatory cytokine releasing (Squires et al., 2007; Wang et al., 2013).

As there is scarce information on inflammatory immune activation in response to an inflammasome-inducer like nigericin in fish, we focused mainly on transcriptomic analysis of pro-inflammatory cytokines and NF-κB in the Japanese pufferfish (*fugu*, *Takifugu rubripes*) head kindey (HK) cells *in vitro*. Further, expression of NOD2 and NLRX1 genes, which were found in GenBank database, was examined as NLR family genes. Additionally to confirm functionality of activated inflammatory immunity, we also assessed phagocytic activity, superoxide anion production (NBT assay) and lysozyme activity in the nigericin-stimulated cells.

2. Materials and methods

2.1. Experimental fish, HK cells stimulation and RNA extraction

Japanese pufferfish (*T. rubripes*) (40 ± 1.2 g; $n = 15$) were obtained from Matsumoto Fisheries Farm, Miyazaki, Japan, and the fish were first acclimatized in an aerated saltwater tank at 20 °C and fed a commercial diet (Sango, Higashimaru Co., Ltd., Kagoshima, Japan) for 2 weeks under a natural photoperiod prior to their use in the study. The health status of experimental fish was checked following the method described earlier (Biswas et al., 2013a). All animal experiments were conducted according to the relevant national and international guidelines, 'Act on Welfare and Management of Animals' (Ministry of the Environment, Japan). Ethics approval from the local IACUC was not sought since this law does not mandate protection of fish. HK cells from the healthy fish were collected as per the protocol described previously (Biswas et al., 2013a). Viable cells in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS) and 1% streptomycin/penicillin (Invitrogen) were adjusted to 1×10^7 cells mL^{-1} after enumeration using trypan blue stain (Life Technologies, NY, USA) and seeded in wells of a 24-well plate (Nunc A/S, Roskilde, Denmark). The cells were treated with three different stimulants, *viz.* bacterial lipopolysaccharide (LPS; Sigma–Aldrich, St. Louis, MO, USA) at 20 μg mL^{-1}, nigericin (Focus Biomolecules, PA, USA) at 30 μM and a combination of nigericin (30 μM) + LPS (20 μg mL^{-1}) and incubated for 1, 4, 12, 24 and 48 h at 25 °C. The NLRP3 inflammasome-mediated IL-1β processing requires a pre-treatment with a pro-inflammatory stimulus such as a TLR agonist, bacterial LPS (Schroder et al., 2012). Caspase-1 activation by the pore-forming toxin and NLRP3 agonist, nigericin, absolutely requires a pre-treatment with LPS (Bauernfeind et al., 2009). Therefore, we conducted this combined stimulation of HK cells with nigericin + LPS to evaluate their synergistic effect on inflammatory response. A control (cells without stimulation) was maintained for each time point. Each treatment and control had three replicates. Incubated cells were harvested at the time points mentioned above, submerged in RNAlater solution (Ambion, Austin, TX, USA) for overnight and finally stored at −80 °C prior to RNA extraction.

Total RNA was extracted from the stimulated HK cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA samples were treated with a recombinant DNase I (RNase-free) for removal of genomic DNA, as per the manufacturer's protocol (Takara Bio Inc., Shiga, Japan). The concentration of all RNA samples were checked quantitative and qualitatively in a NanoDrop spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA).

2.2. Multiplex RT-PCR assay and capillary electrophoresis

In the study, a multiplex reverse transcription- polymerase chain reaction (RT-PCR) assay (GenomeLab Genetic Analysis System, GeXPS; Beckman Coulter, Inc., Brea, CA, USA) was used to analyze expression of 18 cytokine genes simultaneously from a single reaction tube. RNA samples of the stimulated HK cells (1, 4 and 24 h) were used in this assay. Primer design (18-cytokine plex) and multiplex assay were conducted using the multiplex RT-PCR assay panel described previously (Kono et al., 2013). RT and PCR were performed using 18 cytokine universal primers adjusted to amplify target genes (Supplementary Table 1). The PCR products from multiplex RT-PCR were prepared and run in the GeXP Genetic Analysis System for capillary electrophoresis and fragment size analysis as per the previously described protocol (Biswas et al., 2013a). The data were normalized to the external synthetic reference control transcript, kanamycin resistance gene using GeXP profiler (eXpress Analysis) software, with the area-under-the-curve set to 1. The relative expression level of each cytokine gene was calculated by normalization to the reference gene, β-actin using GeXP Quant Tool.

2.3. Quantitative real-time PCR (qPCR)

Extracted total RNA was used for cDNA synthesize *via* reverse transcription from 0.5 μg mRNA using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan). The primer sequences of pro-inflammatory cytokines, IL-1β, IL-6 and IL-18, and NF-κBp65, NOD2 and NLRX1 with 98–105% amplification efficiencies used in qPCR are enlisted in Table 1. The qPCR reaction was conducted using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo Co., Ltd.) for each sample run in triplicate as per the protocol described earlier (Kinoshita et al.,

Table 1
Gene-specific primers used for real-time qPCR analysis.

Gene	GeneBank accession No.	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Product size (bp)
IL-1β	AB704199	TCTTCAACAAGCACATCACTGG	CCAGGAGGGTGAAGATAGTGTT	150
IL-6	NM_001032722	CAAGGATGCTTGTCTACGAAGG	GCTCTTTGATCAGGATGTTGCT	137
IL-18	NM_001032632	GGAATCGTCTCTGTACCCATCT	GTCCACGTCGTCATACTCTTTG	106
NF-κBp65	XM_003966703	TGCAGAGTCAATCGCAACTC	GGAAGAAGCGGACTTCAATG	100
NOD2	DQ060247	CGACTAGGCCAGCTTGCCTTTA	GCGAAGAAACACTGCATGGTCAC	188
NLRX1	XM_003976996	CACATGTGCCACTCTGCATTGCC	GCATCAAAGAACTGTGGTGCTCC	167
β-Actin	U37499	CCAGAAAGACAGCTACGTTGG	GCAACTCTCAGCTCGTTGTAG	147
EF-1α	NM_001037873	GCATGGTTGTCACCTTTGCTCCCG	GTCAGCTCCCTTTGGTGGGTCGTT	191

2014). The comparative threshold cycle (C_T) method ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008) was used to analyze the expression level of 3 cytokine and NF-κBp65 genes using β-actin, and NOD2 and NLRX1 genes using EF-1α as internal control genes.

2.4. Detection of cellular and humoral immune parameters

After isolation of *fugu* HK cells according to the described protocol, stimulation was carried out with LPS (20 μg mL^{-1}), nigericin (30 μM) and a combination of nigericin (30 μM) + LPS (20 μg mL^{-1}). The phagocytic activity of HK cells using flow cytometry was performed at 4, 12 and 24 h post-stimulation (hps) following the method described elsewhere (Biswas et al., 2013b). Superoxide anions produced by phagocytic cells were determined using the reduction of nitroblue tetrazolium (NBT; Sigma–Aldrich, St. Louis, MO, USA) assay at 4, 12, 24 and 48 hps as per the previously described method (Biswas et al., 2013b). Lysozyme activity of stimulated HK cells was assessed at 4, 12, 24 and 48 hps using a bacterium, *Micrococcus lysodeikticus* (MP Biochemicals, Illkirch, France). The optical density of the leukocyte supernatant containing the said bacterium solution was measured after 1 h incubation at 520 nm in a micro-plate photometer (Multiskan FC, Thermo Scientific, DE, USA).

