NF- κ B signaling induces inductive expression of the downstream molecules and IgD gene in the freshwater carp, *Catla catla*

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Abstract

Toll-like receptors (TLRs) in innate immune system act as primary sensors in detecting the microbial components and activate their signaling cascades to induce NF- κ B (nuclear factor NF- κ B) towards the augmentation of immunoglobulin (Ig) synthesis. To gain insights into the efficacy of NF- κ B pathway in immunoglobulin D (IgD) synthesis in the Indian Major Carp *Catla catla*, cloning and sequencing of TLR-signaling downstream molecules [TRAF3 (TNF receptor-associated factor 3), NEMO (nuclear factor-kappa B essential modulator), NF- κ B and BAFF (B cell activating factor)] were performed by infecting the fish with pathogens. mRNA expression analysis of the downstream molecules and IgD showed significant up-regulation of these genes in kidney ($P \leq 0.001$) as compared to spleen ($P \leq 0.05$). To ascertain the role of NF- κ B pathway in IgD synthesis, the primary cell culture of kidney and spleen in monolayer cell suspension was treated with NF- κ B inhibitor (BAY 11-7082) and down-regulation of *BAFF*, *NEMO*, *NF- κ B*, and *IgD* gene was observed. These results highlight the importance of NF- κ B signaling pathway in augmenting the *IgD* gene expression in the freshwater carp, *Catla catla*.

Keywords *Catla catla* · Immunoglobulin · NF- κ B · NEMO · BAFF · TRAF3 · IgD

Introduction

In fish, the innate immune system acts as a potential barrier against wide spectrum of pathogens and play major role in preventing infection and diseases. In the host, the unique molecular signature of the microbes/pathogens such as flagellin (FLA), lipoteichoic acid (LTA), lipopolysaccharides (LPS), peptidoglycan (PGN) and nucleic acids which are specified as pathogen/ microbial-associated molecular pattern (PAMPs/MAMPs) are recognized by various pattern recognition receptors (PRRs) such as TLRs (Toll-like receptors), NLRs (Nod-like receptors) and RLRs (Rig-I like receptors) (Kawai and Akira 2009; Kumar et al. 2009). Various PAMPs are recognized by the TLRs present on the extracellular surface and within the endosome of the innate

immune cells. Recognition and binding of PAMPs by various TLRs trigger either MyD88-dependent or independent signaling pathways to induce innate immune cytokines (Zhang and Ghosh 2001; Bagchi et al. 2007; Kawai and Akira 2011). The recognition of PAMPs by various TLRs on the B-cell surface directly activates the B-cell. The indirect binding of PAMPs by the TLRs on the antigen presenting cells (APCs) such as macrophage and dendritic cells results in the synthesis of B-cell activating factor (BAFF) aiding the survivability of the B-cells (Heer et al. 2007; Barr et al. 2012; Bikker et al. 2014; Soletto et al. 2018). Identification and characterization of several TLRs have been reported in various fish species, viz. *Takifugu rubripes* (Oshiumi et al. 2003), *Danio rerio* (Meijer et al. 2004; Nie et al. 2018), *Paralichthys olivaceus* (Hirono et al. 2004), *Ictalurus punctatus* (Baoprasertkul et al. 2007; Quiniou et al. 2013), *Oncorhynchus mykiss* (Palti et al. 2010; Brietzke et al. 2016), *Epinephelus coioides* (Wei et al. 2011), *Pseudosciaena crocea* (Ao et al. 2016), *Labeo rohita* (Samanta et al. 2012), *Cirrhinus mrigala* (Basu et al. 2012), *Cyprinus carpio* (Fink et al. 2016), *Ctenopharyngodon idella* (He et al. 2016) and *Catla catla* (Patel et al. 2019). The activation of MyD88-dependent TLR-signaling pathways results in the induction of various downstream signaling molecules such as IRAK,

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TAB1, TAB2 and TRAF6 (Rebl et al. 2008; Patel et al. 2019; Li et al. 2019) to induce the synthesis of pro-inflammatory cytokines and Type-I-interferons. TLR comprises of extra-cellular N-terminus with leucine-rich repeat (LRR) region, trans-membrane (TM) domain and intracellular C-terminus with Toll/IL-1 receptor domain (TIR). TIR harbors conserved amino acids which activate MyD88 and engage the downstream molecules and adaptors for activation of NF- κ B and other downstream molecules (Keating et al. 2007; Leibowitz and Yan 2016). NF- κ B, a transcriptional factor, gets activated in response to the engagement of different receptors (TLR, BCR, CD40 and TNFR) (Silverman and Maniatis 2001; Bonizzi and Karin 2004) and plays an important role in defense system by regulating the expression of cytokines and growth factors in the biological systems (Ghosh et al. 1998; Li and Verma 2002; Ghosh and Karin 2002; Michael et al. 2002; Liang et al. 2004; Correa et al. 2004).

The NF- κ B complex comprises of p65, c-Rel, RelB, p50 and p52 molecules that exist in unstimulated cells as homo-dimers or hetero-dimers and are tightly bound to I κ B proteins (Hayden and Ghosh 2004). The complex transcriptional motivating molecule instigates two different signaling pathways, namely classical (canonical) pathway and alternative (non-canonical) pathway. The classical pathway leads to the phosphorylation and degradation of I κ B proteins deactivating NF- κ B complex with the help of IKK. I κ B proteins consist of IKK α and IKK β catalytic subunits and the IKK γ /NEMO regulatory subunit (Baeuerle 1998). The disruptions of gene during the deactivation involve major mediators (IKK β and IKK γ) of the classical NF- κ B signal transduction pathways (Li et al. 2005). Contrary to the classical pathway, inducers of NF- κ B activate alternative pathway without the degradation of I κ B, but are associated with the linkage of TRAFs (TRAF3, TRAF2) for the activation of the NF- κ B-inducing kinase (NIK) (Hayden and Ghosh 2014).

