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Multiple subtypes of TLR22 molecule from *Schizothorax prenanti* present the functional diversity in ligand recognition and signal activation

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ABSTRACT

Evolutionary development has increased the diversity of genotypes and the complexity of gene functions in fish. *TLR22* has been identified as a teleost-specific gene, but its functions are tremendously different among different fish species. Whether the functional diversity relates to the difference of genotypes remains poorly understand. In this study, we cloned and identified three TLR22 molecules from *Schizothorax prenanti* (*S. prenanti*), named as *spTLR22-1*, *spTLR22-2* and *spTLR22-3*. The full-length coding regions of spTLR22s are 2841 bp, 2805 bp and 2868 bp and coding 946 aa, 934 aa and 955 aa, respectively. All spTLR22s are composed of multiple leucine-rich repeat (LRR) domains, a transmembrane structure and a Toll/IL-1 receptor (TIR) region. The phylogenetic analysis showed that three spTLR22s were close to *Cyprinus carpio* TLR22-1, TLR22-2 and TLR22-3, respectively. Among the spTLR22s, they presented not close relationship but remained to belong to TLR22 subfamily. All *spTLR22s* were ubiquitously expressed in all tested tissues, but the expression levels of *spTLR22s* were dominant in immune-related tissues, such as gill and spleen. The expression levels of *spTLR22-1* and *spTLR22-3* were significantly increased after treatment with bacteria, LPS and Poly(I:C). However, *spTLR22-2* seems like no response to these treatments. The luciferase reporter assay demonstrated that all spTLR22s could activate NF-κB signaling pathway, but only spTLR22-1 and spTLR22-2 could activate IFN-β signaling pathway. Interestingly, in the ligand recognition analysis, spTLR22-1 and spTLR22-3 but not spTLR22-2 had the recognized potential to Poly(I:C), and all spTLR22s could not recognize LPS. Both spTLR22-1 and spTLR22-3 significantly up-regulated the expression of anti-viral-related genes (*Mx*, *IFN* and *ISG15*) and down-regulated the expression of anti-inflammatory factor *IL-10* after the overexpression in carp EPC cell line, but spTLR22-2 failed to impact the expression of these genes. Moreover, we found that all spTLR22s localized to the intracellular region. Taken together, our results reveal that spTLR22-1 and spTLR22-3 but not spTLR22–2 may be involved into the anti-viral immune response via IFN-β signaling pathway, and all spTLR22s can activate NF-κB signaling pathway but only spTLR22-1 and spTLR22-3 response to the stimulation of bacteria and LPS.

1. Introduction

The innate immune system plays an essential role in preventing the invasion of microbial pathogens, and is regarded as the primary defense mechanism in animals [1]. In mammals, highly conservative pathogens associated molecular patterns (PAMPs) from microorganism are recognized by pattern recognition receptors (PRRs). Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs) are four major PRRs, which play different roles in preventing the invasion of microbial pathogens [1–4]. However, since multiple genome-wide doubling events, the diversity of genotypes and the complexity of gene functions have been increased in fish. A lot of new PRRs have been identified in fish, but their functions remains poorly understand. Thus, elucidating the mechanism of ligands

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recognition and signaling transduction mediated by fish-specific PRRs, such as TLR18-28, is of great importance and significance for preventing the microbial disease in fish.

To date, 13 TLRs (TLR1-13) have been identified in mammals [5] and at least 22 TLRs (TLR1, 2, 3, 4, 5 M, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 and 28) have been identified in different fish species [6]. TLR family members are type I transmembrane receptor that contain multiple extracellular leucine-rich repeats (LRRs) domains, a transmembrane structure, and a cytoplasmic segment with a Toll/IL-1 receptor (TIR) region [7]. The structural difference of the LRR domains among in TLRs leads to recognize diverse components of microbial pathogens while the relatively conservative TIR region can selectively recruit and activate downstream adaptor molecules, such as myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adaptor inducing interferon-β (TRIF) [8]. For example, TLR2 can recognize lipopeptides from bacteria and zymosan from fungi and activate MyD88-dependent pathway [1]; TLR3 can recognize the double-stranded RNA (dsRNA) and activate TRIF-dependent pathway [9]; TLR4, can recognize the lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria and activate NF-κB- and AP-1-mediated signaling via MyD88-dependent pathway [10]; TLR5, flagellin of bacteria flagella and MyD88-dependent pathway [11]; TLR8, single-stranded viral RNA (ssRNA) and MyD88-dependent pathway [12]; and TLR9, CpG-DNA and MyD88-dependent pathway [13].

