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Toll like receptor induces Ig synthesis in *Catla catla* by activating MAPK and NF-κB signalling

Bhakti Patel[a], Rajanya Banerjee[a], Madhubanti Basu[b], Saswati S. Lenka[b], Mahismita Paichha[b], Mrinal Samanta[b], Surajit Das[a,*]

[a] Laboratory of Environmental Microbiology and Ecology (LEnME), Department of Life Science, National Institute of Technology, Rourkela, 769 008, Odisha, India
[b] Fish Health Management Division, Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, 751002, Odisha, India

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ABSTRACT

The molecular crosstalk of proximal innate immune receptor signaling mediated by Toll-like receptors (TLRs) is crucial in generating an adaptive immune response. The extracellular-signal regulated kinases (ERK) participate in propagating intracellular signals initiated by stimulated TLRs to transcription factors eliciting cytokine release. Although ERK signaling has been extensively studied in mammalian counterparts, very little is known about its existence in carps and its role in augmentation of immunoglobulin (Ig) synthesis. Therefore, to gain insights into the efficacy of MAP kinase cascade in orchestrating fish antigen receptor generation, *Catla catla* fingerlings were induced with various TLR agonists or pathogen associated molecular patterns (PAMPs). Analysis of upstream signaling events revealed that PAMPs stimulated the tissues leading to a significant upregulation ($P < 0.001$, One-way ANOVA) of different TLRs (TLR2, TLR3, TLR4 and TLR5) followed by activation of MyD88 dependent and independent pathway. Activation of ERK and NF-κB mediated cytokine production consequently triggered the enhanced expression of IgZ and IgM as was evident by qRT-PCR analysis, flow cytometry, immunoblotting and ELISA. Pretreatment with ERK inhibitor (UO126) antagonized PAMPs mediated TLR stimulation, leading to sequential downregulation of MyD88/NF-κB/cytokines via interrupting ERK/NF-κB signaling axis. Together these results demonstrate that TLR stimulation triggers IgZ and IgM production via activation of ERK and NF-κB in *C. catla* indicating that NF-κB mediated cytokine production and ERK1/2 signaling is not only functional in fish, but may be crucial for generation of Ig repertoire in lower vertebrates.

1. Introduction

The emergence of adaptive immunity is predominantly the most remarkable hallmark in the vertebrate evolution and is believed to have arisen in order to combat with increasing incidences of microbial threats (Trowsdale and Parham, 2004; Flajnik and Kasahara, 2010). The first line of host defence against the encountered pathogen is however, triggered by the proximal elements of innate immunity (Medzhitov and Janeway, 2000). Stimulation of prototypical innate immune receptors like Toll-like receptors (TLRs) and NOD-like receptors (NLRs) is known to play a crucial role in eliciting an adaptive immune response by triggering generation of immunoglobulin repertoire (Yang et al., 2015; Basu et al., 2016). In mammals, the transduction of these intracellular signals from stimulated receptors at one end to effector cells in the nucleus requires recruitment of several cellular signaling pathways like MAP kinase, PI3K/mTOR and NF-κB (Takeda and Akira, 2004; West et al., 2004; Zaru et al., 2007). Therefore, the crosstalk between innate and adaptive immunity is very crucial for synchronizing a targeted immune response against pathogenic infiltrates.

Similar to mammalian counterparts, teleosts activate TLR signaling in sentinel cells by recognizing unique molecular signatures of microbes popularly known as pathogen associated molecular patterns (PAMPs) like peptidoglycans, zymosan, ds-RNA, lipopolysaccharide and flagellin (Kawai and Akira, 2010; Rebl et al., 2010). PAMP-TLR interaction induces receptor oligomerization and subsequently stimulates intracellular signaling cascade through recruitment of two types of adaptor signaling pathways, myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathway (McGettrick and O'Neill, 2004). Synergistic activation of intracellular MAP kinase pathway and degradation of Iκβ are known to provoke activation of transcription factors like activator protein (AP-1) and NF-κB respectively, which in turn triggers pro-inflammatory cytokine production in B and T cells leading to B cell proliferation (Takeda and Akira, 2004; Mogensen,

* Corresponding author.
E-mail addresses: surajit@nitrkl.ac.in, surajit@myself.com (S. Das).

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2009). However, unlike mammals, teleosts lack bone marrow and lymph nodes that are pivotal centres for B cell lymphopoiesis and cell-mediated immune responses respectively. B cell differentiation and maturation apparently occurs in teleost anterior kidney, while spleen hosts the putative site for lymphocyte mediated responses (Zwollo et al., 2005; Isogai et al., 2009; Sunyer, 2013). Till date, 21 different types of TLRs have been identified in various fish species and each of which differentially contribute to host defence (Troutman et al., 2012; Zhang et al., 2014; Qi et al., 2017; Zhiwei et al., 2017). Although, gene cloning and fundamental studies have greatly uncovered the role of TLR signaling in regulating innate immune system in teleosts (Purcell et al., 2006; Rebl et al., 2010), the mechanistic basis of TLR-induced immunoglobulin expression on exposure to various ligands is yet to be delineated. Therefore, studying molecular interplay of protective responses and intracellular signaling events in piscine lymphoid organs could provide deeper insights into the evolution and development of vertebrate immune system.

The mitogen-activated protein kinase pathway (MAPK) employs three conserved subgroups of serine/threonine kinase subfamily that include extracellular signal-regulated kinase (ERK), p-38 kinase and c-Jun amino-terminal kinase (Seger and Krebs, 1995; Garrington and Johnson, 1999). These subfamily of cytoplasmic kinases are capable of modulating other intracellular proteins and regulate vital functions like cell differentiation, apoptosis, proliferation and immune responses (Chen et al., 2001; Zhang and Dong, 2005; Arthur and Ley, 2013). Recently, the involvement of p-38 kinase and ERK signaling was demonstrated in PAMPs activated TLR signaling cascade in *Salmo salar* cultured phagocytes (Iliev et al., 2013). Additionally, Banerjee et al. (2014) had shown the crucial role of ERK 1/2 phosphorylation in provoking apoptosis in *Aeromonas*-induced head kidney macrophages. Although, enormous studies have been undertaken to elucidate ERK 1/2 signaling in amelioration of pathogenesis in mammals (De Luca et al., 2012; Chakraborty et al., 2014; Manjunatha et al., 2017), very little is known regarding its existence and role in formulating a complex immune response in Indian major carps.

In our previous study, we identified the novel immunoglobulin isotype, IgZ (Patel et al., 2016a) and IgD (Banerjee et al., 2017) in Indian major carp, *C. catla* and studied its tissue specific expression profile on bacterial and parasitic infection. In the present study, emphasis has been laid to investigate the crucial role of MAP kinase pathway during TLR induction and immunoglobulin synthesis in *C. catla*. *C. catla* fingerlings were stimulated *in-vivo* with various TLR agonists with and without ERK inhibitor (U0126).

2. Materials and methods

2.1. Chemicals

Phospho p44/42 MAPK (ERK1/2), Phospho-NF-κB p65, p44/42 MAPK (ERK1/2), NF-κB p65, β-actin, Anti-Rabbit IgG HRP-conjugated peroxidase and ERK inhibitor (U0126) were purchased from Cell Signaling Technology (USA). Anti-MyD88 (CT), Anti-TNF-α (IN) and Anti-TLR-3 (CT) were obtained from Eurogentec (Belgium). Rabbit polyclonal antibodies, anti-IgM (Basu et al., 2016) and anti-IgZ were in-house antibodies and synthesized from *Labeo rohita*. Anti-Rabbit IgG-FITC antibody, 3-Aminopropyl triethoxy silane, paraformaldehyde and Tetra-Methyl Benzidine (TMB) substrate were procured from Sigma-Aldrich (USA). Amersham ECL Western Blotting Detection kit was purchased from GE Healthcare (Germany) and 4′, 6-diamidino-2-phenylin-dole (DAPI) from Himedia (India).

2.2. Experimental animal

Healthy catla fingerlings weighing ~100 g were obtained from the ICAR-Central Institute of Freshwater Aquaculture (CIFA), Kausalyaganga, Bhubaneshwar, India and were stocked in 500 L Fibre-reinforced plastic (FRP) tanks under continuous aeration. Animals were allowed to acclimatize for 4 weeks and were fed *ad libitum* with commercial carp diet twice daily. The optimum water temperature (28–30 °C) and pH conditions (7.4–7.6) were maintained along with routine water exchange throughout the experiment.

2.3. Pathogen infection

For bacterial challenge, *Aeromonas hydrophila* (ATCC 35,654) and *Streptococcus uberis* (ATCC 700,407) were obtained as lyophilised cells from Himedia (India) and were cultured in tryptic soy broth (Himedia, India) and Luria Bertani broth respectively at 37 °C for 18 h with constant shaking. Previous studies have shown that both the strains were pathogenic to carp (Patel et al., 2016a; Basu et al., 2012). The cultured broth was centrifuged at 1000 x g for 5 min and the pellet was washed twice in phosphate buffer saline (PBS) (pH 7.4) and was resuspended in it. Enumeration of bacterial cells was performed by determination of CFU/mL (Patel et al., 2016b).

2.4. In-vivo ligand and inhibitor treatment

To study the interaction of TLR and ligand interaction, *C. catla* fingerlings were divided into two separate groups. The first group of fish were treated with various TLR agonists- Peptidoglycan (PGN), Polyinosinic:polycytidylic acid (Poly I:C), Lipopolysaccharide (LPS) and Flagellin (Flag) via intra-venous route (Table 1A). Control group of fish in each case were treated with 100 µL of endotoxin free water. The second group of fish were pre-treated intra-peritoneally with ERK inhibitor (U0126). After 2 h incubation, the second group of fish were challenged separately with *A. hydrophila*, *S. uberis* and all TLR agonists (Table 1B). After 4 h and 8 h of TLR agonists treatment and 72 h of bacterial (*A. hydrophila* and *S. uberis*) infection, control and treated fish were sacrificed and blood, anterior kidney, spleen, gill and intestine were isolated for further experimental purposes.

Table 1
Experimental design (Treatment groups).