These pathways lead to the production of inflammatory cytokines and synthesize immunoglobulins (Igs) by regulating B-cells (Breitfeld et al. 2000; Cerutti et al. 2012). The B-cell activating factor (BAFF) has been reported to play the crucial role in the defense system of the host by regulating the functions of B-cells. Antigen presenting cells such as monocytes/macrophages and dendritic cells secrete BAFF, which not only modulates survivability of the B-cell but also performs other B-cell functions (Smulski and Eibel 2018). The lipopolysaccharides (LPS) binds to TLR4 and stimulates myeloid cells and monocytes, promoting B-cell regulation and proliferation (Nardelli et al. 2001) and favors immunoglobulin class switch recombination (Litinskiy et al. 2002). On the other hand, peptidoglycan (PGN) ligand binds TLR2 (Samanta et al. 2012; De Oliveira Nascimento et al. 2012) and triggers dendritic cells for the stimulation of BAFF which enhances B-cell activation (Jego et al. 2003; Cerutti et al. 2012). These findings suggest that TLR might

play a crucial role in the production of BAFF through the myeloid cells via NF- κ B complex in fish.

Various innate immune signaling studies have been carried out extensively in murine and higher vertebrates (Kawai and Akira 2007) which clearly showed the role of downstream molecules in activation of TLR signaling pathways, but it remained still unknown in the primordial vertebrates such as fish. In our previous study, cloning of IgD in the freshwater carp, *Catla catla* and its gene expression level in various tissues in response to live pathogens and ligand stimulations have been reported (Banerjee et al. 2017). In the present study, the identification of the downstream molecules involved in signaling pathway was first ascertained; and the role of NF- κ B signaling pathway in activating the B-cell for the expression of IgD gene has been investigated in *C. catla*.

Materials and methods

Maintenance of experimental fish, bacterial challenge and sampling

Healthy *Catla catla* fish (~50 gm) were stocked in 500-L FRP tanks with proper aeration and were acclimatized in the tank for 4 weeks with ad libitum commercial carp diet. The water temperature (28°–30 °C) and pH conditions (7.4–7.6) were maintained with routine exchange of water throughout the experiment. Gram-negative bacterium *Aeromonas hydrophila* (ATCC 35654) and Gram-positive bacterium *Streptococcus uberis* (ATCC 700407) (HiMedia, India) were cultured in Luria Bertani broth (HiMedia, India) at constant shaking for 16 h at 120 rpm. Both the overnight grown cultures were centrifuged for 5 min at 3000 rpm and the pellet was washed with 1X phosphate buffer saline (PBS; pH 7.4). The obtained fresh pellet was re-suspended in PBS. For the infection study, colony forming unit (CFU) assay was done using tenfold serial dilutions to determine the viable cells. Healthy fish ($n=3$) was injected with 100 μ L of PBS (control), whereas, 4×10^6 CFU/fish of *A. hydrophila* and 1×10^6 CFU/fish of *S. uberis* was injected in infected group ($n=3$) (Banerjee et al. 2019). Infection in fish was done intra-peritoneally (i.p.). The infected fish were killed after 24 h, 48 h and 72 h. Immunologically relevant tissues (liver, kidney and spleen) were collected aseptically in TRIzol reagent (Life Technologies, USA). The isolated tissues were kept in – 80 °C for further experimental purposes. This study in catla was approved by the ethics committee of the ICAR-CIFA (approval number ICAR-CIFA/Eth/02/2016).

Isolation of RNA and first strand cDNA synthesis

The isolated tissues from the treated and control fish were used for the extraction of RNA using TRIzol reagent. The tissues were homogenized vigorously and 200 µL of chloroform was added followed by centrifugation at $12,000 \times g$ for 15 min. Three different phases were obtained out of which the upper clear aqueous phase containing RNA was treated with 500 µL isopropanol (Sigma-Aldrich, USA) followed by washing using chilled ethanol. The isolated RNA was quantified in Bio-photometer (Eppendorf, Germany) and 2 µg RNA from each sample was used for cDNA synthesis. To eradicate the genomic DNA contamination, 1 µL of DNaseI was added to the RNA followed by the addition of stop solution (Sigma-Aldrich, USA). After the deactivation of DNaseI, the treated RNA was reverse transcribed to cDNA using RevertAid cDNA synthesis kit (Thermo Scientific, USA). The first strand cDNA synthesis was done following the manufacturer's procedure. 1 µL of random hexamer and Oligo dT each was added to the DNase treated RNA and was incubated at 65 °C for 5 min. Then the reaction mixture containing the first-strand reaction buffer (5X), dNTP mix, reverse transcriptase and RNase inhibitor was added following the kits protocol and the reverse transcription was carried out by incubating the mixture at 25 °C for 5 min, 42 °C for 60 min, followed by 70 °C for 10 min in a thermal cycler (Bio-Rad, USA).