2.5. Statistical analysis

One-way analysis of variance followed by Tukey's tests were run to find out any difference in cytokine, NF-κBp65, NOD2 and NLRX1 gene expressions, phagocytic activity, NBT and lysozyme activity assay values between different stimulations at each sampling time using SPSS for Windows v. 17.0 program (SPSS Inc., Chicago, IL, USA). The accepted level of significance was $P < 0.05$.

3. Results

3.1. Cytokine gene expressions

Transcriptional responses analyzed by multiplex RT-PCR assay for 18 cytokine genes in the stimulated HK cells of the Japanese pufferfish are presented is Table 2. Further, fold changes in expression levels analyzed by real-time qPCR for certain cytokine genes (IL-1β, IL-6 and IL-18) in the stimulated HK cells and leukocytes are illustrated in Fig. 1.

3.1.1. Pro-inflammatory cytokines

In multiplex RT-PCR assay, there were constitutively higher expressions ($P < 0.05$) of the pro-inflammatory IL-1β and IL-6 genes in the HK cells treated with LPS, nigercin and nigericin + LPS (Table 2). IL-18 gene was constitutively expressed in the nigericin and nigercin + LPS treated HK cells of the pufferfish (Table 2). Although the pro-inflammatory cytokine, TNF-N (TNF-Novel) had increased transcript levels at 1 and 24 hps with nigericin, 4 hps with LPS, and 24 hps with nigericin + LPS stimulations, in HK cells, TNF-α gene expression remained almost unchanged with all kinds of stimulations (Table 2). In real-time qPCR analysis, the pro-inflammatory cytokine genes, IL-1β, IL-6 and IL-18 were higher expressed ($P < 0.05$) mainly by the combined nigericin + LPS stimulation in HK cells (Fig. 1).

3.1.2. Other cytokines

IFN-γ gene expression was significantly increased ($P < 0.05$) in HK cells by nigericin treatment at all the time points, and LPS and nigercin + LPS at 4 and 24 hps (Table 2). There were no specific and significant trends in other cytokine expressions observed in the stimulated HK cells.

3.2. Expression of NF-κBp65 gene

In real-time qPCR analysis, NF-κBp65 mRNA level in HK cells was elevated ($P < 0.05$) by nigericin treatment at 4, 12 and 48 hps, and by nigericin + LPS stimulation at 1, 4 and 48 hps (Fig. 2).

3.3. Expression of NOD2 and NLRX1 genes

A conspicuous elevated expression of NOD2 gene was observed in HK cells stimulated with LPS, nigericin and nigericin + LPS at all the time points (Fig. 3). However, NLRX1 gene expression was enhanced at all the time points by LPS stimulation only, not by nigericin and nigericin + LPS stimulations (Fig. 3).

3.4. Cellular and humoral immune responses in stimulated HK cells

In the stimulated HK cells, phagocytic activity was enhanced ($P < 0.05$) by all three types of stimulations at 4, 12 and 24 hps (Fig. 4). On the contrary, the production of superoxides assessed by NBT reduction, was significantly higher at 4 and 24 hps in the stimulation with nigericin only (Fig. 5). In this study, lysozyme activity was noticed to be elevated later than phagocytosis and superoxide anion production at 24 hps by nigericin and combined nigericin + LPS, however, it continued to be increased until 48 hps in the pufferfish HK cells induced with both the stimulations (Fig. 6).

4. Discussion

Inflammatory response in fish seems to be little different from that in mammals. For instance, fish have several orthologs of IL-1β cytokine functioning in ways similar to those in mammals. Pro-inflammatory stimulants and microbial infections up-regulate IL-1β transcription in fish leukocytes, and recombinant IL-1β causes an acute-phase inflammatory response (Koussounadis et al., 2004; Martin et al., 2007). Although, caspase-1 dependent IL-1β processing occurs in fish (Reis et al., 2012; Vojtech et al., 2012) corresponding to that in mammalian species, an association

Table 2
Relative expression (to β-actin) of cytokine genes at different time points in the Japanese pufferfish head kidney cells stimulated with LPS (20 μg mL^{-1}), Nigericin (30 μM) and Nigericin (30 μM) + LPS (20 μg mL^{-1}). Values with dissimilar superscript letters are significantly different ($P < 0.05$) at a time point.