Group A: Bacterial and TLR agonists treatment
Control (C)
A. hydrophila infected at 8×10^6 CFU/100 gm of fish for 72 h (AH)
S. uberis infected at 2×10^6 CFU/100 g of fish for 72 h (SU)
Peptidoglycan infected at 100 µg/100 g of fish for 4 h (PGN 1)
Peptidoglycan infected at 100 µg /100 g of fish for 8 h (PGN 2)
PolyI:C infected at 600 µg /100 g of fish for 4 h (PolyI:C 1)
PolyI:C infected at 600 µg /100 g of fish 8 h (PolyI:C 2)
Lipopolysaccharide infected at 40 µg/100 gm of fish 4 h (LPS 1)
Lipopolysaccharide infected at 40 µg /100 gm of fish 8 h (LPS 2)
Flagellin infected at 2.5 µg/100 g of fish 4 h (Flag 1)
Flagellin infected at 2.5 µg/100 g of fish 8 h (Flag 2)
Group B: ERK inhibitor (U0126) and TLR agonists treatment
Control (C)
ERK inhibitor (U0126) stimulation only at 15 µM/100 g of fish (ERKI)
A. hydrophila infected at 8×10^6 CFU/100 gm of fish for 72 h (AH)
Pre-treated with U0126 + *A. hydrophila* infected at 8×10^6 CFU/100 gm of fish (AH + I)
S. uberis infected at 2×10^6 CFU/100 g of fish for 72 h (SU)
Pre-treated with U0126 + *S. uberis* infected at 2×10^6 CFU/100 g of fish (SU + I)
Peptidoglycan infected at 100 µg/100 g of fish for 8 h (PGN)
Pre-treated with U0126 + Peptidoglycan infected at 100 µg/100 g of fish (PGN + I)
PolyI:C infected at 600 µg/100 g of fish 8 h (PolyI:C)
Pre-treated with U0126 + PolyI:C infected at 600 µg/100 g of fish (PolyI:C + I)
Lipopolysaccharide infected at 40 µg/100 gm of fish 8 h (LPS)
Pre-treated with U0126 + Lipopolysaccharide infected at 40 µg/100 gm of fish (LPS + I)
Flagellin infected at 2.5 µg/100 g of fish 8 h (Flag)
Pre-treated with U0126 + Flagellin infected at 2.5 µg/100 g of fish (Flag + I)

Table 2
Primers used for cloning and real-time PCR study.

Primers	Sequence 5'→3'	T_m°C	Amplicon size (bp)	Accession number
TLR2 FW	TCTTGATTGGCTCGAGAAGC	50	306	KY089042
TLR2 RV	CATCCTCCATTTGCCCTATGT			
TLR3 FW	GCTCCACAGGGTTGAAGACA	53	310	MF766464
TLR3 RV	GCACGGCCAAGCTTTAGAAT			
TLR4 FW	TAGCATCCCACTTCATGTTC	60	374	KY089036
TLR4 RV	GCTGTCAATGTCAAAGTCTG			
TLR5FW	CCTCATGCTGATGCAACAAAC	51	346	MF766466
TLR5 RV	GTCTTCCTCACAGATGCTCTT			
MyD88 FW	CAGAGTGACTTCCAGTTTGTGC	51	245	KY089039
MyD88 RV	CCACCATCCTCTTGCACCTT			
TRAF6 FW	CAGTTGACAATGAGGTGCTG	53	328	MF766465
TRAF6 RV	CACACTGTATTGGCGAAAGG			
ERK FW	CAACAGACCCATCTTCCCTG	57	318	KY089038
ERK RV	GTGAAGGGTTCCTCAGCTAC			
NF-B FW	TTTACAGGAGCGGCGGATAC	59	453	KY089040
NF-B RV	GTGCGAAACACGATAGCCAC			
TNFα FW	CCAGGCTTTCACTTCAGG	50	181	FN543477
TNFα RV	GCCATAGGAATCGGAGTAG			
IL10 FW	CGCAGTGCAGAAGAGTCGAC	57	310	GU256643
IL10 RV	CCCGCTTGAGATCCTGAAATAT			
IgM FW	TCATGATGATAAAGATGTAATGCGT	55	165	MG859932
IgM RV	TAATTTCCCGCCTTGTGCTC			
IgZ FW	AACCACAGACACCAACCCTG	57	265	KT808879
IgZ RV	AACAGTTTTCCCCGGTGTGT			
βactin FW	AGACCACCTTCAACTCCATCATG	55	200	EU184877
βactin RV	TCCGATCCAGACAGAGTATTTACG			

2.5. qRT-PCR assay

To evaluate the transcript expression profile of crucial TLR-induced signaling cascade, mRNA expression in various cell receptors, adaptors, transducers, transcription factors, cytokines and immunoglobulins was analysed by qRT-PCR. Total RNA was extracted using TRIzol reagent (Life Technologies, USA) following Patel et al. (2016b). The integrity of extracted RNA was checked by observing characteristic 28S and 18S rRNA bands on 1% agarose gel. 2 µg of RNA was reverse transcribed to cDNA by RevertAid cDNA synthesis kit (Thermo Scientific, USA) following manufacturer's instruction. Quantitative RT-PCR was carried out in Mastercycler ep realplex (Eppendorf, Germany) using 2X SYBR Select Mix (Applied Biosystems, USA). Each 10 µL of PCR reaction consisted of 2X SYBR green, specific forward and reverse primer (0.75 µM) and 1 µL cDNA as template and autoclaved Milli-Q water to a final volume of 10 µL. The standard cycling conditions were 50 °C for 2 min, initial denaturation of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 50–57 °C for 15 s and 72 °C for 1 min, followed by dissociation curve analysis to verify the amplification of a single product. The differential expression of immune-related genes and their amplification cycle conditions are given in Table 2. Relative expression of target genes was calculated using $2^{-\Delta\Delta CT}$ method taking β-actin as internal control (Pfaffl, 2001).

2.6. Immunoblotting

To determine the proteomic expression profile of TLR-induced signalling cascade, whole cell lysate of anterior kidney was isolated from treated *C. catla* fingerlings using RIPA lysis buffer following Rucka et al. (2013). The protein lysates were electrophoresed (100 V, 2 h) in 12% denaturing gel and thereafter, SDS separated proteins were transferred (50 V, 1.5 h) to PVDF membrane (GE Healthcare, Germany). Non-specific binding was blocked in PBS containing 5% BSA and 0.01% tween 20 for 1 h. The membranes were then immunoblotted with primary antibodies against Phospho p44/42 MAPK (ERK1/2), Phospho-NF-κB p65, p44/42 MAPK (ERK1/2), NF-κB p65, Anti-MyD88 (CT), Anti-TNF-α (IN), Anti-TLR-3 (CT), anti-IgM and anti-IgZ overnight at 4 °C followed by secondary anti-rabbit IgG HRP-conjugated peroxidise antibody incubation for 2 h at room temperature. Immunodetection was performed by chemiluminescent western blotting detection reagents (Amersham; GE Healthcare, Germany). The membranes were then stripped with 0.2 M NaOH for 10 min followed by washing, blocking, and incubation with control antibody.

2.7. ELISA

Wells of ELISA polystyrene plates were coated with 200 µL of isolated protein samples in triplicates for overnight at 4 °C following He et al. (2017). The microtitre plates were then rinsed with PBS twice and blocked with 2% BSA and 0.01% tween 20. The protein samples were incubated with primary antibody followed by secondary antibody as described previously. To start the reaction, 100 µL of a chromogenic substrate, TMB solution was added into the plates. The reaction was stopped by addition of equal volume of 2 M sulphuric acid to the plates and absorbance was measured at 450 nm in a microplate reader (Perkin Elmer, USA).

2.8. Flow cytometry

To analyse activated protein positive cell populations, the treated *C. catla* anterior kidney and spleen were processed for single-cell preparations following Abos et al. (2013) and with slight modifications. 100 mg of both the tissues were resuspended in 5 mL of Leibovitz-15 (L-15) medium supplemented with 5% FBS, 1% penicillin-streptomycin solution. Thereafter, the muscle layers were separated with the help of a sterilised blade and the cell clump was crushed and passed through a cell strainer (100 µm, Himedia). The single cell suspensions thus formed were evaluated under a microscope and were then digested in a mixture of 0.25% Trypsin-EDTA solution for about 30 min. After enzymatic digestion, the cells were washed with 5 mL of PBS and centrifuged at 1000 rpm for 5 min followed by blocking in chilled incubation buffer (1% BSA and 0.05% Triton X-100) for 1 h. Thereafter the cells were incubated with primary antibody for 3 h at room temperature followed by washing with PBS and incubation with Anti-Rabbit IgG-FITC antibody for 20 min A FACS Accuri equipped with a 100-mm nozzle was used (BD Biosciences, CA) for data acquisition and 10,000 cells were

acquired. The cell population was evaluated in FITC/FL1 channel using BD Accuri C6 software.

2.9. Confocal microscopy

In order to investigate NF-κB expression on TLR induction, frozen 5 μM sections of anterior kidney were thawed, air dried, and fixed in 4% paraformaldehyde solution following Yang et al. (2015). The fixed sections were washed with 1X PBS thrice and blocked thereafter with 1% BSA and 0.05% Triton X-100 in PBS for 1 h at room temperature. The kidney sections were washed with PBS thrice and thereafter stained with anti phospho-NF-κB antibody (Cell Signaling Technology, USA) for 2 h at room temperature followed by Anti-Rabbit IgG FITC antibody (Sigma-Aldrich) treatment for 1 h. For nucleus staining, the treated sections were exposed to DAPI for 15 min. followed by PBS washing and DPX mounting. The sections were observed under confocal microscope SP8 (Leica, Germany).

2.10. Statistical analysis

Data are represented as mean ± S.D. from 3 to 5 independent experiments. The data were analyzed using GraphPad Prism software (version 5.01) for determining statistical significance of difference between control verses treatment groups and among treatment conditions. Significance was determined by one-way analysis of variance (One-way ANOVA) with Tukey's post-hoc test for multiple comparisons. $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Stimulation of TLRs on bacterial and PAMPs treatment

To investigate the effect of bacterial and different TLR agonists treatment in immunologically relevant tissues of *C. catla*, two separate studies were undertaken. The first study was designed to examine the tissue specific mRNA expression pattern of TLR2, TLR3, TLR4 and TLR5 on stimulation with detrimental fish pathogens-*Aeromonas hydrophila* and *Streptococcus uberis* at indicated time points (Table 1A) in kidney, gill, spleen and intestine. qRT-PCR analysis revealed that the maximum induction of TLRs was observed post 72 h of bacterial infection, hence selected this time point for subsequent studies on TLRs (Supplementary file Fig. S1). The next step was to determine a comparative effect of stimulation with bacterial pathogens- *A. hydrophila* and *S. uberis* and a panel of TLR agonists- PGN, Poly I:C, LPS and Flag in lymphocytes and crucial primary and secondary lymphoid organs in fish, kidney and spleen. Significant increase ($P < 0.05$) in the transcript expression levels of TLR2, TLR3, TLR4 and TLR5 was observed (Fig. 1A-D) suggesting that *C. catla* kidney tissues can respond to TLR agonists. TLR2 expression was observed to be considerably high in PGN treated tissue after 8 h followed by *S. uberis* treatment post 72 h (Fig. 1A), whereas TLR3 mRNA levels were relatively peaked in Poly I:C treated tissue (Fig. 1B). Interestingly, TLR4 was found to be significantly upregulated (47.9-fold, $P < 0.001$) after 8 h of LPS treatment, followed by PGN treated *C. catla* group (13.3-fold, $P < 0.001$) (Fig. 1C). Relatively higher transcript expression of TLR5 was recorded in flagellin treated tissues ($P < 0.01$) followed by *A. hydrophila* infection ($P < 0.05$) (Fig. 1D). To confirm qRT-PCR findings, both ELISA and immunoblotting assay were performed post 8 h of treatment conditions to study TLR3 protein expression. As depicted in (Fig. 1E), ELISA measured higher TLR3 protein levels in cell lysates isolated from Poly I:C treated kidney tissue as compared to control. However, relatively lower TLR3 expression was recorded in other treatment conditions. Further, immunblotting result (Fig. 1F) corroborated with the qRT-PCR and ELISA findings. Flow cytometric analysis exhibited a significant difference ($P < 0.05$) in the percent of $TLR3^+$ cells in Poly I:C treated kidney cell suspensions as compared to bacterial and other TLR agonists stimulation, validating the protein expression results (Fig. 1G). Similar ELISA and flow cytometric results were obtained in lymphocytes and spleen on estimation of TLR3 expression in titres and cell suspensions (Supplementary file Fig. S2), indicating endosomal TLR3 is a cognate receptor of ds-RNA analogs like Poly I:C in *C. catla* like mammalian counterparts.