Cloning of TRAF3, NEMO and NF-κB gene of *Catla catla*

NF-κB signaling cascade is triggered by activation of various downstream molecules such as TRAF3, NEMO (NEMO1 and NEMO2) and NF-κB. To clone the downstream signaling molecules, various primers of the downstream signaling

molecules were designed from the homologous species based on the conserved region of different carps and then the designed primers (Table 1) were used to amplify the genes. 1 µL cDNA was taken as a template and the PCR was performed in a total volume of 25 µL with the following conditions: initial denaturation at 94 °C for 5 min followed by 45 cycles of denaturation (95 °C), annealing (50–60 °C) and extension (72 °C) along with final extension at 72 °C for 10 min. The PCR product was loaded in 2% agarose gel for checking the desired amplicon size. The amplicon was excised and purified with agarose gel extraction kit (Roche, Germany) and was cloned in pGEM-T Easy vector (Promega, USA). The positive clones were sequenced and the consensus sequence was checked in NCBI BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) to find its similarity with the homologous genes.

Primary culture of catla kidney and spleen cells

Primary culture of kidney and spleen was carried out following Samanta et al. (2012). Briefly, spleen and kidney were surgically isolated from healthy catla fish (~100 gm), washed with 1X PBS and was then transferred to RPMI-1640 medium (HiMedia, India). Then tissues were minced with the help of sterilized blade. The clumps of tissues were further passed through 100-µm cell strainer (HiMedia, India). The cell suspension in the medium was centrifuged at 3000 rpm for 5 min at 4 °C. The pellet obtained after centrifugation was washed twice and further dissolved in the RPMI-1640 medium containing 10% FBS and antibiotic (1% penicillin–streptomycin solution) (HiMedia, India). The suspended cells were then plated in six-well cell-culture plates followed by incubation at 28 °C. On the 4th day, the medium of the cells was half replaced with fresh medium, and on the 7th day monolayer formation was observed in some parts.

Table 1 Primers used for cloning and real-time PCR study of downstream signaling molecules

Name of the genes	Primer sequence 5' → 3'	Tm (°C)
<i>BAFF</i>	Fw: 5'-GTCGGGGTGAAAGGAGAAGG-3' Rv: 5'-TCTCTTTTTCGGACCACTC-3'	60
<i>NEMO1</i>	Fw: 5'-GCACTGAAGAACGAGAGGCT-3' Rv: 5'-CCTGATGGTTCAGCACAGGT-3'	60
<i>NEMO2</i>	Fw: 5'-CGAACGACAAAACCTGGCTGG-3' Rv: 5'-TAGCAAGGGTATCCGCCTCT-3'	60
<i>TRAF3</i>	Fw: 5'-GAGCACCTAAGCCGCAAATG-3' Rv: 5'-CAGCAAGACGACTGCTGAGA-3'	62
<i>NF-κB</i>	Fw: 5'-TTTACAGGAGCGGCGGATAC-3' Rv: 5'-GTGCGAAACACGATAGCCAC-3'	59
<i>IgD</i>	Fw: 5'-CAACATCACCTGGCTGCTTG-3' Rv: 5'-GCACAGGCCATATTCCATGC-3'	60
<i>β-actin</i>	Fw: 5'-AGACCACCTTCAACTCCATCATG-3' Rv: 5'-TCCGATCCAGACAGAGTATTACG-3'	55

NF- κ B complex inhibition in primary cell culture

To investigate the role of NF- κ B complex in downstream signaling cascade, experiment was performed with the primary cell culture of kidney and spleen. The primary cell culture was washed with antibiotic free medium and then either PGN (20 μ g/ml) or LPS (10 μ g/ml) (Sigma-Aldrich, USA) was added to it. PGN/LPS (PAMPs)-treated primary cell culture was then incubated for 4 h and 8 h. To study the role of NF- κ B, primary culture was treated with 2.0 μ M BAY 11-7082 (NF- κ B inhibitor) (InvivoGen, USA) for 2 h prior to the stimulation with PGN and LPS (Mori et al. 2002; Samanta et al. 2012). The control and treated primary culture were used for RNA isolation and cDNA synthesis.

mRNA transcript expression of BAFF, NEMO, NF- κ B and IgD

To examine the expression of *BAFF*, *NEMO*, *NF- κ B* and *IgD* gene in the presence of PAMPs (LPS/PGN) + BAY 11-7082 (NF- κ B inhibitor), RNA was isolated from the primary cultures followed by cDNA synthesis and qRT-PCR assay. PCR was carried out in 10 μ l volume which was comprised of 2X SYBR Select Mix (Applied Biosystems, USA), gene-specific forward and reverse primer (0.75 μ M), 1 μ l cDNA template and Milli-Q water to make up the final volume. The PCR conditions were initial denaturation at 95 $^{\circ}$ C for 2 min followed by 40 cycles of 95 $^{\circ}$ C for 15 s, 50–60 $^{\circ}$ C for 15 s and 72 $^{\circ}$ C for 1 min. This cycle was followed by an analysis of the dissociation curve to verify the amplification of the PCR product. The mRNA expression levels of the above-mentioned genes were normalized with the internal control β -actin. The calculation of relative expression was done using $2^{-\Delta\Delta C_t}$ method (Pfaffl 2001). The obtained data from the analysis of qRT-PCR were expressed as mean of three individual experiment + standard error (S.E).