Gene	1 h post stimulation				4 h post stimulation				24 h post stimulation			
	Control	LPS	Nigericin	Nig+LPS	Control	LPS	Nigericin	Nig+LPS	Control	LPS	Nigericin	Nig+LPS
Pro-inflammatory cytokines												
IL-1β	1.96±0.04^{a}	4.00±0.07^{b}	11.45±0.02^{c}	10.36±0.18^{c}	1.93±0.10^{a}	3.21±0.14^{b}	7.44±0.02^{c}	6.80±0.03^{c}	1.35±0.04^{a}	4.64±0.13^{c}	2.83±0.02^{b}	2.92±0.01^{b}
IL-6	1.63±0.03^{a}	2.40±0.03^{b}	2.23±0.10^{b}	1.81±0.15^{a}	1.42±0.12^{a}	5.21±0.57^{d}	2.62±0.05^{c}	2.07±0.15^{b}	1.37±0.18^{a}	2.24±1.34^{c}	0.91±0.08^{a}	1.89±0.20^{b}
IL-18	1.07±0.19^{a}	1.38±0.05^{a}	2.12±0.13^{b}	1.65±0.24^{a}	0.69±0.13^{a}	2.59±0.18^{c}	1.90±0.22^{b}	3.03±0.02^{d}	0.93±0.36^{a}	2.63±0.04^{b}	2.30±0.14^{b}	2.70±0.17^{b}
TNF-α	0.56±0.24^{b}	0.12±0.03^{a}	0.61±0.01^{b}	0.60±0.04^{b}	1.46±0.35^{b}	0.13±0.02^{a}	0.05±0.01^{a}	0.69±0.07^{b}	0.21±0.01^{a}	1.58±0.58^{b}	0.28±0.02^{a}	0.28±0.03^{a}
TNF-N	1.51±0.05^{b}	0.26±0.05^{a}	3.17±0.37^{c}	0.42±0.03^{a}	1.45±0.04^{b}	2.08±0.13^{c}	0.41±0.06^{a}	0.60±0.09^{a}	1.43±0.23^{b}	0.25±0.05^{a}	2.16±0.45^{c}	3.93±0.03^{c}
Other cytokines												
IL-10	0.31±0.02	1.21±0.03	0.89±0.00	0.88±0.02	0.19±0.01^{a}	2.00±0.07^{c}	1.41±0.01^{b}	3.22±0.02^{c}	0.41±0.05^{a}	4.81±0.78^{c}	2.23±0.04^{b}	5.22±0.08^{d}
IL-4/13A	0.99±0.05	0.86±0.03	1.09±0.03	1.05±0.10	0.74±0.04	1.02±0.05	0.85±0.01	0.87±0.02	0.42±0.06	1.27±2.36	0.32±0.03	1.15±0.07
IL-4/13B	0.68±0.04^{a}	1.88±0.34^{b}	2.37±0.02^{c}	1.13±0.13^{a}	0.81±0.02^{a}	0.42±0.01^{a}	1.46±0.24^{b}	0.57±0.08^{a}	0.94±0.34^{a}	1.28±2.04^{a}	4.49±1.14b	4.20±0.10c
IL-12p35	1.02±0.15	1.32±0.02	3.02±0.19	1.70±0.22a	1.41±0.06b	4.20±0.50a	1.98±0.05b	2.19±0.06b	1.77±0.13	5.13±0.50a	2.71±0.09^{b}	5.22±1.04^{a}
IL-12p40	1.67±0.02^{b}	1.27±0.01^{b}	3.73±0.01^{c}	0.44±0.02^{a}	1.10±0.09^{a}	2.22±0.02^{c}	1.62±0.30^{b}	0.50±0.01^{a}	0.77±0.44^{a}	1.00±0.14^{a}	1.83±0.02^{b}	1.93±0.02^{b}
I-IFN-1	1.14±0.14^{a}	0.73±0.58^{a}	1.65±0.20^{b}	1.31±0.21^{a}	0.43±0.11^{a}	1.82±0.05^{b}	0.51±0.11^{a}	1.38±0.13^{c}	0.66±0.37^{b}	0.20±0.10^{a}	0.80±0.32^{b}	1.46±0.03^{b}
IFN-γ	1.32±0.85^{a}	1.12±0.01^{a}	4.86±0.68^{b}	1.89±0.03^{a}	1.78±0.03^{a}	8.98±0.04^{c}	2.87±0.03^{b}	8.94±0.06^{c}	1.02±0.09^{a}	8.80±1.01^{b}	8.78±0.06^{b}	6.16±0.02^{b}
IL-2	1.09±0.04^{a}	1.02±0.02^{a}	3.68±0.03^{b}	0.70±0.02^{a}	1.21±0.01^{a}	2.00±0.02^{b}	0.91±0.01^{a}	0.98±0.01^{a}	1.59±0.04^{a}	1.75±1.91^{b}	1.93±0.03^{b}	1.96±0.24^{b}
IL-7	0.20±0.01^{a}	0.18±0.08^{a}	1.13±0.71^{b}	1.23±1.08^{b}	0.21±0.05^{a}	0.53±0.19^{a}	1.16±0.72^{b}	4.46±0.16^{c}	1.11±0.08^{b}	0.26±0.08^{a}	0.28±0.01^{a}	1.15±0.02^{b}
IL-15	1.29±0.02^{a}	4.61±0.63^{c}	1.73±0.04^{b}	4.34±0.03^{c}	0.59±0.28	0.32±0.02	0.32±0.01	0.48±0.01	1.43±0.06	0.70±0.06	1.05±0.01	1.16±0.01
IL-21	0.65±0.14^{a}	0.60±0.08^{a}	1.10±0.11^{a}	1.56±0.03^{b}	0.68±0.25	0.93±0.11	0.78±0.14	1.28±0.15	0.88±0.14^{a}	5.65±1.82^{c}	0.89±0.15^{a}	2.72±0.04^{b}
TGF-β1	1.14±0.03	0.95±0.15	1.10±0.06	1.10±0.19	0.92±0.02	1.10±0.04	0.80±0.06	1.14±0.04	0.97±0.17	0.95±0.67	0.74±0.08	1.05±0.01
CSF-1β	2.89±0.30^{c}	1.50±0.61^{b}	6.00±1.75^{d}	0.46±0.07^{a}	1.92±0.57^{b}	2.73±0.42^{c}	0.92±0.13^{a}	1.86±0.20^{b}	1.28±0.73^{a}	0.91±0.34^{a}	1.41±0.12^{b}	3.96±0.24^{c}

Boxes highlighted in yellow, green and pink indicate the increased expression level more than 2, 5 and 10 times, respectively.

of caspase-1 in the fish inflammasome could not be confirmed (Angosto et al., 2012). Therefore, towards obtaining some insights into the inflammatory response in the Japanese pufferfish we conducted this stimulation experiment using nigericin, which indirectly induces inflammasome and caspase-1 activation for the processing and release of IL-1β by causing an outflow of intracellular K^+. This is the first report on inflammatory cytokine-mediated immune responses in fish to the inflammasome-inducing agent, nigericin. In this study, the up-regulated cytokine genes, and elevated cellualar and humoral immune parameters in HK cells of the Japanese pufferfish in response to nigericin stimulation indicated induction of inflammatory responses by this immune-stimulatory chemical.

4.1. Pro-inflammatory cytokine expressions

In the process of infection and inflammatory response, the kidney as an immuno-competent organ transcripts pro-inflammatory cytokines, IL-1β, IL-6, IL-18, TNF-α and TNF-N. In this study, most of these pro-inflammatory cytokines were up-regulated in the HK cells treated with nigericin. Here, an early and significantly elevated expression of IL-1β was observed in the HK cells at 1 hps, and the expression sustained until 24 hps at a decreasing level. Nigericin activates NLRP3 inflammasome that finally processes IL-1β maturation through involvement of caspase-1 and ASC (Stout-Delgado et al., 2012). Extracelluar nigericin stimulates K^+ efflux that triggers the pore formation by pannexin-1, allowing delivery of microbial products into cytosol to activate inflammasome (Kanneganti et al., 2007; Pelegrin and Surprenant, 2007). Therefore, higher transcript level of IL-1β indicated likely occurrence of caspase-1 mediated IL-1β processing resulted from the combined treatment of nigericin and LPS. Similarly, in a previous study, LPS priming induced NLRP3 expression that is essential for NLRP3 inflammasome activation in mammalian cells (Bauernfeind et al., 2009). Expression of caspase-1 specific activity and IL-1β processing from a 30-kDa to 22-kDa forms were identified in zebrafish (*Danio rerio*) leukocytes in response to infection with a bacterial pathogen, *Francisella noatunensis* (Vojtech et al., 2012). On the contrary, gilthead seabream (*Sparus aurata*) macrophages during stimulation with nigericin released IL-1β without caspase-1 processing (Angosto et al., 2012). At this juncture, it is difficult to conclude that IL-1β processing in pufferfish cells occurred due to inflammasome activation and needs further clarification by conducting functional studies involving inflammasome molecules. IL-1β is produced by macrophages, monocytes and lymphocytes and acts as a key molecule in acute response to microbial invasion and tissue injury (Low et al., 2003), and downstream release of other cytokines (Engelsma et al., 2002). Therefore, this acute and sustained IL-1β expression in response to nigericin and LPS treatments would have mediated the inflammatory response and tissue damage (Arend, 2002), and also influenced the subsequent cytokine release cascade (Biswas et al., 2012).

IL-6 is generally regarded as a pro-inflammatory cytokine and performs a diverse immune functions such as stimulation or inhibition of inflammatory responses, recruitment of neutrophils to infection sites, tissue repairs, and shifting from innate to adaptive immunity by replacing neutrophils with monocytes and lymphocytes (McGeough et al., 2012). During the cytokine release cascade, IL-1β is one of the most potent activators of IL-6 production. Increased acute-phase and sustained expression of IL-6 in this study may be related to the inflammatory immune response. However, from mammalian experimental models it was suggested that IL-6 is a reliable marker of inflammation, with no direct role in inflammasome-mediated immunity (Lamkanfi et al., 2009; McGeough et al., 2012).