3.2. TLR induction triggers MyD88 dependent and independent pathway

It is well established that TLR-induced signaling leads to recruitment of either MyD88, TICAM1 or both the adaptor based signal transduction. When checked for tissue specific mRNA expression profile of MyD88 on bacterial treatment, significant upregulation ($P < 0.001$) was recorded post 72 h of infection in almost all four immunologically relevant tissues mentioned earlier (Supplementary file Fig. S3 A, S3B). Next step was to investigate which type of adaptor signaling is activated on differential TLR induction in kidney, MyD88 mRNA, corresponding protein expression and TICAM1 mRNA levels were evaluated in bacterial and TLR agonists treatment groups. qRT-PCR analysis revealed significant upregulation of MyD88 of around 19.0-fold in flagellin treated (Flag 2, $P < 0.001$) kidney samples, followed by 18.04-folds in (PGN 2, $P < 0.001$) after 8 h treatment (Fig. 2A). As depicted (Fig. 2B), MyD88 protein levels were considerably higher in all the treatment groups in relation to control. However, Poly I:C treated samples showed relatively lower MyD88 expression as compared to bacterial and other ligand treated groups. Flow cytometric analysis correlated the mRNA and protein results exhibiting significant increase in $MyD88^+$ cell population upto 19.3% on PGN stimulation followed by 15.7% and 11.6% on Flag and LPS stimulation post 8 h respectively (Fig. 2C). Equal levels of increase in $MyD88^+$ cell population was recorded in bacterial treated groups as compared to control, whereas no significant change was measured in Poly I:C treated cell suspensions. Additionally, both lymphocytes and spleen exhibited similar trend of MyD88 protein expression on analysis of ELISA and flow cytometric results (Supplementary file Fig. S3C-F). On the other hand, TICAM1 mRNA levels were found to be significantly upregulated in the Poly I:C treated group (12.2-fold, $P < 0.001$) followed by relatively lower expression on LPS treatment (Fig. 2D). As anticipated, relatively low expression of TICAM1 was recorded in bacterial and other treatment groups.

3.3. TLR-mediated innate immune signaling stimulates MAPK (ERK) signaling pathway

To clarify whether TLR-induced signaling cascade is dependent on MAP kinase (MAPK) pathway, both the phosphorylated and total ERK protein expression levels were determined. Among the several downstream events associated with TLR induction, TNF receptor-associated factor (TRAF6) is considered an essential signal intermediate involved in cytokine production. Therefore, a time dependent kinetics of tissue specific TRAF6 and ERK mRNA expression on bacterial stimulation was evaluated by qRT-PCR analysis (Supplementary file Fig. S4). A comparative study of ERK transcript expression in kidney post bacterial and TLR agonists treatment at indicated time points (treatment group 1) demonstrated that ERK expression was considerably higher in all the treatment groups and significantly increased ($P < 0.001$) with the increasing ligand stimulation time (Fig. 3A). However, the results indicated that TLR agonists predominantly aggravated ERK expression with respect to control as compared to (AH) and (SU) treatment. ELISA results depicted a dramatic increase in the phospho-ERK (pERK) levels in all the treatment conditions with significant upregulation in flagellin (Flag) ($P < 0.01$) followed by peptidoglycan (PGN) treated group ($P < 0.01$) in kidney (Fig. 3B), though no changes in total-ERK levels with respect to control were recorded (Supplementary file Fig. S5). Immunoblotting assay further corroborated with the qRT-PCR data, indicating relatively higher pERK expression in each of the ligand stimulated groups. In contrary, negligible changes in the total-ERK

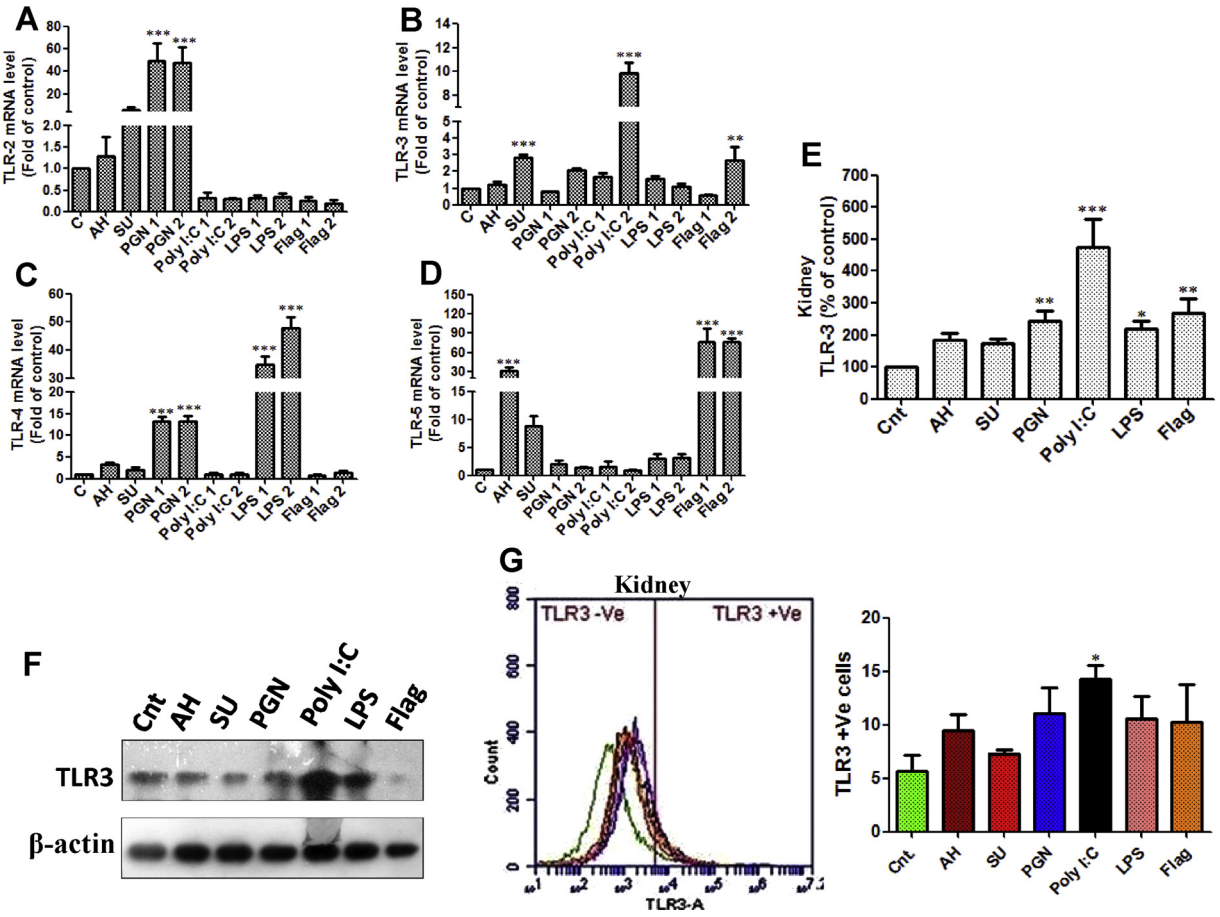

Fig. 1. TLR induction on PAMPs stimulation in *C. catla* kidney. qRT-PCR assay showing mRNA expression of (A) TLR2, (B) TLR3, (C) TLR4, (D) TLR5; where C-control; AH-*Aeromonas hydrophila* (1×10^6 CFU/100 g fish) after 72 h; SU-*Streptococcus uberis* (1×10^6 CFU/100 g of fish) after 72 h; PGN 1-Peptidoglycan (4 h incubation); PGN 2 (8 h of incubation); Poly I:C 1-(4 h incubation); Poly I:C 2-(8 h incubation); LPS 1-Lipopolysaccharide (4 h of incubation); LPS 2 (8 h of incubation); Flag 1-Flagellin (4 h of incubation); Flag 2 (8 h of incubation). TLR3 protein expression after 72 h of bacterial and 8 h of TLR agonists treatment was evaluated by (E) ELISA in terms of percent of control, and (G) Immunoblotting, where equal loading of samples was verified by accessing β-actin levels, and (H) $TLR3^+$ cell population detection by flow cytometric analysis in indicated treatment conditions. Data was represented as mean ± S.D. (bars in the graph), n = 3. Statistical analysis with respect to calibrator was determined by Ono-way ANOVA with $***P < 0.001$, $**P < 0.01$ and $*P < 0.05$ as significance levels.

expression was observed, correlating ELISA result (Fig. 3C). Further, flow cytometric results exhibited a marked increase of pERK expression in Flag (16.9%) and PGN-stimulated kidney cell suspensions (15.8%) followed by LPS (13.8%) and Poly I:C (12.8%) treatment (Fig. 3D). As anticipated, no changes in the tERK expression in the cell suspensions with respect to control was recorded (Fig. 3E). Similar trend of pERK expression was recorded in spleen cell suspensions and lymphocytes, though protein expression levels were relatively higher in lymphocytes as compared to kidney and spleen (Supplementary file Fig. S5).