Deducing the downstream signaling pathway of NF- κ B through in-silico functional protein interaction network

The co-relations among proteins were determined by STRING (<https://string-db.org/>) software (Szklarczyk et al. 2015). Amino acid sequences of the identified proteins using BLASTp searches were evaluated against *Danio rerio*. The representation of protein–protein interaction network of signal transduction was analysed with the confidence scores of 0.9 in the experiments, database and text mining.

Statistical analysis

Data are represented as mean $\pm$ S.E. from three independent experiments. The data were analyzed using GraphPad

Prism (version 5.01) for determining statistical significance of difference between control versus treatment groups. Significance was determined by one-way analysis of variance (ANOVA).

Results

Cloning of downstream molecules of NF- κ B-dependent and NF- κ B-independent pathway

The identification and confirmation of downstream molecules of NF- κ B-dependent and NF- κ B-independent pathway were performed using TA cloning, sequencing and BLAST analysis (Table 2). *NEMO*, *TRAF3* and *NF- κ B* genes showed higher percentage of sequence similarity with *Cyprinidae* family species. The nucleotide sequence of these genes has been submitted in the GenBank database with the accession numbers MG893207.1 (NEMO1), MG893208.1 (NEMO2), MK134709.2 (TRAF3) and KY089040 (NF- κ B).

Basal level expression of TRAF3, NEMO, NF- κ B and IgD genes

The basal level expression of *TRAF3*, *NEMO*, *NF- κ B* and *IgD* gene in liver, kidney and spleen was investigated by the qRT-PCR assay. Among the examined tissues, liver was chosen as calibrator. The basal level expression of *TRAF3* gene in kidney and spleen tissues was ~2-fold and ~1.8-fold, respectively (Fig. 1a). Similarly, the basal level expression

Table 2 Identification of the downstream molecules and the similarity with *Cyprinidae* family

Downstream molecules	Length (bp)	Accession number	Similarity with <i>Cyprinidae</i> family (%)
TRAF3	200	MK134709.2	<i>C. carpio</i> —91.50 <i>C. auratus</i> —88.56 <i>C. idella</i> —86.15 <i>D. rerio</i> —82.50
NEMO1	275	MG893207.1	<i>C. auratus</i> —92.73 <i>C. carpio</i> —92.00 <i>D. rerio</i> —90.18 <i>C. idella</i> —95.60
NEMO2	327	MG893208.1	<i>C. auratus</i> —93.56 <i>C. carpio</i> —92.94 <i>D. rerio</i> —88.69 <i>C. idella</i> —93.21
NF- κ B	453	KY089040	<i>C. auratus</i> —93.76 <i>C. idella</i> —92.49 <i>D. rerio</i> —89.30

Fig. 1 Basal level gene expression profile of (a) *TRAF3* (b) *NEMO* (c) *NF-κB* and (d) *IgD* in kidney and spleen. Expression of gene transcripts in both the tissues was represented as a ratio relative to β-actin (internal control). Liver was chosen as calibrator and the rela-

tive expression was expressed as fold changes from the calibrator. All the data are represented as mean ± S.E. ($n=3$). Significant difference with respect to the calibrator was determined by one-way ANOVA with *** $P<0.001$, ** $P<0.01$ and * $P<0.05$ as significance levels

of *NEMO* gene was ~1.8-fold in kidney and ~1.2-fold in spleen tissues (Fig. 1b). *NF-κB* gene expression in kidney and spleen tissues was ~1.3-fold and ~1.8-fold, respectively (Fig. 1c). The basal level expression of *IgD* gene in kidney and spleen tissue was ~9.8-fold and ~1.3-fold, respectively (Fig. 1d).

Modulation of TRAF3, NEMO, NF-κB and IgD gene expression by bacterial infection

TRAF3, NEMO, NF-κB, and IgD mRNA transcript expression was analyzed in kidney and spleen at various time intervals following infection with *A. hydrophila* and *S. uberis*. *TRAF3* gene expression in *A. hydrophila*-infected tissues of kidney and spleen were analyzed at all the time points. In the *A. hydrophila*-infected fish, *TRAF3* gene expression in the kidney tissues was ~7.8-fold at 48 h, while it was ~2- and ~3-fold at 24 and 72 h, respectively. *TRAF3* gene expression in the spleen tissues was enhanced (~3.0-fold) at 24 h and then moderately decreased at 48 h followed by significant increase to ~6.0-fold at 72 h (Fig. 2a). *NEMO* gene expression was up-regulated (~1.8-fold) at 24 h in the tissues of kidney and was down-regulated at 48 and 72 h

by ~0.02-fold and ~0.01-fold, respectively. However, in the tissues of spleen, the expression gradually decreased with time (~0.3-fold at 24 h; ~0.09-fold at 48 h and ~0.01-fold at 72 h) (Fig. 2b). The expression of *NF-κB* gene in the tissues of kidney was significantly higher (~14.8-fold) at 24 h post-infection, but a gradual down-regulation (~0.1-fold and ~0.2-fold) was noted at 48 h and 72 h post-infection. In spleen, the *NF-κB* gene expression was down-regulated at all the tested time points. (Fig. 2c). Expression of *IgD* gene in the tissue of kidney showed gradual increase (~1.8-fold to ~4.8-fold) up to 48 h but again declined at 72 h (~0.8-fold). In contrast to the kidney, *IgD* gene expression was down-regulated at 24, 48 and 72 h post-infection (Fig. 2d).