The cell-mediating pleiotropic cytokine, IL-18 is produced by monocytes/macrophages and participates in clearance of intracellular pathogens through the induction of IFN-γ, and of viruses by activation of cytotoxic T cells (Arend et al., 2008; Gracie et al., 2003). In response to intracellular pathogen, ASC and caspase-1

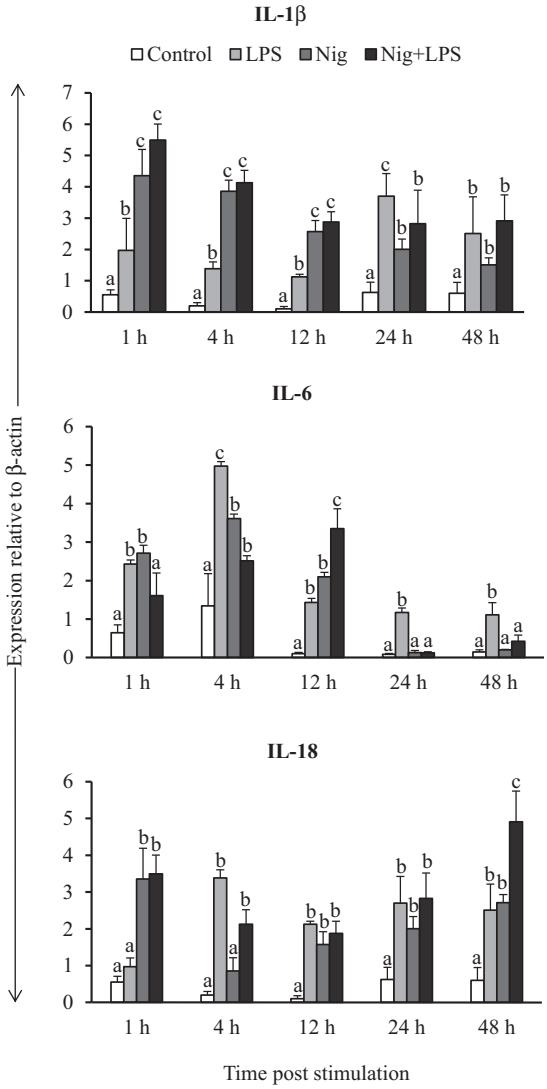

Fig. 1. Relative expression (mean ± SD; $n = 3$) of pro-inflammatory cytokine genes (IL-1β, IL-6 and IL-18) at different time points in the Japanese pufferfish head kidney cells stimulated with LPS (20 μg mL^{-1}), Nigericin (30 μM) and a combination of Nigericin (30 μM) + LPS (20 μg mL^{-1}). Bars with different superscript letters indicate a significant difference ($P < 0.05$) in expression levels between stimulated cells and unstimulated control cells at a time point.

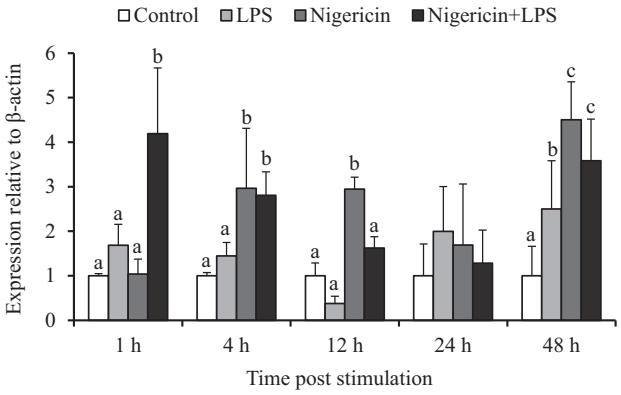

Fig. 2. Relative expression (mean ± SD; $n = 3$) of nuclear factor (NF)-κBp65 gene at different time points in the Japanese pufferfish head kidney cells stimulated with LPS (20 μg mL^{-1}), Nigericin (30 μM) and a combination of Nigericin (30 μM) + LPS (20 μg mL^{-1}). Bars with different superscript letters indicate a significant difference ($P < 0.05$) in expression levels between stimulated cells and unstimulated control cells at a time point.

in the NLR pathway play a pivotal role in the release of IL-18 (Pedra et al., 2007). In this study, an increased transcription of IL-18 was observed in pufferfish HK cells mainly in response to nigericin and combined nigericin + LPS stimulations. Similarly, increased NLRP3, caspase-1, ASC, IL-1β and IL-18 activities in mouse dendritic cells treated with nigericin (10 μM) indicated NLRP3 inflammasome activation (Stout-Delgado et al., 2012). Therefore, in the present study, elevated expressions of IL-1β and IL-18 in nigericin-treated cells suggest an inflammatory immune response through probable activation of inflammasome.

TNFs, produced mainly by macrophages/monocytes and T lymphocytes have a variety of functions in the regulation of inflammation and cellular immune responses including the stimulation of respiratory burst activity and phagocytosis (Zou et al., 2003). In our experiment, between two TNF genes, only the fish-specific TNF-N was up-regulated by the nigericin and combined nigericin + LPS treatments in fish HK cells. This elevated TNF-N induction may be due to the activation of IL-18 (Puren et al., 1998). Our results suggest the acute and synergistic induction of pro-inflammatory cytokine transcriptions as conseuqunces of nigericin and LPS treatments.

4.1.1. Other cytokine expressions

Among the other cytokines, only IFN-γ expression was increased by the treatment of nigercin and nigericin + LPS. IFN-γ mainly participates in cell mediated and adaptive immunity (Zou and Secombes, 2011) and induces intracellular pathogen killing ability of leukocytes (van de Veerdonk et al., 2011). IL-18 causes induction of Th1 response to produce IFN-γ (Dinarello, 1999). Therefore, the increased IFN-γ transcription in the present experiment might have resulted from IL-18 induction by the inflammasome-inducing agent. Remarkably, susceptibilty to infection in ASC and caspase-1-deficient mice was increased due to the absence of IL-18 secretion and reduced IFN-γ levels (Pedra et al., 2007).

4.2. NF-κBp65 gene expression

The transcription factor, NF-κB family modulates expression of hundreds of genes that are related to various cellular activities, such as pro liferation, differentiation and death, as well as innate and adaptive immune responses (Rahman and McFadden, 2011). In mammals, the NF-κB family is composed of five related transcription factors *viz.* p50, p52, p65 (also called RelA), c-Rel, and RelB that share an N-terminal DNA-binding/dimerization domain, known as the Rel homology domain to form homo- and heterodimers (Hayden and Ghosh, 2011). NF-κB dimers regulate gene expression by binding to a variety of related target DNA sequences called κB sites. In mammalian cells, NF-κB activation by TLRs as well as by cytokine receptors such as the TNF-receptor not only controls IL-1β gene transcription, but additionally turns on NLRP3 transcription, thereby licensing NLRP3 inflammasome activation (Bauernfeind et al., 2009; Bryant and Fitzgerald, 2009). We noticed an elevation in NF-κBp65 gene expression in pufferfish HK cells treated with nigericin and nigericin + LPS. Therefore, this NF-κBp65 activation might have modulated IL-1β gene expression that was increased by the said stimulations in an inflammasome-dependent or independent manner.