3.4. TLR-induced signaling cascade differentially activate NF-κB pathway

Since, both the MyD88 dependent and TICAM1 dependent pathway on TLR stimulation could lead to NF-κB activation and its subsequent translocation to the nucleus, pNF-κB expression at transcript and protein levels were evaluated. Time dependent mRNA expression study of NF-κB in different tissues demonstrated importance of kidney as a crucial organ for immune activation against bacterial infection (Supplementary file Fig. S6). Induction of kidney with indicated TLR agonists (treatment group 1, Table 1) showed significant upregulation ($P < 0.001$) of NF-κB expression, correlating with the TLR-induced ERK expression observed earlier (Fig. 4A). Protein expression results recorded by ELISA showed a marked increase of phosphorylated NF-κB (pNF-κB) in both bacterial and TLR ligand treated *C. catla* kidney (Fig. 4B). Relatively lower expression of pNF-κB was recorded post 8 h of Poly I:C stimulation as compared to PGN ($P < 0.001$), LPS ($P < 0.01$) and Flag ($P < 0.01$) treatment. Likewise, pNF-κB protein levels peaked in all the treatment groups particularly in all ligand stimulated lymphocytes and spleen. Similar to tERK expression, no noticeable difference in the total-NF-κB expression was observed as compared to untreated group (Supplementary file Fig. S6C-E). Consistent with ELISA and qRT-PCR data, immunostaining analysis of treated and untreated group of *C. catla* kidney cryosections showed marked increase in the immunofluorescence intensity of the pNF-κB levels after all the TLR ligand stimulation followed by bacterial infection as compared to control (Fig. 4C). As shown in Fig. 4D, flow cytometric results corroborated with transcript and protein expression levels. Both bacterial and ligand treatment followed similar trend of pNF-κB expression in lymphocyte fractions and spleen cell subsets with no significant changes in tERK expression (Supplementary file Fig. S6).

3.5. Activation of cytokine expression on TLR-induction

To elucidate the contribution of TLR-induced ERK and NF-κB activation in modulating inflammatory pathway, the expression of various cytokines and enzymes associated with inflammation (TNF-α, IL-10, COX-2 and iNOS) were examined. *A. hydrophila* and *S. uberis* stimulation induced significant upregulation ($P < 0.05$) of TNF-α, COX-2, iNOS and downregulation ($P < 0.01$) of IL-10 in time dependent and tissue specific mRNA expression kinetics (Supplementary file Fig. S7).

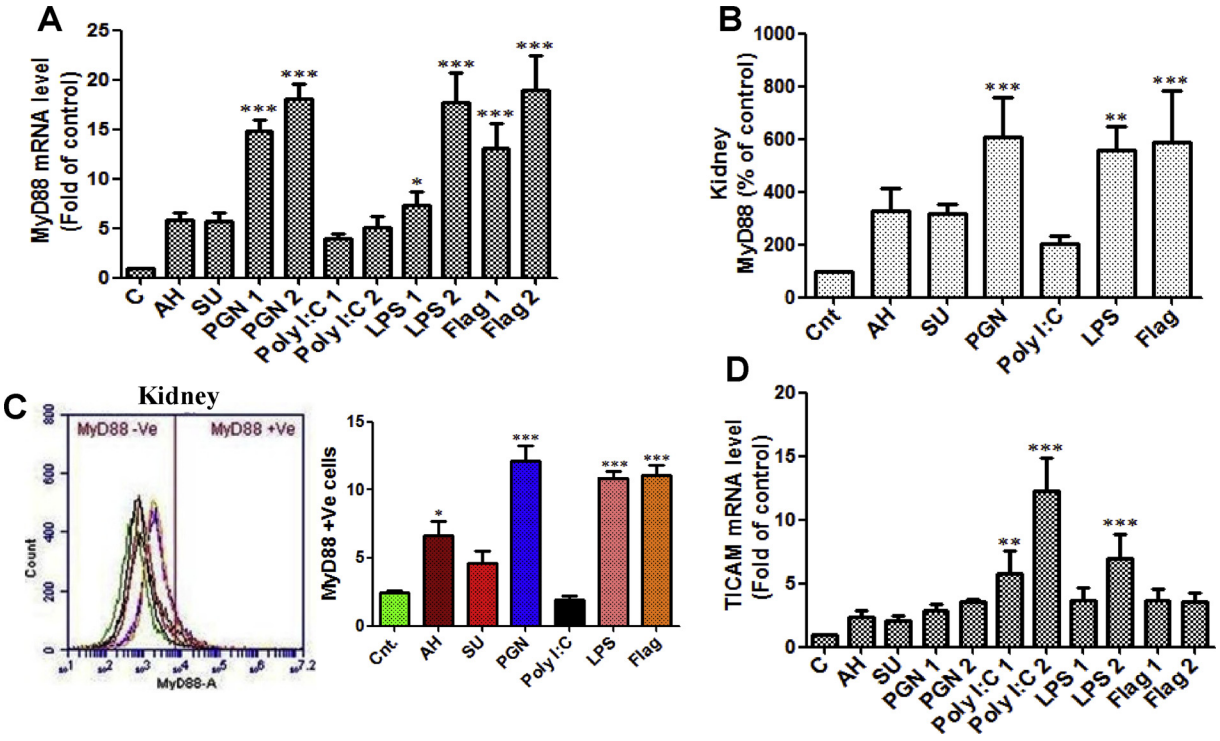

Fig. 2. Stimulation of MyD88 dependent and independent pathway on TLR induction in *C. catla* kidney. (A) qRT-PCR analysis showing mRNA expression of MyD88, where C-control; AH-*A. hydrophila*; SU-*S. uberis*; PGN 1-Peptidoglycan (4 h incubation); PGN 2 (8 h of incubation); Poly I:C 1-(4 h incubation); Poly I:C 2-(8 h incubation); LPS 1-Lipopolysaccharide (4 h of incubation); LPS 2 (8 h of incubation); Flag 1-Flagellin (4 h of incubation); Flag 2 (8 h of incubation), (B) ELISA showing MyD88 protein expression, (C) Flow cytometric analysis showing MyD88$^+$ cell fractions, and (D) qRT-PCR analysis evaluating TICAM1 mRNA expression on bacterial and TLR agonists exposure. Data was represented as mean ± S.D. (bars in the graph), n = 3. Statistical analysis with respect to control was determined by One-way ANOVA with $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ as significance levels.

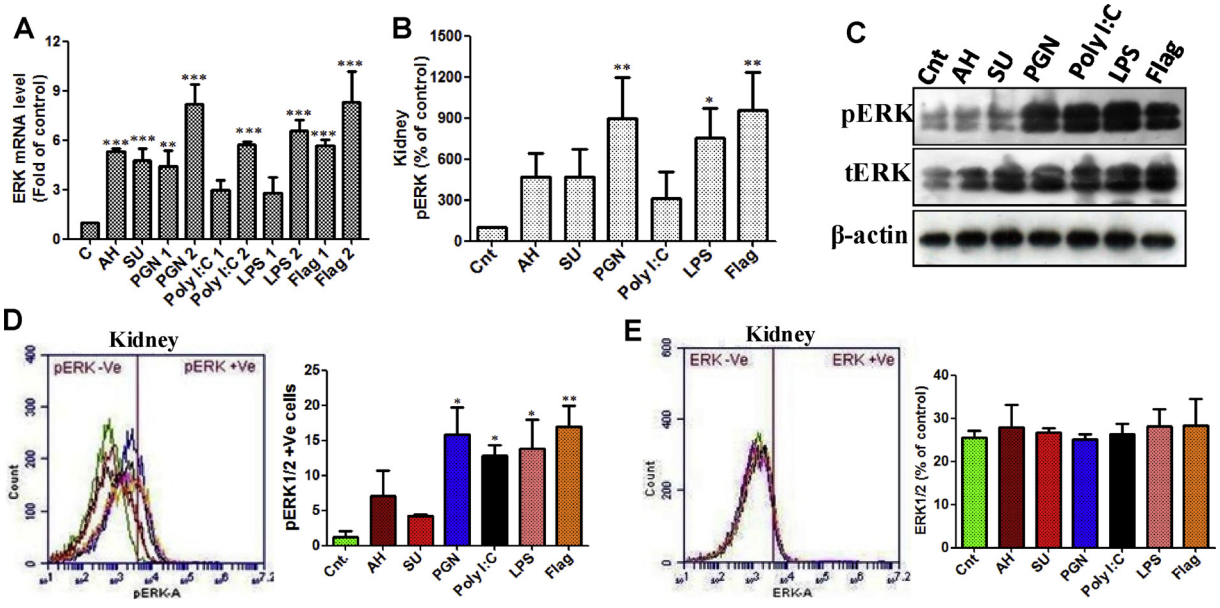

Fig. 3. TLR induction triggers ERK signaling pathway in *C. catla* kidney. (A) Quantification of ERK levels post stimulation of TLR-induced pathway indicating increased ERK mRNA expression in all treatment conditions, where C-control; AH-*A. hydrophila*; SU-*S. uberis*; PGN 1-Peptidoglycan (4 h incubation); PGN 2 (8 h of incubation); Poly I:C 1-(4 h incubation); Poly I:C 2-(8 h incubation); LPS 1-Lipopolysaccharide (4 h of incubation); LPS 2 (8 h of incubation); Flag 1-Flagellin (4 h of incubation); Flag 2 (8 h of incubation). Detection of phosphorylated ERK (pERK) protein expression after 72 h of bacterial and 8 h of TLR agonists treatment by (B) ELISA, and (C) Immunoblotting analysis, where equal loading of treated samples was verified by accessing total ERK (tERK) and β-actin levels. Flow cytometric analysis of catla kidney exposed to 72 h of bacterial and 8 h of TLR agonists showing cell populations of (D) pERK, and (E) tERK. Data was represented as mean ± S.D. (bars in the graph), n = 3. Statistical analysis with respect to control was determined by One-way ANOVA with $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ as significance levels.