The expression of these genes was also investigated in the *S. uberis*-infected tissues of *C. catla*. It is interesting to note that variation in *TRAF3* gene expression followed the similar trend as observed in *A. hydrophila*-infected tissues of kidney and spleen. In the kidney tissues, the *TRAF3* gene expression was increased by ~1.8-fold at 24 h, followed by ~7-fold at 48 h and then ~5-fold at 72 h. Tissues of spleen also showed the variation in *TRAF3* gene expression at all the time points (~3.0-fold, 24 h; ~2.0-fold, 48 h; ~4.0-fold, 72 h) (Fig. 3a). The *NEMO* gene expression in kidney was

Fig. 2 Modulation of gene expression of (a) *TRAF3* (b) *NEMO* (c) *NF-κB* and (d) *IgD* in the *Aeromonas hydrophila* challenged fish post 24 h, 48 h and 72 h of infection. β -Actin was used as the internal control for normalizing the values of the target genes. All the

data are represented as mean $\pm$ S.E. ($n=3$). Significant difference with respect to the control was determined by one-way ANOVA with *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ as significance levels

Fig. 3 Modulation of gene expression of (a) *TRAF3* (b) *NEMO* (c) *NF-κB* and (d) *IgD* in the *Streptococcus uberis* challenged fish post 24 h, 48 h and 72 h of infection. β -Actin was used as the internal control for normalizing the values of the target genes. All the data

are represented as mean $\pm$ S.E. ($n=3$). Significant difference with respect to the control was determined by One-way ANOVA with *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ as significance levels

marginally enhanced (~ 1.2 -fold) at 24 h and thereafter it decreased. In spleen, there was down-regulation at all time points as compared to the control (Fig. 3b). NF- κ B mRNA transcripts in the kidney tissue were up-regulated only at 24 h (~ 1.9 -fold). In the spleen tissue, down-regulation was observed at all the time points (Fig. 3c). The expression of IgD mRNA transcript was also investigated in the infected tissues at different time intervals. In the kidney tissue, the IgD expression was significantly up-regulated at 24 h (~ 2.2 -fold), followed by its down-regulation at 48 h (~ 0.8 -fold)

and 72 h (~ 0.1 -fold). In the spleen tissue, expression of IgD gene was found to be down-regulated over the time (~ 0.5 -fold, 24 h; ~ 0.38 -fold, 48 h; ~ 0.4 -fold, 72 h) (Fig. 3d).

PAMP-mediated inductive expression of BAFF, NEMO and IgD genes is regulated by NF- κ B

The primary culture of kidney and spleen was prepared and maintained till the 7th day (Fig. 4). The expression of BAFF, NEMO, NF- κ B and IgD gene was investigated by

Fig. 4 Proliferation of primary-cultured kidney and spleen cells of healthy *C. catla*. Red arrow indicates the initiation of monolayer formation

qRT-PCR after stimulating the kidney and spleen cell culture with PAMPs (PGN or LPS). It is well established that the process of TLR signaling eventually leads to the synthesis of cytokines such as BAFF. To investigate whether NF- κ B molecule affects the role of BAFF in B-cell regulation, kidney and spleen cell-suspension were treated with LPS or PGN along with the presence/absence of BAY 11-7082 (Fig. 5a,b). PAMP (LPS/PGN)-treated culture showed an up-regulation at 4 h (~10/43.7-fold) and 8 h (~57/54-fold) in *BAFF* gene expression when compared to the control. Furthermore, LPS/PGN + BAY 11-7082-treated kidney primary culture depicted an inhibition in *BAFF* gene expression at 4 h (~7.3/0.5-fold) and 8 h (~0.61/8.9-fold) when compared to the PAMP-treated culture (Fig. 5a). LPS/PGN + BAY 11-7082-treated spleen primary culture was also used for investigating the expression of BAFF. The transcriptomic BAFF expression was up-regulated in LPS/PGN-treated primary culture at 4 h (~12.7-fold/~40.9-fold) and 8 h (~22-fold/~19.8-fold), respectively. It was noted that BAFF gene expression was significantly inhibited in LPS or PGN stimulation in presence of BAY 11-7082 treated spleen primary cell-suspension culture at 4 h (LPS + BAY 11-7082: ~0.17-fold; PGN + BAY 11-7082: ~0.31-fold) and 8 h (LPS + BAY 11-7082: ~3.94-fold; PGN + BAY 11-7082: ~0.54-fold) (Fig. 5b).

The interaction of BAFF with BAFF-R receptor induces NF- κ B-independent (canonical) and NF- κ B-dependent (non-canonical) pathway. NEMO molecule is known to play a role in NF- κ B-independent pathway leading to the activation of genes involving in cell survivability, inflammation and activation of other pathways. To re-establish the fact that canonical pathway is independent of NF- κ B complex in fish, expression of *NEMO* gene was analyzed in kidney primary cell-suspension culture which was treated with LPS in the presence/absence of BAY 11-7082. *NEMO* gene expression in PAMP (LPS/PGN)-treated culture showed an up-regulation at 4 h (~5.7/26-fold) and 8 h (~23.6/39-fold) post-treatment when compared to the control. However, kidney primary culture treated with PAMPs (LPS/PGN) + BAY 11-7082 showed an inhibition of *NEMO* gene expression at 4 h (~3.8/1.6-fold) and 8 h (~0.26/2.84-fold), respectively, in the LPS/PGN-treated culture (Fig. 5c). LPS/PGN-treated spleen primary culture showed an up-regulation of *NEMO* gene at 4 h (~2.9/1.4-fold). However, at 8 h post-infection, LPS-treated culture showed down-regulation (~0.28-fold) and the PGN-treated spleen primary culture showed the up-regulation of *NEMO* gene expression at 8 h (~2.66-fold) when compared to the control. In case of LPS + BAY 11-7082-treated spleen cell-suspension culture, the inhibitor did not suppress the *NEMO* mRNA expression after 8 h (~5.93-fold). But at 4 h, the *NEMO* gene expression was found to be decreased to ~0.7-fold with respect to the PAMP-treated culture (Fig. 5d). PGN + BAY 11-7082-treated spleen