4.3. NOD2 and NLRX1 gene expressions

Some of the NLR genes discovered in mammals are partially conserved in other vertebrates including fish. Among NLRs, NLRP1,

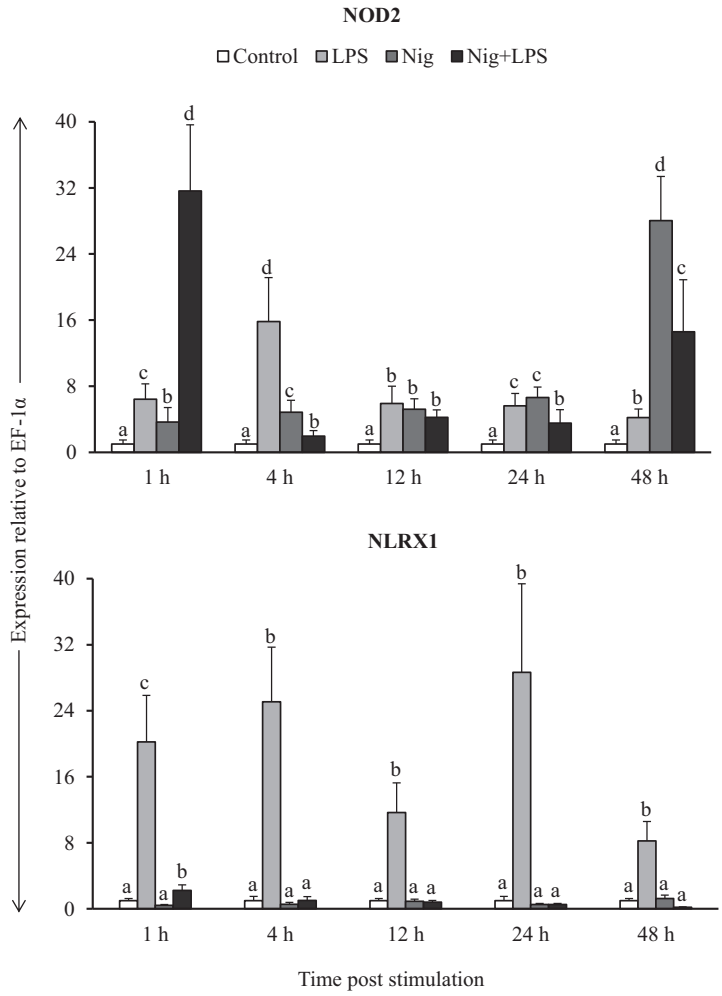

Fig. 3. Relative expression (mean ± SD; $n = 3$) of NOD2 and NLRX1 genes at different time points in the Japanese pufferfish head kidney cells stimulated with LPS (20 μg mL^{-1}), Nigericin (30 μM) and a combination of Nigericin (30 μM) + LPS (20 μg mL^{-1}). Bars with different superscript letters indicate a significant difference ($P < 0.05$) in expression levels between stimulated cells and unstimulated control cells at a time point.

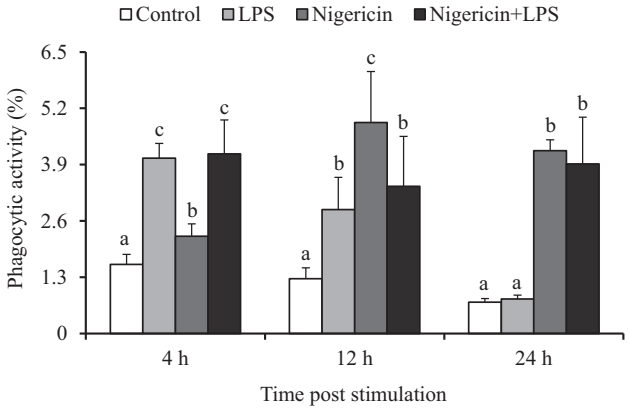

Fig. 4. Phagocytic activity (mean ± SD; $n = 3$) in the Japanese pufferfish head kidney cells stimulated with LPS (20 μg mL^{-1}), Nigericin (30 μM) and a combination of Nigericin (30 μM) + LPS (20 μg mL^{-1}). Phagocytic activity indicates the percentage of cells that phagocytosed yellow-green fluorescent latex beads detected using flow cytometry. Bars with different superscript letters indicate a significant difference ($P < 0.05$) in phagocytic activity at a time point.

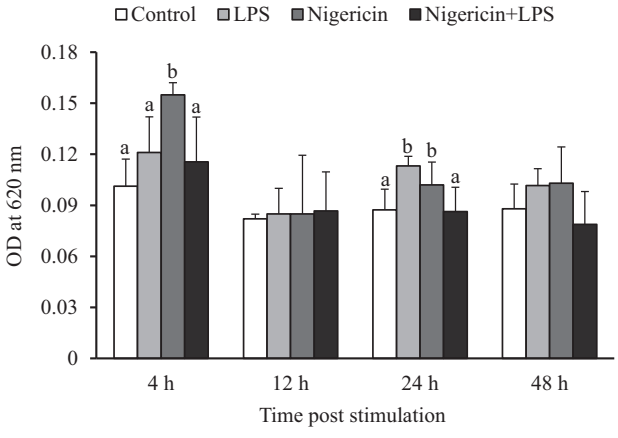

Fig. 5. Production of superoxide anion in the Japanese pufferfish head kidney cells stimulated with LPS (20 μg mL^{-1}), Nigericin (30 μM) and a combination of Nigericin (30 μM) + LPS (20 μg mL^{-1}). Superoxide anion production was measured as optical density (OD) (mean ± SD; $n = 3$) at 620 nm in the nitroblue tetrazolium (NBT) assay. Bars with different superscript letters indicate a significant difference ($P < 0.05$) in OD values at a time point.

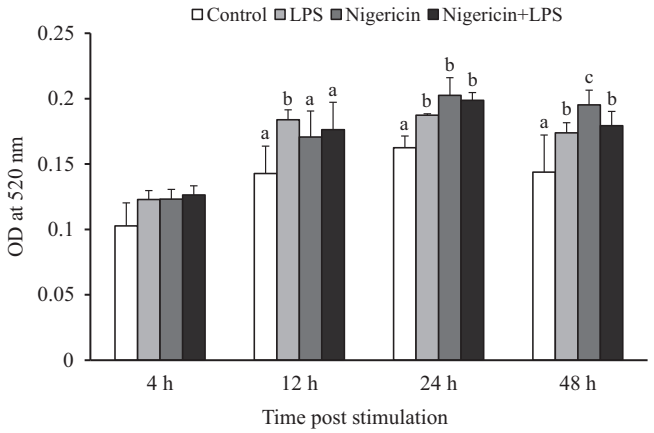

Fig. 6. Lysozyme activity (LA) in the Japanese pufferfish head kidney (HK) cells stimulated with LPS (20 μg mL^{-1}), Nigericin (30 μM) and a combination of Nigericin (30 μM) + LPS (20 μg mL^{-1}). LA indicates the bactericidal capacity of the HK cells against a bacterium, *Micrococcus lysodeikticus*. LA was measured as optical density (OD) (mean ± SD; $n = 3$) at 520 nm of the HK cell supernatant containing the said bacterium after 1 h incubation. Bars with different superscript letters indicate a significant difference ($P < 0.05$) in OD values at a time point.

NLRP3, NLRC4, NLRP6 and NLRP12 are known to be inflammasome candidate proteins in mammals with NLRP3/NALP3 is the most common and best-studied member (Rathinam et al., 2012). However, NLRP in fish is an enigmatic. Although, fish possesses a pyrin domain (PYD) containing NLRs known as NLRCs, their function as inflammasome proteins has not yet been confirmed. Therefore, we could not find any such gene identified from fugu. Moreover, no information on NLR molecules from fugu is available except NOD2 and NLRX1. Therefore, we have chosen NOD2 and NLRX1 genes found in GenBank database for expression analysis to further validate the inflammatory response triggered by nigericin.