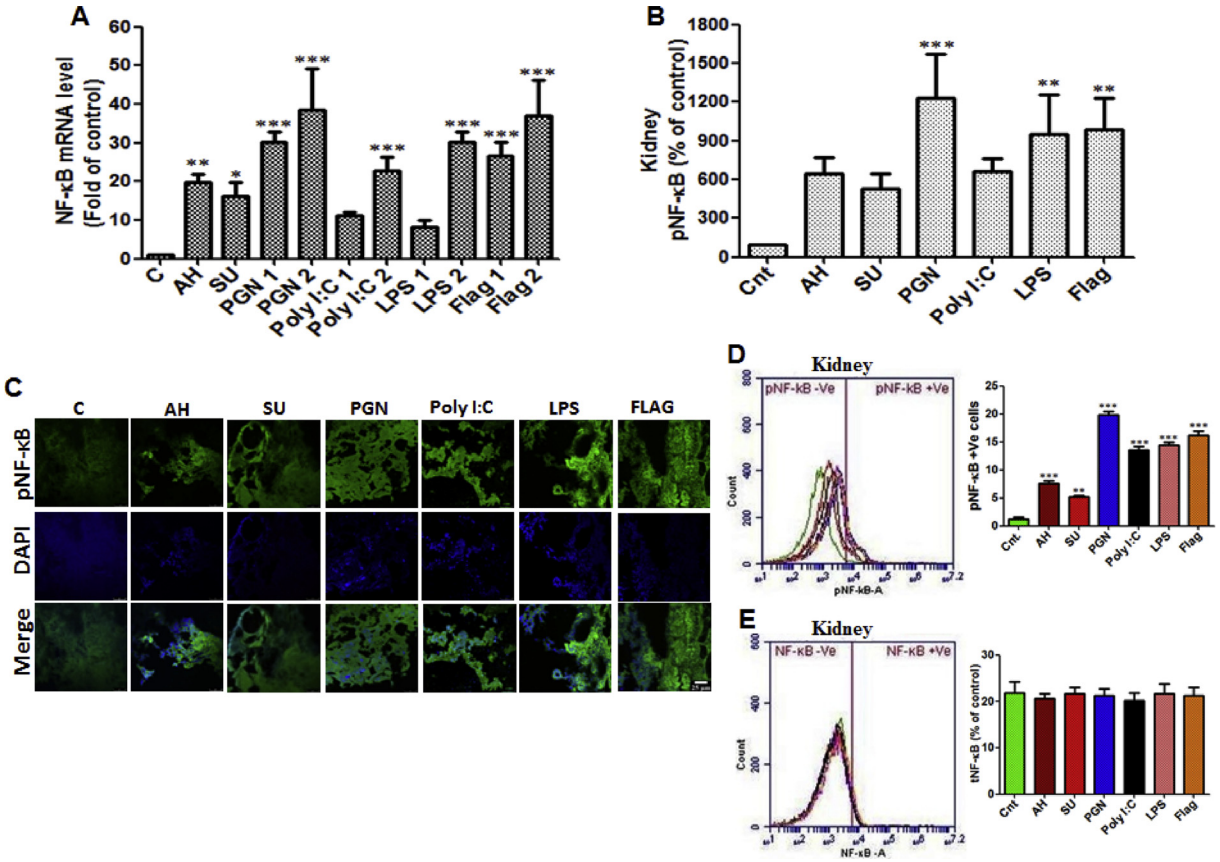

Fig. 4. TLR stimulation differentially activates NF-κB signaling in *C. catla* kidney. (A) qRT-PCR assay showing dramatic increase in NF-κB mRNA levels post bacterial and TLR agonists treatment conditions, where C-control; AH-*A. hydrophila*; SU-*S. uberis*; PGN 1-Peptidoglycan (4 h incubation); PGN 2 (8 h of incubation); Poly I:C 1-(4 h incubation); Poly I:C 2-(8 h incubation); LPS 1-Lipopolysaccharide (4 h of incubation); LPS 2 (8 h of incubation); Flag 1-Flagellin (4 h of incubation); Flag 2 (8 h of incubation), (B) ELISA showing protein expression of phosphorylated NF-κB (pNF-κB) on bacterial and TLR agonists treatment in terms of percent of control, (C) Confocal microscopy analysis of catla kidney after 8 h of indicated bacterial and TLR agonists treatment showing increased expression of NF-κB in all the treatment groups. DAPI was used to counterstain nucleus (blue). Scale Bar = 25 μm. Flow cytometric analysis illustrating expression of (D) pNF-κB, and (E) tERK on 72 h of bacterial and 8 h of TLR agonists treatment. Data was represented as mean ± S.D. (bars in the graph), n = 3. Statistical analysis with respect to control was determined by One-way ANOVA with $***P < 0.001$, $**P < 0.01$ and $*P < 0.05$ as significance levels (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Induction of kidney with a panel of indicated TLR agonists significantly upregulated TNF-α expression ($P < 0.01$) in a time dependent manner (Fig. 5A). As depicted in Fig. 5B, ELISA analysis revealed relatively higher TNF-α protein levels in all the TLR agonists stimulated groups as compared to bacterial treated groups. Flagellin (Flag) induction witnessed dramatic increase ($P < 0.001$) in TNF-α expression followed by PGN ($P < 0.01$), LPS ($P < 0.01$) and Poly I:C ($P < 0.05$) stimulation in kidney. Similar results were observed in FCM analysis exhibiting significant upregulation ($P < 0.05$) of cytokine expression in all the treatment conditions (Fig. 5C). Additionally, both ELISA and flow cytometric analysis in lymphocytes and spleen revealed correlation with the findings obtained in kidney, indicating that induction of TNF-α expression might be an outcome of transactivation of ERK and NF-κB on stimulation with various TLR agonists (Supplementary file Fig. S8).

3.6. TLR-induction differentially stimulate immunoglobulins in C. Catla lymphoid organs

It was investigated whether NF-κB-induced cytokine expression is prerequisite to B cell activation and subsequent immunoglobulin expression. qRT-PCR analysis demonstrated considerable increase in IgM mRNA expression on PGN (24.8-fold, $P < 0.001$), Flag (18.3-fold, $P < 0.001$), Poly I:C (7.8-fold, $P < 0.05$) induction in kidney (Fig. 6A). In comparison to *S. uberis* (SU), *A. hydrophila* (AH) treatment witnessed significant upregulation of IgM expression ($P < 0.05$) post 72 h. However, SU treatment induced relatively higher IgZ mRNA expression as compared to (AH) stimulation (Fig. 6D). In response to TLR ligand treatment, IgZ expression followed the similar trend of mRNA induction as that of IgM. In contrary, relatively moderate IgM and IgZ expression were detected post 4 h of all TLR agonists and post 8 h of LPS-stimulation with respect to control. TLR agonists-stimulated *C. catla* kidney revealed significant upregulation of IgM ($P < 0.001$) and IgZ ($P < 0.01$) protein levels as compared to bacterial stimulation ($P < 0.01$; $P < 0.05$) respectively on ELISA analysis (Fig. 6B, E). Further, flow cytometric analysis revealed almost 32% increase in IgM^+ cell population on PGN treatment followed by 28.6% increase in flagellin stimulated cell suspensions (Fig. 6C). Additionally, Poly I:C ($P < 0.01$) and LPS ($P < 0.05$) treated samples recorded exactly similar amounts of IgM^+ cell fractions, which were significantly higher than that of control. Similarly, IgZ^+ cell population was found to be higher on PGN stimulation ($P < 0.001$) followed by flagellin ($P < 0.01$), Poly I:C ($P < 0.01$) LPS ($P < 0.01$) treatment as compared to control (Fig. 6F). Further, IgM and IgZ protein expression analysis in lymphocytes and spleen by ELISA and flow cytometry corroborated with results obtained in *C. catla* kidney (Supplementary file Fig. S9).

3.7. Inhibition of ERK and NF-κB signaling on U0126 treatment

To further elucidate that ERK signaling is crucial in TLR-induced

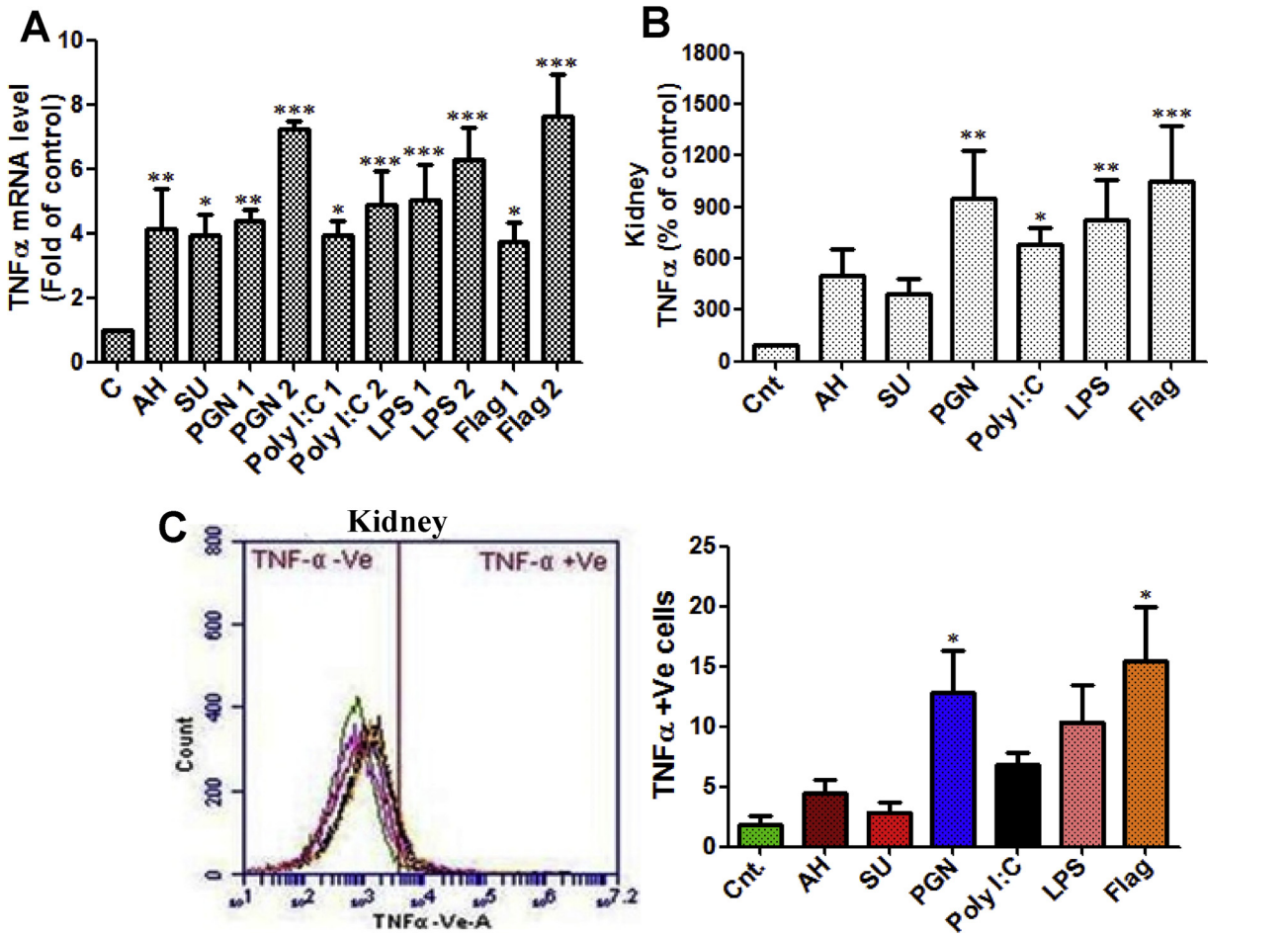

Fig. 5. Assessment of TLR-induced TNF-α expression in *C. catla* kidney. (a) qRT-PCR analysis depicting relative mRNA expression of TNF-α on bacterial and TLR agonists stimulation, where C-control; AH-*Aeromonas hydrophila*; SU-*Streptococcus uberis*; PGN 1-Peptidoglycan (4 h incubation); PGN 2 (8 h of incubation); Poly I:C 1-(4 h incubation); Poly I:C 2-(8 h incubation); LPS 1-Lipopolysaccharide (4 h of incubation); LPS 2 (8 h of incubation); Flag 1-Flagellin (4 h of incubation); Flag 2 (8 h of incubation), (B) Detection of TNF-α protein expression by ELISA post stimulation with bacteria and TLR agonists in terms of percent of control, and (C) FCM analysis potraying increased TNF-α expression in *C. catla* kidney cell suspensions after 72 h of bacterial and 8 h of TLR agonists treatment. Data was represented as mean ± S.D. (bars in the graph), n = 3. Statistical analysis with respect to control was determined by One-way ANOVA with $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ as significance levels.