culture showed an elevated *NEMO* expression at 4 h (~1.87-fold) and at 8 h (~5.94-fold) when compared to the PGN-treated culture in the absence of inhibitor.

The NF- κ B-dependent pathway was subsequently investigated by examining the expression of *NF- κ B* gene. LPS/PGN-treated kidney and spleen suspension culture when compared with the respective control showed an up-regulation in *NF- κ B* gene expression (~5.73/25.7-fold, 4 h; ~23.6/39.8-fold, 8 h) and (~5.11/13.2-fold, 4 h; ~5.94/4.72-fold, 8 h). Primary culture of kidney and spleen was further treated in the presence of BAY 11-7082 and it was observed that LPS + BAY 11-7082-treated kidney primary culture showed the decreased (~3.97-fold, 4 h; ~0.26-fold, 8 h) *NF- κ B* gene expression when compared to the LPS-treated primary culture (Fig. 5e). Spleen primary culture treated with LPS + BAY 11-7082 also showed low level of gene expression (~1.72-fold, 4 h; ~0.46-fold, 8 h) (Fig. 5f). Similarly, PGN + BAY 11-7082-treated kidney and spleen primary culture showed an inhibitory effect in the *NF- κ B* gene expression (~1.69/2.09-fold, 4 h; ~2.84/1.23-fold, 8 h) at all the time points when compared with PGN-treated kidney and spleen primary culture (Fig. 5e,f).

To investigate whether NF- κ B inhibits downstream cascade and subsequently the *IgD* gene expression, qRT-PCR was performed. LPS/PGN-treated kidney and spleen primary culture showed significant upregulation of *IgD* gene expression at both 4 h (~4.45/40.44-fold and ~16.6/15.7-fold) and 8 h (~17.7/56.9-fold and ~11.6/1.18-fold) post-treatment when compared to their respective controls (Fig. 5g,h). A considerable decrease (~3.41-fold, 4 h; ~0.25-fold, 8 h) in *IgD* mRNA expression was found, when kidney cell suspension culture was treated with LPS + BAY 11-7082. PGN + BAY 11-7082-treated kidney primary culture also showed substantial inhibition (~0.16-fold, 4 h; ~0.23-fold, 8 h) in both the time intervals on comparing with PGN-treated kidney culture (Fig. 5g). Similarly, spleen cell suspension culture treated with/without LPS + BAY 11-7082 showed significant inhibition at 4 h (~1.24-fold) and 8 h (~0.98-fold) post-treatment when compared to LPS-treated culture. A significant decrease (~0.59-fold, 4 h; ~1.05-fold, 8 h) of *IgD* mRNA expression was also observed in spleen primary culture in the PGN + BAY 11-7082 treatment when compared to the PGN stimulated primary culture (Fig. 5h).

Protein–protein interaction network of the signaling pathway involved in *C. catla*

Protein sequences of TLR2, TLR4, TRAF3, BAFF (TNFSF13b), NF- κ B (re1, re1a, re1b) subunits and NF- κ B were connected to each other in a form of network with the help of predicted functional partners (CD40, TBK1, MyD88, MAVS and TRAF family member-associated NF- κ B activator) (Fig. 6a). The maximum number of lines in the

Fig. 5 Differential gene expression of (*BAFF*, *NEMO*, *NF-κB* and *IgD*) in primary cell culture of kidney (a, c, e and g) and spleen (b, d, f and h). Primary cell culture was stimulated with PGN and LPS for 4 h and 8 h in presence (treated) or absence (control) of BAY 11-7082. β -actin was used as the internal control for normal-

izing the values of the target genes. All the data are represented as mean $\pm$ S.E. ($n=3$). Significant difference with respect to the control was determined by one-way ANOVA with *** $P<0.001$, ** $P<0.01$ and * $P<0.05$ as significance levels

Node ID with real protein name

tlr2: CD282
 tlr4a: CD284
 myd88: MYD88
 nfkb2: p100
 rel: c-Rel
 rela: p65
 relb: l-Rel
 cd40: p50
 tbk1: TANK Binding Kinase 1
 mavs: IFN-B Promoter Stimulator 1
 traf3: TRAF3
 ENSDARG0000067723: TRAF2
 tnfsf13b: BAFF
 ighd: IgD

(a)

Nodes:

Network nodes represent proteins

splice isoforms or post-translational modifications are collapsed, i.e. each node represents all the proteins produced by a single, protein-coding gene locus.

Node Color

colored nodes:
query proteins and first shell of interactors

white nodes:
second shell of interactors

Node Content

empty nodes:
proteins of unknown 3D structure

filled nodes:
some 3D structure is known or predicted

Edges:

Edges represent protein-protein associations

associations are meant to be specific and meaningful, i.e. proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding each other.