NOD2, a member of NLR family comprises caspase recruitment domain (CARD)-containing effector-binding domains. In mammals, NOD2 recognizes bacterial components through its leucine-rich repeats (LRR), then oligomerizes through its NOD domain and recruits receptor-interacting protein 2 (RIP2) by an electrostatic interaction of the CARD domain (Brooks et al., 2011). This incidence steers activation and translocation of NF-κB into the nucleus through a signaling cascade for transcription of pro-inflammatory cytokines (Abbott et al., 2007; Chen et al., 2010; Hasegawa et al., 2008; Hu et al., 2010; Inohara et al., 2000; Inohara and Núñez, 2003; Rosenzweig et al., 2009; Windheim et al., 2007). Similarly in fish, overexpression of CARD domain of rainbow trout NOD2 significantly induced pro-inflammatory cytokines, including IL-1β through interaction with RIP2 kinase (Chang et al., 2011). Although, it is difficult to ascertain the exact reason behind an increased expression of NOD2 in the present study, the intracellular LPS entered through the cell-membrane pores created by the pore-forming agent, nigericin (Bauernfeind et al., 2009) probably caused this as LPS has already been known to induce an increased expression of NOD2 in grass carp (Chen et al., 2010) and goldfish macrophages (Xie et al., 2013). Moreover, induction of NOD2 in all the stimulations in this study would have elicited NF-κB and subsequent pro-inflammatory cytokine up-regulations.

NLRX1 localizes to the outer membranes of mitochondria through an N-terminal mitochondrial targeting (MT) sequence (Arnoult et al., 2009; Moore et al., 2008; Tattoli et al., 2008). NLRX1 is an inducer of pro-inflammatory response through TNF-α activation that brings about a production of reactive oxygen species (ROS) (Arnoult et al., 2009; Tattoli et al., 2008). On the contrary, NLRX1 can also act as an anti-inflammatory regulator by negatively regulating RIG-1 and TLRs through interaction with the mitochondrial antiviral signaling adaptor (MAVS) and TRAF6-IKK, respectively (Allen et al., 2011; Moore et al., 2008). Nigericin, which is an indirect inducer of inflammasome and caspase-1 by causing intracellular K^+ efflux, did not have any effect on NLRX1 in our study. However, LPS stimulated its enhanced transcription in HK cells. Consistent to our result, LPS stimulation could rapidly induce NLRX1 protein ubiquity in mice cells (Xia et al., 2011). Therefore, nigericin caused NLRX1-independent inflammatory immune response in pufferfish HK cells. However, LPS induced NLRX1 expression would suggest its probable participation in fish defense against Gram-negative bacterial infections.

4.4. Cellular and humoral immune responses

As indicators of inflammatory response, cellular and humoral immune parameters were measured in the pufferfish HK cells stimulated with nigericin, and a combination of nigericin and LPS. Among these parameters, phagocytic activity was increased more consistently in the treated cells. Phagocytosis initiates an early activation of the inflammatory response before antibody production, and is mediated by phagocytic cells such as neutrophils, monocytes and macrophages in fish (Balcázar et al., 2007). Release of IL-1β is dependent on both phagocytosis and assembly of the NLRP3 inflammasome in chitosan-stimulated murine macrophages (Bueter et al., 2011). Therefore, the elevated IL-1β transcripts in the stimulated cells may be a consequence of the increased phagocytosis and activation of inflammasome. Production of ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) is preceded by phagocytic process to kill microbes in vertebrate hosts (Harikrishnan et al., 2011). ROS plays an important role in the activation of inflammasome (Bauernfeind et al., 2011; Bryant and Fitzgerald, 2009; Martinon et al., 2009). Various NLRP3 activators including nigericin cause ROS production in mammalian monocytes (Hewinson et al., 2008). The production of superoxides assessed by NBT reduction in the stimulation with nigericin might have triggered inflammasome activation. Lysozyme is a humoral non-specific mucolytic defense protein that has leukocytic origin and wide distribution in fish (Lie et al., 1989). It dissolves the peptidoglycan layers of Gram-positive bacterial cell wall by hydrolyzing β (1→4) linkage between N-acetylmuramic acid and N-acetylglucosamine (Saurabh and Sahoo, 2008). In addition, it facilitates phagocytosis by activating polymorphonuclear leukocytes and macrophages (Saurabh and Sahoo, 2008). In this study, lysozyme activity was noticed to be elevated later than phagocytosis and superoxide anion production at 24 hps, however, it continued to be increased until 48 hps in pufferfish HK cells induced with both types of stimulations. From these results, it was evident that inflammatory immune response was mounted in nigericin treated pufferfish HK cells.

In this preliminary study, the inflammasome-inducing agent, nigericin triggered an elevated transcription of pro-inflammatory cytokines, especially IL-1β and IL-18 indicating probable activation of inflammasome components for processing of these cytokines. In addition, an increased expression of NF-κBp65 and NOD2 genes, and induction of cellular and humoral immune responses also supported activation of inflammatory response. Further characterization of NLR candidates and functional analysis of the inflammasome for the processing of IL-1β and IL-18 from the Japanese pufferfish would elucidate the evolution of inflammasome functioning in fish.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2014.04.010.

References

Abbott, D.W., Yang, Y., Hutti, J.E., Madhavarapu, S., Kelliher, M.A., Cantley, L.C., 2007. Coordinated regulation of Toll-like receptor and NOD2 signaling by K63-linked polyubiquitin chains. Mol. Cell. Biol. 27, 6012–6025.

Allen, I.C., Moore, C.B., Schneider, M., Lei, Y., Davis, B.K., Scull, M.A., Gris, D., Roney, K.E., Zimmermann, A.G., Bowzard, J.B., Ranjan, P., Monroe, K.M., Pickles, R.J., Sambhara, S., Ting, J.P.Y., 2011. NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-IMAVS and TRAF6-NF-κB signaling pathways. Immunity 34, 854–865.

Angosto, D., López-Castegón, G., López-Muñoz, A., Sepulcre, M.P., Arizcum, M., Meseguer, J., Mulero, V., 2012. Evolution of inflammasome function in vertebrates: inflammasome and caspase-1 trigger fish macrophage cell death but are dispensable for processing of IL-1β. Innate Immun. 18, 815–824.

Arend, W.P., 2002. The balance between IL-1 and IL-1Ra in disease. Cytokine Growth Factor Rev. 13, 323–340.

Arend, W.P., Palmer, G., Gabay, C., 2008. IL-1, IL-18, and IL-33 families of cytokines. Immunol. Rev. 223, 20–38.

Arnoult, D., Soares, F., Tattoli, I., Castanier, C., Philpott, D.J., Girardin, S.E., 2009. An N-terminal addressing sequence targets NLRX1 to the mitochondrial matrix. J. Cell Sci. 122, 3161–3168.

Balcázar, J.L., de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Gironés, O., Muzquiz, J.L., 2007. Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). FEMS Immunol. Med. Microbiol. 51, 185–193.

Bauernfeind, F.G., Horvath, G., Stutz, A., Alnemri, E.S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B.G., Fitzgerald, K.A., Hornung, V., Latz, E., 2009. Cutting edge: NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J. Immunol. 183, 787–791.