and NF-κB mediated Ig secretion in *C. catla*, ERK and NF-κB stimulation was assessed in presence of ERK antagonist, U0126. Since, kidney is an essential haematopoietic organ involved in regulation of immune response in fish, subsequent experiments pertaining to inhibition study were carried out in *C. catla* kidney. qRT-PCR results revealed that U0126 pretreatment prior to bacterial and TLR agonists stimulation markedly reduced the mRNA expression of ERK in all conditions (Supplementary file Fig. S10 A). To validate the mRNA expression results, ELISA and immunoblotting assay were performed. A significant attenuation of phosphorylated ERK levels were observed in each of the U0126 pre-treated conditions as compared to infected conditions only on analysing ELISA results (Fig. 7A). Immunoblotting assay showed a marked reduction in the phosphorylated ERK (pERK) expression in inhibitor pre-treated, bacterial and ligand stimulated cell lysates (Fig. 7B). No changes in expression of tERK and β-actin in any of the treatment conditions with respect to control were determined. Further, U0126 pretreated LPS (LPS + I), PGN (PGN + I) and Flag (Flag + I) induced kidney cell suspensions detected considerably reduced ERK expression upto 7.9%, 6.1% and 5.5% respectively (Supplementary file Fig. S10C). Moderate level of ERK inhibition was observed in other indicated pretreatment groups.

To explore whether U0126 triggered ERK abrogation affects NF-κB signaling cascade, verification of phosphorylated and total NF-κB (pNF-κB, tNF-κB) expression on similar U0126 pretreatment and pathogen treatment conditions was determined. NF-κB mRNA expression was found to be alleviated in each of the U0126-pretreated samples except in case of ERK inhibited *S. uberis* induced (SU + I) treatment (Fig. 7C). Protein expression analysis exhibited dramatic reduction of pNF-κB in all the ERK inhibited treatment groups as assessed by ELISA (Fig. 7D). However, no change in the expression was recorded in control and only ERK Inhibitor (ERKI) treated samples, indicating U0126 is not conferring any change in the basal ERK levels. This suggested the crucial role of ERK in bacterial and TLR agonists-induced pathology. pNF-κB expression results obtained in kidney suspensions correlated with the findings of qRT-PCR and ELISA observed previously (Supplementary file Fig. S11B). Additionally, tERK and tNF-κB protein expression results determined by ELISA and flow cytometry exhibited no respective changes with respect to control (Supplementary file Fig. S10B; D, Supplementary file Fig. S11 A; C).

3.8. Abrogation of ERK-mediated augmentation of cytokine production on ERK inhibition

To investigate whether ERK and NF-κB inhibition is pre-requisite to cytokine production in catla kidney, mRNA expression of TNF-α was evaluated by qRT-PCR, ELISA and FCM analysis. U0126 pretreatment considerably downregulated TNF-α expression at mRNA levels in all treatment groups, which was correlated with the ERK and NF-κB

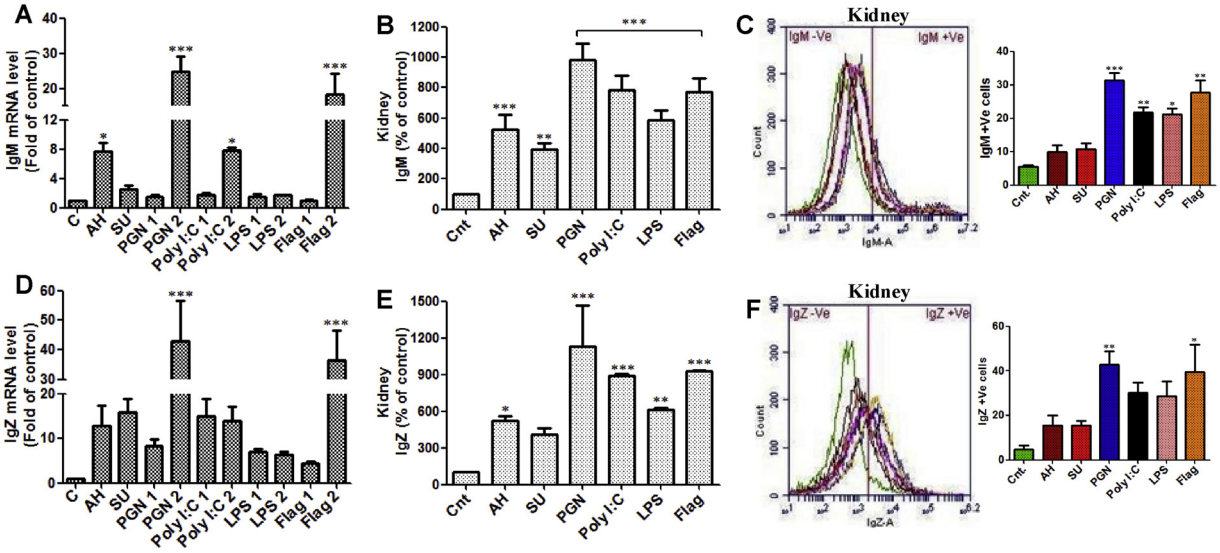

Fig. 6. Differential induction of immunoglobulin expression on TLR stimulation in *C. catla* kidney. Quantification of mRNA expression of (A) IgM, and (D) IgZ was monitored in all the treatment conditions, where C-control; AH-*A. hydrophila*; SU-*S. uberis*; PGN 1-Peptidoglycan (4 h incubation); PGN 2 (8 h of incubation); Poly I:C 1-(4 h incubation); Poly I:C 2-(8 h incubation); LPS 1-Lipopolysaccharide (4 h of incubation); LPS 2 (8 h of incubation); Flag 1-Flagellin (4 h of incubation); Flag 2 (8 h of incubation). ELISA showing protein expression of (B) IgM and (E) IgZ on bacterial and TLR agonists treatment in terms of percent of control. Flow cytometry analysis of (C) IgM^+ and (F) IgZ^+ *C. catla* kidney cell population in indicated treatment conditions after 8 h of stimulation. Data was represented as mean ± S.D. (bars in the graph), n = 3. Statistical analysis with respect to control was determined by One-way ANOVA with $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ as significance levels.

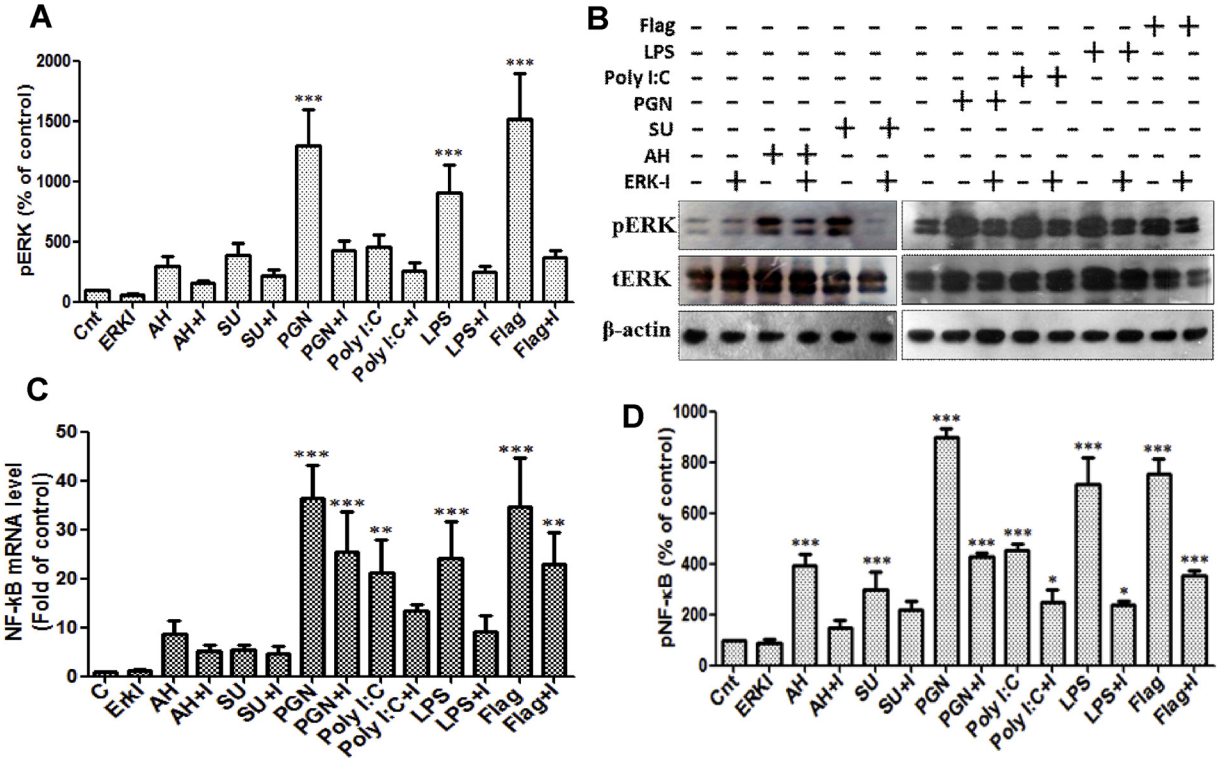

Fig. 7. Abrogation of TLR-induced ERK signaling on U0126 treatment in *C. calta* kidney. (A) Alleviation in phosphorylated-ERK (pERK) protein expression assessed by ELISA in terms of percent of control, (B) Immunoblotting analysis exhibiting inhibition of pERK protein expression in all the U0126 pretreatment conditions. Expression of tERK and β-actin was performed to verify equal loading of protein. (C) qRT-PCR analysis showing attenuation in transcript expression of NF-κB in all the U0126-pretreated conditions, and (D) Detection of phosphorylated NF-κB (pNF-κB) protein levels on ERK inhibition by ELISA in terms of percent of control, C-control; AH-*A. hydrophila*; SU-*S. uberis*; PGN Peptidoglycan; Poly I:C; LPS-Lipopolysaccharide, Flag-Flagellin after 8 h of treatment. Data was represented as mean ± S.D. (bars in the graph), n = 3. Statistical analysis with respect to control was determined by One-way ANOVA with $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ as significance levels.