Known Interactions

from curated databases

experimentally determined

Predicted Interactions

gene neighborhood

gene fusions

gene co-occurrence

Others

textmining

co-expression

protein homology

Your Input:

tnfsf13b	Tumor necrosis factor (ligand) superfamily, member 13b (268 aa)
rel	V-rel reticuloendotheliosis viral oncogene homolog (596 aa)
tlr4al	Toll-like receptor 4a, like (816 aa)
rela	V-rel reticuloendotheliosis viral oncogene homolog A (588 aa)
traf3	TNF receptor-associated factor 3 (573 aa)
tlr2	Toll-like receptor 2 (818 aa)
nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100 (902 aa)
ighd	Immunoglobulin heavy constant delta (1543 aa)
relb	V-rel reticuloendotheliosis viral oncogene homolog B (582 aa)

Predicted Functional Partners:

		Neighborhood	Gene Fusion	Coexpression	Cooccurrence	Experiments	Databases	Textmining	[Homology]	Score
cd40	CD40 molecule, TNF receptor superfamily member 5; CD40 antigen (286 aa)									0.999
tbk1	TANK-binding kinase 1 (727 aa)									0.997
myd88	Myeloid differentiation primary response protein MyD88; Adapter protein involved in the Toll-like receptor and ...									0.996
mavs	Mitochondrial antiviral signaling protein (585 aa)									0.995
ENSDARG0000067723	TRAF family member-associated NFKB activator (171 aa)									0.993

(b)

Fig. 6 Protein–protein interaction network through STRING software. (a) Protein–protein interaction network of signal transduction immune-related protein. In this network, nodes are the proteins; line and number of lines represent predicted interactions and strength of

predicted functional interaction between proteins respectively, (b) screenshot of the STRING shows the supplied set of immune-related proteins involved in signalling pathway. It shows details of protein abbreviation, node colour and interaction of edges

networking structure attaching one protein molecule to the other depicts the high interaction between each other. The color codes of the nodes and interaction of edges are presented in Fig. 6b.

Discussion

Pattern recognition receptor (PRR), especially TLR signaling cascade, is highly conserved across the broad range of vertebrates (Staros 2005; Konat et al. 2006). Various studies in teleosts suggest the crucial role of TLR signaling pathways in orchestration of immune responses (Kawasaki and Kawai 2014; Salvi et al. 2016; Patel et al. 2019). However, studies on detailed molecular interaction of cellular signaling in regulation of adaptive immunity in fish are very limited (Litman et al. 2010; Zhu et al. 2014; García-Valtanen et al. 2017). PAMPs recognized by cognate TLR receptors induce innate immunity through the synthesis of various cytokines (Enghard et al. 2007). The cytokines are small molecules that bind to the specified receptors to instigate the B-cell activity such as B-cell proliferation and survivability (Domingo-Gonzalez et al. 2016). Among these, the two closely related cytokines belonging to TNFSF members, namely BAFF and APRIL are known to play a profound role in B-cell regulation and its survivability (Banerjee et al. 2020). The synergistic relationship between the TLR and B-cell receptor and its engagement in the functional level ensures an appropriate antigen-specific activation in an invaded infection. B-cell differentiation including immunoglobulin (Ig) class switch recombination depends on the integration of signals from the TLRs, B-cell receptor, TNF superfamily members and cytokine receptors (Pone et al. 2010). This is crucial for exhibiting adaptive immune responses. To validate this, primary and secondary lymphoid organs (kidney and spleen) of *C. catla* were used for the present work. In a previous study by Patel et al. (2019), TLR (2–5) showed higher expressions in these mentioned lymphoid organs in response to live pathogens and PAMPs.

The gene transcript of TRAF3, NEMO and NF- κ B were analyzed in the live bacterial-infected *C. catla* tissues. Expression of TRAF3, a signal transduction adaptor molecule, was analysed in *A. hydrophila*- and *S. uberis*-infected *C. catla*. Comparatively high level of TRAF3 mRNA expression in kidney was recorded in *S. uberis*-infected fish, whereas spleen had a variation in the expression during different time intervals. This expression of TRAF3 in *S. uberis*-infected *C. catla* is consistent with the data in *Lutjanus sanguineus* which showed a wide variation in TRAF3 expression in kidney and spleen (Cai et al. 2015). *S. agalactiae*-infected *O. niloticus* also had shown similar TRAF3 expression (Han et al. 2019). Similar study was performed in *Patinopecten yessoensis*, a marine mollusk that

showed significant expression of TRAF3 when infected with *Micrococcus luteus* (Wang et al. 2015). The TRAF3 expression was also noted to be higher in kidney and spleen in *A. hydrophila*-infected catla in the present study. A similar result was interpreted in *Vibrio anguillarum*-infected marine mollusk (*Patinopecten yessoensis*), which exhibited the peak in TRAF3 gene expression in the immunological tissues during the initial time interval followed by slight reduction in TRAF3 expressions with the advancement of time (Wang et al. 2015; Cheng et al. 2016). Differential expression of TRAF molecule indicates a higher sensitivity of the innate defense system to the Gram-negative bacteria (Toubiana et al. 2013; Zhang et al. 2013; Wang et al. 2015). Diverse levels of TRAF expression might be due to the various levels of innate responses of hosts in combating the Gram-positive or Gram-negative bacteria.