Bauernfeind, F.G., Bartok, E., Rieger, A., Franchi, L., Núñez, G., Hornung, V., 2011. Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. J. Immunol. 187, 613–617.

Biswas, G., Korenaga, H., Takayama, H., Kono, T., Shimokawa, H., Sakai, M., 2012. Cytokine responses in the common carp, *Cyprinus carpio* L. treated with baker's yeast extract. Aquaculture 356–357, 169–175.

Biswas, G., Korenaga, H., Nagamine, R., Kawahara, S., Takeda, S., Kikuchi, Y., Dashnyam, B., Yoshida, T., Kono, T., Sakai, M., 2013a. Cytokine mediated immune responses in the Japanese pufferfish (*Takifugu rubripes*) administered with *Lactobacillus paracasei* spp. *paracasei* (06TCa22) isolated from the Mongolian dairy product. Int. Immunopharmacol. 17, 358–365.

Biswas, G., Korenaga, H., Nagamine, R., Takayama, H., Kawahara, S., Takeda, S., Kikuchi, Y., Dashnyam, B., Kono, T., Sakai, M., 2013b. Cytokine responses in the Japanese pufferfish (*Takifugu rubripes*) head kidney cells induced with heat-killed probiotics isolated from the Mongolian dairy products. Fish Shellfish Immunol. 34, 1170–1177.

Brooks, M.N., Rajaram, M.V.S., Azad, A.K., Amer, A.O., Valdivia-Arenas, M.A., Park, J., Núñez, G., Schlesinger, L.S., 2011. NOD2 controls the nature of inflammatory response and subsequent fate of *Mycobacterium tuberculosis* and *M. bovis* BCG in human macrophages. Cell. Immunol. 13, 402–418.

Bryant, C., Fitzgerald, K.A., 2009. Molecular mechanisms involved in inflammasome activation. Trends Cell Biol. 19, 455–464.

Bueter, C.L., Lee, C.K., Rathinam, V.A.K., Healy, G.J., Taron, C.H., Specht, C.A., Levitz, S.M., 2011. Chitosan but not chitin activates the inflammasome by a mechanism dependent upon phagocytosis. J. Biol. Chem. 286, 35447–35455.

Chang, M., Wang, T., Nie, P., Zou, J., Secombes, C.J., 2011. Cloning of two rainbow trout nucleotide-binding oligomerization domain containing 2 (NOD2) splice variants and functional characterization of the NOD2 effector domains. Fish Shellfish Immunol. 30, 118–127.

Chen, G., Shaw, M.H., Kim, Y.G., Núñez, G., 2009. NOD-like receptors: role in innate immunity and inflammatory disease. Annu. Rev. Pathol. 4, 365–398.

Chen, W.Q., Xu, Q.Q., Chang, M.X., Nie, P., Peng, K.M., 2010. Molecular characterization and expression analysis of nuclear oligomerization domain proteins NOD1 and NOD2 in grass carp, *Ctenopharyngodon idella*. Fish Shellfish Immunol. 28, 18–29.

Dinarello, C.A., 1999. IL-18: a Th1-inducing, proinflammatory cytokine and a new member of the IL-1 family. J. Allergy Clin. Immunol. 103, 11–24.

Engelsma, M.Y., Huising, M.O., van Muiswinkel, W.B., Flik, G., Kwang, J., Savelkoul, H.F.J., Verburg-van Kemenade, B.M.L., 2002. Neuroendocrine-immune interactions in fish: a role for interleukin-1. Vet. Immunol. Immunopathol. 87, 467–479.

Franchi, L., Muñoz-Planillo, R., Núñez, G., 2012. Sensing and reacting to microbes through the inflammasomes. Nat. Immunol. 13, 325–332.

Gracie, J.A., Robertson, S.E., McInnes, I.B., 2003. Interleukin-18. J. Leukoc. Biol. 73, 213–224.

Hansen, J.D., Vojtech, L.N., Laing, K.J., 2011. Sensing disease and danger: a survey of vertebrate PRRs and their origins. Dev. Comp. Immunol. 35, 886–897.

Harikrishnan, R., Kim, M.-C., Kim, J.-S., Balasundaram, C., Heo, M.-S., 2011. Immunomodulatory effect of probiotics enriched diets on *Uronema marinum* infected olive flounder. Fish Shellfish Immunol. 30, 964–971.

Hasegawa, M., Fujimoto, Y., Lucas, P.C., Nakano, H., Fukase, K., Núñez, G., Inohara, N., 2008. A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-κB activation. EMBO J. 27, 373–383.

Hayden, M.S., Ghosh, S., 2011. NF-κB in immunobiology. Cell Res. 21, 223–244.

Hewinson, J., Moore, S.F., Glover, C., Watts, A.G., MacKenzie, A.B., 2008. A key role for redox signaling in rapid $P2X_7$ receptor-induced IL-1β processing in human monocytes. J. Immunol. 180, 8410–8420.

Hu, C., Sun, L., Hu, Y., Lu, D., Wang, H., Tang, S., 2010. Functional characterization of the NF-κB binding site in the human NOD2 promoter. Cell. Mol. Immunol. 7, 288–295.

Inohara, N., Núñez, G., 2003. NODs: intracellular proteins involved in inflammation and apoptosis. Nat. Rev. Immunol. 3, 371–382.

Inohara, N., Koseki, T., Lin, J., Peso, L., Lucas, P.C., Chen, F.F., Oqura, Y., Núñez, G., 2000. An induced proximity model for NF-κB activation in the Nod1/RICK and RIP signaling pathway. J. Biol. Chem. 275, 27823–27831.

Kanneganti, T.D., Lamkanfi, M., Kim, Y.G., Chen, G., Park, J.H., Franchi, L., Vandenabeele, P., Núñez, G., 2007. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. Immunity 26, 433–443.

Kinoshita, S., Biswas, G., Kono, T., Hikima, J., Sakai, M., 2014. Presence of two tumor necrosis factor (*tnf*)-α homologs on different chromosomes of zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). Mar. Genomics 13, 1–9.

Kono, T., Takayama, H., Nagamine, R., Korenaga, H., Sakai, M., 2013. Establishment of a multiplex RT-PCR assay for the rapid detection of fish cytokines. Vet. Immunol. Immunopathol. 151, 90–101.

Koussounadis, A.I., Ritchie, D.W., Kemp, G.J., Secombes, C.J., 2004. Analysis of fish IL-1β and derived peptide sequences indicates conserved structures with species-specific IL-1 receptor binding: implications for pharmacological design. Curr. Pharm. Des. 10, 3857–3871.

Laing, K.J., Purcell, M.K., Winton, J.R., Hansen, J.D., 2008. A genomic view of the NOD-like receptor family in teleost fish: identification of a novel NLR subfamily in zebrafish. BMC Evol. Biol. 8, 42.

Lamkanfi, M., Malireddi, R.K.S., Kanneganti, T.D., 2009. Fungal zymosan and mannan activate the cryopyrin inflammasome. J. Biol. Chem. 284, 20574–20581.

Lie, Ø., Evensen, Ø., Sørensen, A., Frøysadal, E., 1989. Study on lysozyme activity in some fish species. Dis. Aquat. Org. 6, 1–5.

Locovei, S., Scemes, E., Qiu, F., Spray, D.C., Dahl, G., 2007. Pannexin1 is part of the pore forming unit of the $P2X_7$ receptor death complex. FEBS Lett. 581, 483–488.