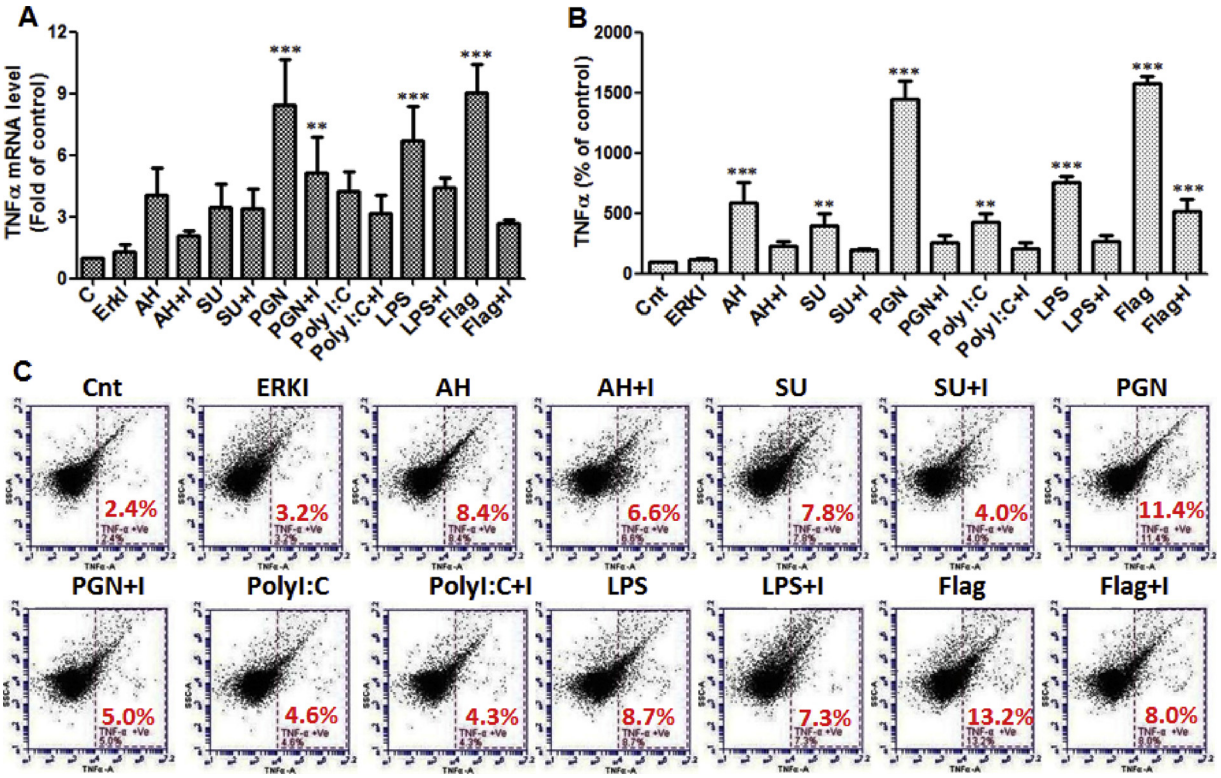

Fig. 8. ERK inhibition attenuates TLR-induced TNF-α expression in *C. calta* kidney. (A) Determination of TNF-α mRNA expression by qRT-PCR analysis illustrating alleviation in all ERK inhibitor (U0126) pretreated conditions, (B) ELISA showing effects of U0126 pretreatment in attenuating TLR-induced TNF-α protein expression in terms of percent of control, and (C) Monitoring TNF-α expression in U0126 pretreated and untreated kidney cell suspensions by FCM analysis, where C-control; AH-*A. hydrophila*; SU-*S. uberis*; PGN Peptidoglycan; Poly I:C; LPS-Lipopolysaccharide, Flag-Flagellin after 8 h of treatment. Data was represented as mean ± S.D. (bars in the graph), n = 3. Statistical analysis with respect to control was determined by One-way ANOVA with $***P < 0.001$, $**P < 0.01$ and $*P < 0.05$ as significance levels.

inhibition results observed previoulsy (Fig. 8A). In parallel, ELISA results showed that U0126 administration led to significant reduction in NF-κB-induced TNF-α production in each of pretreatment groups, (Fig. 8B). Consistently, flow cytometric analysis revealed that ERK-inhibited PGN (PGN + I) and Flag (Flag + I) treatment abrogated TNF-α expression more predominantly as compared to other treatment groups (Fig. 8C). Similar levels of protein expression of TNF-α were observed in control and ERK inhibitor treated kidney cell suspensions.

3.9. Alleviation in ERK-dependent immunoglobulin expression on U0126 treatment

In order to determine whether U0126-induced ERK inhibition, interrupts with immunoglobulin expression, a comparative study of mRNA and corresponding protein levels was performed. The expression analysis of the universal immunoglobulin isotype, IgM and newly identified isotype, IgZ before and after U0126 pre-treatment was carried out. qRT-PCR analysis exhibited considerable downregulation of IgM and IgZ in each of the ERK inhibitor pre-treated group as compared to only infected group of kidney. However, control and ERK inhibitor treated (ERKI) mRNA expressed similar levels of immunoglobulin expression (Fig. 9A, C). The results were confirmed at the protein levels by ELISA, which were consistent with the above findings (Fig. 9B, D). Further, flow cytometric results depicted that recorded levels of IgM^+ expression in kidney sell suspensions decreased to 5.2%, 3.8%, 23.8%, 5.2%, 6.8% in (AH + I), (SU + I), (PGN + I), (PolyI:C + I), (LPS + I), (Flag + I) respectively (Supplementary file S12 A). Similarly, IgZ^+ expression markedly reduced in each of U0126 pretreated groups as compared to their respective bacterial and TLR agonists treated groups (Supplementary file S12B). In contrary, no changes in the expression were observed in control and U0126 treated (ERKI) conditions, validating the mRNA and protein expression data.

4. Discussion

The critical role of TLR-induced MAPK and NF-κB signaling pathway in inflammation, regulation of tumorigenesis and orchestrating an immune response has been well established (Arthur and Ley, 2013; Yang et al., 2015; Udden et al., 2017). However, the molecular interplay of cellular signaling events regulating primordial adaptive immunity is poorly understood in the paraphyletic group of fish (Flajnik and Kasahara, 2010). The seminal discoveries about the functioning of teleost immune system has contributed in providing insights into the evolution of vertebrate immune system and elucidating new paradigms in mammalian immunology (Sunyer, 2013). Given the central role of TLRs in initiating an adaptive immune response, the purpose of the present study is to elucidate- (i) bacterial and TLR agonists stimulated TLR signaling pathway in various *Catla catla* lymphoid organs and lymphocytes, to speculate (ii) whether ERK pathway is functional and operative in fish and to understand (ii) the role of ERK in regulation of TLR-induced immune response against different pathogenic invasions. Hence, the present study provides the first evidence of the recruitment of TLR-triggered ERK signaling pathway in regulation of teleost immunoglobulin expression.

Recognition of PAMPs by cognate TLR receptors in the sentinel cells is a crucial event in activation of innate immune responses and inflammation (Banchereau and Steinman, 1998; Mogensen, 2009). In the present study, TLR2, TLR3, TLR4 and TLR5 were found to be broadly expressed in all the examined immune relevant tissues. However, TLR2 and TLR5 exhibited profound induction in kidney with respect to other tissues indicating the crucial haematopoietic functions of catla kidney in absence of bone marrow in teleosts. Similar report of tissue specific

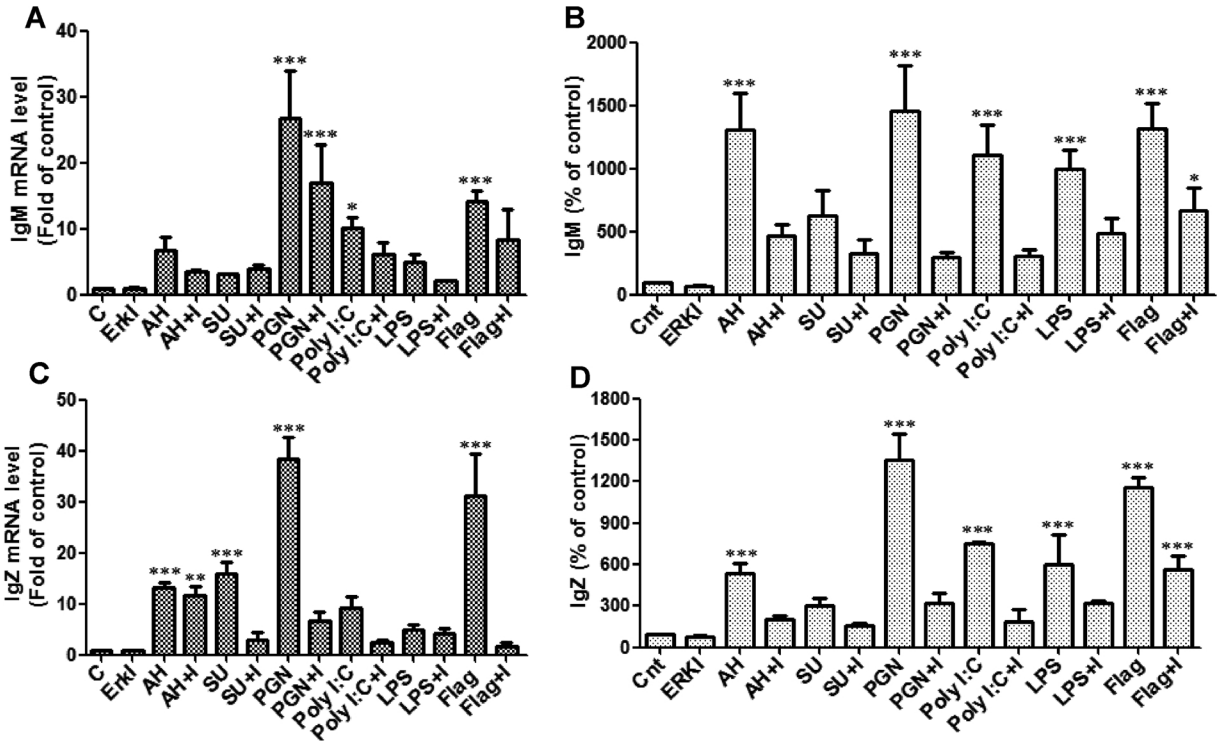

Fig. 9. ERK inhibitor (U0126) pretreatment interrupts the TLR-induced immunoglobulin expression in *C. catla* kidney. qRT-PCR analysis indicating mRNA expression of (A) IgM, and (C) IgZ in the indicated treatment conditions. Control was taken as calibrator and relative expression of target genes were evaluated with respect to calibrator. ELISA showing protein expression of (B) IgM and (D) IgZ after ERK inhibitor (U0126) pre-treatment and pathogen treatment conditions, where C-control; AH-*A. hydrophila*; SU-*S. uberis*; PGN Peptidoglycan; Poly I:C; LPS-Lipopolysaccharide, Flag-Flagellin after 8 h of treatment. Data was represented as mean ± S.D. (bars in the graph), n = 3. Statistical analysis with respect to control was determined by One-way ANOVA with $***P < 0.001$, $**P < 0.01$ and $*P < 0.05$ as significance levels.