Further, the expression of NEMO molecule was studied, and it was found to be involved in NF- κ B-independent pathway. The live bacteria-infected catla fish showed higher expression in kidney at 24 h, whereas spleen had a gradual down-regulation with the increase in the time. Earlier studies on NEMO-deficient 293T and MEF cells demonstrated the complete absence of NF- κ B activation (Pescatore et al. 2016). This highlights the importance of NEMO in triggering NF- κ B independent pathway which helps in subsequent induction of type I-interferons (IFNs). The ubiquitin binding-defective NEMO which failed to restore TBK1 and IRF3 phosphorylation was confirmed through an in vitro study (Zeng et al. 2009; Fang et al. 2017). NEMO binds neither to TBK1 nor IKK ϵ (Chariot et al. 2002; Zhao et al. 2007). How NEMO manages to activate TBK1 and IKK ϵ is still not well known. Furthermore, in *C. catla*, NF- κ B mRNA expression was analyzed in both the bacterial-infected tissues and a gradual elevation was found. Similar result was exhibited in the tissues of *Cyprinus carpio* when infected with Gram-negative bacterium *Aeromonas sorbia* (Byadgi et al. 2018). Chen and Lin (2001) studied NF- κ B expression in liposaccharides stimulated murine J774 macrophages and depicted an abrogation in the NF- κ B activity and established the fact that NF- κ B helps in cellular signaling transduction. Fallahi-Sichani et al. (2012) also emphasized that the containment of bacteria in the granuloma level can be achieved with a balance between the NF- κ B-mediated bacterial killing activities and the NF- κ B-mediated anti-inflammation. This balance can be controlled with the combination of molecular scale biochemical process involving IKK activity and stability of mRNA transcripts associated with NF- κ B-mediated responses.

The Gram-positive and Gram-negative bacteria-derived products such as PGN and LPS, respectively, were used to stimulate kidney and spleen cell culture. The role of NF- κ B signaling pathway via B-cell regulation was investigated using NF- κ B inhibitor (BAY 11-7082). Karki and Igwe

(2013) established that TLR4 induces NF- κ B signaling in human embryonic kidney-293 (HEK-293) cell line and TLR4 signaling is induced in the presence of irreversible inhibitor (BAY11-7082) which inactivates NF- κ B. These results indicated essential anatomical and physiological changes with respect to the activation of immune system in animals. Patel et al. (2019) reported that the TLR signaling recruits adaptor proteins such as MyD88, TICAM, TRAF6 and NF- κ B leading to the synthesis of cytokines and immunoglobulins. The contribution of MAPK (ERK) and NF- κ B cell signaling pathways in TLR-induced augmentation of inflammatory pathway is well documented in several studies (Zaru et al. 2007; Yang et al. 2015; Patel et al. 2019). Thus, the role of NF- κ B in *C. catla* was investigated in the present study to decipher the TLR-induced intracellular signaling. Stimulation of lymphoid organs through various pathogens is known to produce cytokines which connect the innate and adaptive immunity. These cytokines help in the regulation of B-cells via the induction of TLR pathway. This is well established in the MyD88-deficient mice (Enghard et al 2007; Mackay and Schneider 2009; Granja et al. 2017).

In *C. catla*, when NF- κ B was inhibited with BAY 11-7082 treatment in the presence of ligands PGN and LPS, it was found that the mRNA transcriptomic expression of the entire downstream signaling molecule was reduced. Similar study was performed by Samanta et al. (2012) which showed the inhibition of NF- κ B expression in luciferase activity in *L. rohita*. Several reports (Pasare and Medzhitov 2005; Rawlings et al. 2012) suggested that TLR-induced cytokine activation provokes B cell proliferation and subsequent B cell differentiation corroborating with the present study. Several other studies reported that NF- κ B inhibitor (BAY 11-7082) restrains κ B kinase (IKK) which targets the pathways to identify the responses of the signaling molecules (Samanta et al. 2012; Strickson et al. 2013). BAY 11-7082 strongly suppresses TNF- α , translocation of p65 (major subunit of nuclear factor- κ B), and its upstream signaling events such as phosphorylation of I κ B α , IKK, and Akt molecules. However, BAY 11-7082 neither inhibits IKK-related kinases (IKK α , IKK β) nor activates the IRAK1 and IRAK4 (Strickson et al. 2013). Cell death was induced in leukaemic and lymphoma cells in the presence of this inhibitor. It also prevents the recruitments of protein to the DNA damage sites by inhibition of ubiquitin complex rather than inhibiting NF- κ B. BAY 11-7082 also played the role in inhibition of NALP3 inflammasome along with combined inhibition of NF- κ B, JNK and other branches of MyD88 pathway by targeting the components of ubiquitin system (Juliana et al. 2010). The present study corroborates with the report of *L. rohita*, where the inhibitory treatment with BAY 11-7082 had proved the blockage of IL-8, but no such changes were visualized in the TLR receptor (Samanta et al. 2012). TLR induction leads to activation of inflammatory

cytokines in a dose-dependent manner in mice (Ojaniemi et al. 2003), human and fish (Schaefer et al. 2004; Bird et al. 2005; Purcell et al. 2006) proving the role of NF- κ B in the inflammatory responses. Thus, the present study elucidates the importance of NF- κ B signaling cascade in IgD synthesis in the Indian major carp, *Catla catla*.

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Author contributions SD and MS designed the experimental plan, analysed the data, interpreted the data and finalized the manuscript. RB executed the experimental work, accumulated the data and drafted the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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