Low, C., Wadsworth, S., Burrells, C., Secombes, C.J., 2003. Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. Aquaculture 221, 23–40.

Mariathasan, S., Weiss, D.S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W.P., Weinrauch, Y., Monack, D.M., Dixit, V.M., 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 440, 228–232.

Martin, S.A., Zou, J., Houlihan, D.F., Secombes, C.J., 2007. Directional responses following recombinant cytokine stimulation of rainbow trout (*Oncorhynchus mykiss*) RTS-11 macrophage cells as revealed by transcriptome profiling. BMC Genomics 8, 150.

Martinon, F., Pétrilli, V., Mayor, A., Tardivel, A., Tschopp, J., 2006. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 440, 237–241.

Martinon, F., Mayor, A., Tschopp, J., 2009. The inflammasomes: guardians of the body. Annu. Rev. Immunol. 27, 229–265.

McGeough, M.D., Pena, C.A., Mueller, J.L., Pociask, D.A., Broderick, L., Hoffman, H.M., Brydges, S.D., 2012. Cutting edge: IL-6 is a marker of inflammation with no direct role in inflammasome-mediated mouse models. J. Immunol. 189, 2707–2711.

Moore, C.B., Bergstralh, D.T., Duncan, J.A., Lei, Y., Morrison, T.E., Zimmermann, A.G., Accavitti-Loper, M.A., Madden, V.J., Sun, L., Ye, Z., Lich, J.D., Heise, M.T., Chen, Z., Ting, J.P.-Y., 2008. NLRX1 is a regulator of mitochondrial antiviral immunity. Nature 451, 573–577.

Mulder, I.E., Wadsworth, A., Secombes, C.J., 2007. Cytokine expression in the intestine of rainbow trout (*Oncorhynchus mykiss*) during infection with *Aeromonas salmonicida*. Fish Shellfish Immunol. 23, 747–759.

Pedra, J.H., Sutterwala, F.S., Sukumaran, B., Ogura, Y., Qian, F., Montgomery, R.R., Flavell, R.A., Fikrig, E., 2007. ASC/PYCARD and caspase-1 regulate the IL-18/IFN-γ axis during *Anaplasma phagocytophilum* infection. J. Immunol. 179, 4783–4791.

Pelegrin, P., Surprenant, A., 2007. Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1β release through a dye uptake-independent pathway. J. Biol. Chem. 282, 2386–2394.

Pétrilli, V., Papin, S., Dostert, C., Mayor, A., Martinon, F., Tschopp, J., 2007. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death Differ. 14, 1583–1589.

Puren, A.J., Razeghi, P., Fantuzzi, G., Dinarello, C.A., 1998. Interleukin-18 enhances lipopolysaccharide-induced interferon-γ production in human whole blood cultures. J. Infect. Dis. 178, 1830–1834.

Rahman, M.M., McFadden, G., 2011. Modulation of NF-κB signalling by microbial pathogens. Nat. Rev. Microbiol. 9, 291–306.

Rathinam, V.A.K., Vanaja, S.K., Fitzgerald, K.A., 2012. Regulation of inflammasome signaling. Nat. Immunol. 13, 333–342.

Reis, M.I.R., do Vale, A., Pereira, P.J.B., Azevedo, J.E., dos Santos, N.M.S., 2012. Caspase-1 and IL-1β processing in a teleost fish. PLoS One 7, e50450.
Rosenzweig, H.L., Kawaguchi, T., Martin, T.M., Planck, S.R., Davey, M.P., Rosenbaum, J.T., 2009. Nucleotide oligomerization domain-2 (NOD-2) induced uveitis: dependency on IFN-γ. Invest. Opthalmol. Vis. Sci. 50, 1739–1745.
Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defence molecule of fish innate immune system. Aquaculture Res. 39, 223–239.
Savan, R., Sakai, M., 2006. Genomics of fish cytokines. Comp. Biochem. Physiol. Part D: Genomics Proteomics 1, 89–101.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C_T method. Nat. Protoc. 3, 1101–1108.
Schroder, K., Tschopp, J., 2010. The inflammasomes. Cell 140, 821–832.
Schroder, K., Sagulenko, V., Zamoshnikova, A., Richards, A.A., Cridland, J.A., Irvine, K.M., Stacey, K.J., Sweet, M.J., 2012. Acute lipopolysaccharide priming boosts inflammasome activation independently of inflammasome sensor induction. Immunobiology 217, 1325–1329.
Squires, R.C., Muehlbauer, S.M., Brojatsch, J., 2007. Proteasomes control caspase-1 activation in anthrax lethal toxin-mediated cell killing. J. Biol. Chem. 282, 34260–34267.
Stout-Delgado, H.W., Vaughan, S.E., Shirali, A.C., Jaramillo, R.J., Harrod, K.S., 2012. Impaired NLRP3 inflammasome function in elderly mice during Influenza infection is rescued by treatment with nigericin. J. Immunol. 188, 2815–2824.
Tattoli, I., Carneiro, L.A., Jéhanno, M., Magalhaes, J.G., Shu, Y., Philpott, D.J., Arnoult, D., Girardin, S.E., 2008. NLRX1 is a mitochondrial NOD-like receptor that amplifies NF-κB and JNK pathways by inducing reactive oxygen species production. EMBO Rep. 9, 293–300.
van de Veerdonk, F.L., Netea, M.G., Dinarello, C.A., Joosten, L.A.B., 2011. Inflammasome activation and IL-1β and IL-18 processing during infection. Trends Immunol. 32, 110–116.
Vojtech, L.N., Scharping, N., Woodson, J.C., Hansen, J.D., 2012. Roles of inflammatory caspases during processing of zebrafish interleukin-1β in *Francisella noatunensis* infection. Infect. Immun. 80, 2878–2885.
Wang, H., Xing, Y., Mao, L., Luo, Y., Kang, L., Meng, G., 2013. Pannexin-1 influences peritoneal cavity cell population but is not involved in NLRP3 inflammasome activation. Protein Cell 4, 259–265.
Windheim, M., Lang, C., Peggie, M., Plater, L.A., Cohen, P., 2007. Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide. Biochem. J. 404, 179–190.
Xia, X., Cui, J., Wang, H.Y., Zhu, L., Matsueda, S., Wang, Q., Yang, X., Hong, J., Songyang, Z., Chen, Z.J., Wang, R.F., 2011. NLRX1 negatively regulates TLR-induced NF-κB signaling by targeting TRAF6 and IKK. Immunity 34, 843–853.
Xie, J., Hodgkison, J.W., Katzenback, B.A., Kovacevic, N., Belosevic, M., 2013. Characterization of three Nod-like receptors and their role in antimicrobial responses of goldfish (*Carassius auratus* L.) macrophages to *Aeromonas salmonicida* and *Mycobacterium marinum*. Dev. Comp. Immunol. 39, 180–187.
Zhu, L., Nie, L., Zhu, G., Xiang, L., Shao, J., 2013. Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. Dev. Comp. Immunol. 39, 39–62.
Zou, J., Secombes, C.J., 2011. Teleost fish interferons and their role in immunity. Dev. Comp. Immunol. 35, 1376–1387.
Zou, J., Peddie, S., Scapigliati, G., Zhang, Y., Bols, N.C., Ellis, A.E., Secombes, C.J., 2003. Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss*. Dev. Comp. Immunol. 27, 813–822.