distribution of TLR22 was observed in orange-spotted grouper (Ding et al., 2012). Further, comparatively higher levels of TLR mRNA expression were recorded on PAMPs treatment in catla kidney on stimulation with their respective cognate ligands with respect to bacterial treatment. In contrary, the transcript expression of TLR4 was slightly enhanced on PGN treatment. Earlier studies in mice have demonstrated the wider specificities of TLR4 towards recognizing viral motifs and plant products along with bacterial motifs (Kawasaki et al., 2000; Kurt-Jones et al., 2000). Most commercially available LPS (if not ultrapure) are usually contaminated by either other bacterial components like lipoproteins or peptidoglycans and are reported to stimulate both TLR2 along with TLR4. Therefore, ultrapure LPS is greatly encouraged over other purity levels. However, in the present study we observed dramatic increase in the expression of TLR4 alone on LPS treatment with no significant change in TLR2 expression. The LPS used in the present study was previously reported to stimulate TLR4 (Zhang et al., 2009; Yang et al., 2014; Clift et al., 2017; Müller et al., 2017; Papathanassiu et al., 2017). TLR4 and LPS interaction in fish species has not been clearly understood. Earlier reports suggested that few fish species lacked TLR4 gene while zebrafish TLR4 is unresponsive to LPS stimulation due to lack of co-stimulatory molecules CD14 and MD2 (Sullivan et al., 2009). However, several studies have recently indicated that LPS could recognize piscine TLR4 receptor and induce inflammatory signalling (Jiang et al., 2015; Giri et al., 2016; Samanta et al., 2017). The above results indicated that despite few crucial anatomical and physiological changes with respect to immune activation sites in fish, the PAMP-TLR affinities are conserved and much stronger adjuvants in fish TLR induction in comparison to pathogens. Therefore, the participation of TLRs during fish pathogenesis is much similar to that of mammalian counterparts in many aspects.

PAMPS recognition induces conformational changes in the TIR domain of TLRs that subsequently allows homo or heterophillic interaction of TIR domain with newly recruited adaptor proteins-MyD88, TICAM1, TICAM2 and TIRAP (Rebl et al., 2010). Therefore, in the present study MyD88 and TICAM1 expression were evaluated in bacterial and PAMP stimulated catla kidney. The results were largely consistent with the earlier findings implicating that MyD88 is a universal adaptor protein involved in the signal transduction of several TLRs including TLR2, TLR5 (Lee et al., 2011; Whang et al., 2011). Additionally, considerably higher mRNA expression of TICAM1 as compared to MyD88 on Poly I:C treatment correlated with the previous reports regarding the TLR3 affinity for TICAM1 adaptor signaling (Kawasaki and Kawai, 2014). However, the significant upregulation of both MyD88 and TICAM1 in LPS-treated kidney indicated that TLR4-induced signaling in fish is likely to follow both MyD88 dependent and independent pathways. Recently, Srivastava et al., (2017) have shown the critical role of TLR4-induced MyD88 dependent signalling in regulation of anti-inflammatory responses in zebrafish. Therefore, the above results indicate that both MyD88 dependent and independent pathways of TLR-induced signaling cascade together regulate activation of *C. catla* lymphocytes and lymphoid organs. Piscine homologues of cardinal elements (IRAK-4, TRAF-4, TRAF-6) involved in TLR signaling pathway have been identified in several fish genomes and EST databases (Kedinger et al., 2005; Phelan et al., 2005; Wei et al., 2014). The present study demonstrated the activation of TRAF6 tissue specific mRNA expression in response to bacterial treatment in *C. catla*. Similar expression of TRAF6 was observed in swimming crab, *Portunus trituberculatus* on LPS treatment (Zhou et al., 2015). Wei et al. (2014) showed the contribution of TRAF6 in response to various PAMPs treatment in *Epinephelus tauvina* and elucidated its involvement in shaping immune responses.

The contribution of MAPK (ERK) and NF-κB cell signaling pathways in TLR-induced augmentation of inflammatory pathway is well documented in several studies (Zaru et al., 2007; Yang et al., 2015). In order

to delineate the essential role of MAP kinase pathway in TLR-induced intracellular signaling in fish, the existence of ERK in *C. catla* was first ascertained by cloning and sequencing analysis (data not shown). Further, the time dependent qRT-PCR analysis of ERK and NF-κB mRNA levels showed elevation which was consistent with the earlier report (Chen and Lin, 2001). Mu et al., (2010) had previously reported that *Aeromonas hydrophila*-induced TLR signaling in large yellow croaker involves MAPK pathway. Consistent with the above report, phosphorylated ERK and NF-κB levels were found to peak in all the treatment groups. Earlier report suggested that ERK activation has consequently led to NF-κB activation in mice (Chandrakesan et al., 2010). Kang et al. (2010) have demonstrated the crucial contribution of p38-MAPK pathway in eliciting immune responses. It is well documented that the transcription factor NF-κB is a nuclear factor capable of binding to Ig kappa light-chain of pathogen encountered B cells (Sen and Baltimore, 1986). The tight regulation of NF-κB phosphorylation and ubiquitination is therefore required to prevent dysregulation of immune responses (Sun and Ley, 2008). Thus, the dramatic increase in the ERK and NF-κB phosphorylated levels observed in the present study implied their crucial role in the pathophysiology associated with TLR signaling pathway.

Stimulation of lymphocytes and lymphoid organs are known to induce the production of cytokines that are crucial in orchestrating the crosstalk between innate and adaptive immunity. It is likely that both ERK and NF-κB are able to synergize to augment TLR-induced cytokine stimulation. Herein, the present study corroborated with earlier findings that TLR induction culminates to activation of inflammatory cytokines in a dose dependent manner in humans (Schaefer et al., 2004), mice (Ojaniemi et al., 2003) and fish (Bird et al., 2005; Purcell et al., 2006). Additionally, a higher expression of the inducible nitric oxide synthase (iNOS) in bacterial treated *C. catla* was consistent with the earlier findings depicting that pathogen predisposition by TLRs involves activation of multivariant genes and proteins comprising of iNOS and anti-microbial peptides (Arthur and Ley, 2013). However, exuberant production of pro-inflammatory cytokines may culminate to immunopathological complications. In that context, the secretion of anti-inflammatory cytokines like TGF-β and IL-10 are documented to play a key role in negatively regulating TLR signalling (Liew et al., 2005). The present findings of IL10 transcript expression in various tissues post bacterial infection are in agreement with the above report. Further, aggravation of TNF-α expression post multivariant PAMPs and bacterial treatment clearly demonstrated its key role in inflammation. Taken together, the results indicate that carp TLRs can differentially potentiate activation of inflammatory cytokines.

Several studies suggested that TLR-induced cytokine activation provokes B cell proliferation and subsequent B cell differentiation (Pasare and Medzhitov, 2005; Rawlings et al., 2012). In that context, the present work reports the higher expression of IgM and IgZ in catla lymphocytes and lymphoid organs for the first time depicting that TLR-mediated TNF-α may have a role in B cell activation. Recently, Munawara et al. (2017) reported that cytokines could preferentially modulate expression of complement receptor immunoglobulin (CRIg) in human macrophages to regulate anti-microbial immunity. Interestingly, both IgM and IgZ mRNA and protein expression were found to be differentially regulated by various PAMPs induced TLR signaling. Thus, the results corroborated with previous research findings indicating that TLR-induced TNF-α expression may be responsible for differentially regulating immunoglobulin expression in teleosts.

Blocking MAPK pathway in sentinel cells have pointed towards the critical role of serine threonine kinase subfamily proteins of MAP kinase family in TLR and alternative B cell signaling (Richards et al., 2001). However, the relevance of these findings in primitive vertebrate class of fish needs to be defined. Therefore, to determine that TLR-induced MAPK pathway is crucial for immunoglobulin synthesis, PAMP-triggered phosphorylation of ERK and NF-κB activation was studied in presence of ERK inhibitor (U0126). The present study demonstrated that U0126 treatment inhibited ERK cellular signaling pathway, which in turn abrogated ERK-triggered cytokine stimulation. Further, the results reported for the first time that blocking ERK signaling resulted in amelioration of immunoglobulin expression in catla kidney. U0126 and PD98059 are extensively used in several studies as specific MEK inhibitors (Hotokezaka et al., 2002; Yap et al., 2011). Previous report in splenic B cells and immature B cell line, WEHi-231 have revealed that ERK inhibition could potentially interrupt with B cell signaling corroborating the present findings (Richards et al., 2001). Therefore, the results imply that TLR-ERK-NF-κB axis regulates stimulation of haematopoietic organs and cells for Ig secretion in freshwater fish, *Catla catla*.

5. Conclusion

The present study elucidates the crucial role of TLR induction in IgM and IgZ expression involving ERK and NF-κB signaling in Indian major carp, *C. catla*. Further, ERK inhibitor (U0126) pretreatment was found to alleviate the ERK/NF-κB stimulated cytokine and IgZ and IgM expression profile although not completely inhibiting it. This indicated that TLR-induced regulation of cytokine expression and augmentation of immunoglobulin synthesis in *C. catla* is enriched in a MAPK/ERK-dependent mechanism. Thus, the present study establishes ERK as an important therapeutic target and provides avenues for drugs or immunostimulants to modulate ERK signaling for facilitating enhancement of immunoglobulin synthesis in the paraphyletic group of fish.

Ethical statement

This article does not contain any studies with human participants and all experiments involving animals were performed in accordance with the approved guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Govt. of India.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2018.11.012.

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