TLR22, which belongs to TLR11 subfamily, was first document in goldfish (*Carassius auratus*) [14]. Subsequently, it was identified in zebrafish (*Danio rerio*) [15], rainbow trout (*Oncorhynchus mykiss*) [16], grass crap (*Ctenopharyngodon idella*) [17], Atlantic salmon (*Salmo salar*) [18], turbot (*Scophthalmus maximus*) [19], common crap (*Cyprinus carpio L.*) [20], etc. In rainbow trout, the expression levels of *TLR22* gene are significantly upregulated after treatment with formalin-inactivated *Aeromonas salmonicida* [16]. In grass carp, *TLR22* gene is induced following grass carp reovirus (GCRV) infection [17]. Consistently, the response to the stimulation of bacteria and Poly(I:C) are also observed in turbot and common carp [19,20]. In addition, X. Ding et al. [21] proved that TLR22 can be an inflammatory equalizer by suppressing the NF-κB signaling pathway and selective activating of MAPK signaling pathway.

Schizothorax prenanti (*S. prenanti*) is an important cold-water economic fish, which is mainly distributed in the upper reaches of Yangtze river. But the intensive feeding easily causes the infection of microbial pathogens, such as *Aeromonas hydrophila* [22] and reovirus [23], and outbreaks diseases. Previous transcriptome study reveals that multiple subtypes of TLR22 may involve in the process of anti-bacterial immune response in *S. prenanti* [24], but their structures and functions are still unclear. In this study, we cloned and identified three subtypes of *TLR22* coding sequence (CDS) from *S. prenanti* (named as *spTLR22-1*, *spTLR22-2* and *spTLR22-3*). To further understand their functions, we compared the similarity and difference of the tissue distribution, the gene expression after challenge with bacteria or ligands, the induced luciferase activity, the subcellular localization and the expression of downstream cytokines in *Epithelioma papulosum cyprinid* (EPC) cell line.

2. Materials and methods

2.1. Fish breeding and A. hydrophila, LPS and Poly(I:C) challenge

S. prenanti (mean weight of approximately 300 g) were purchased from Sichuan Ya-fish Company (Ya'an, china). The fish were fed in 70 × 50 × 46 cm^3 fiberglass tanks at the simulated natural conditions including natural photoperiod (12 L:12 D) and water temperature (20 °C). All challenges and treatments were performed after at least two weeks of accommodation.

Aeromonas hydrophila (*A. hydrophila*) was incubated in Luria Bertani (LB) medium at 37 °C with overnight shaking. LPS from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and its biological activity was assessed by Limulus Amebocyte Lysate (LAL) assay. Fish were injected intraperitoneally with *A. hydrophila* (10^8 CFU per fish), 5 mg per kg body weight of E.coli. LPS (9.65 × 10^6 EU/mg) and isochoric PBS. Each treatment has 8 biological repetitions (n = 8). After treating different times (6, 12, 24 and 48 h), the fish were euthanized by using 300 mg/L MS222 (yuanye Bio. Co. Ltd., Shanghai, China), and the spleen and head kidney were collected and stored in −80 °C until for total RNA extraction.

In the Poly(I:C) challenge, Poly(I:C) was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) and dissolved in sterile PBS. Fish was injected intramuscularly with Poly(I:C) (5 mg per kg body weight) and isochoric PBS. Each treatment has 8 biological repetitions (n = 8). After treating different times (6, 12 and 24), all fish were dealt with the same method as above.

2.2. Isolation of S. prenanti HKLs and LPS/Poly(I:C) stimulation

The isolation and culture of *S. prenanti* head kidney leukocytes (HKLs) were performed according to previous descriptions [25]. The isolated HKLs were stimulated with 10 μg/mL LPS, 10 μg/mL Poly (I:C) and isopyknic PBS in 24-well culture plate. Then, at 3, 6, 12, 24 and 48 h after treatment, the cells were collected and stored in −80 °C until subsequent RNA extraction.

2.3. RNA extraction and cDNA synthesis

The total RNA of tissues and cells was extracted by RNAiso Plus reagent (Takara Bio, Co. Ltd., Dalian, China), and its concentration and purity was examined by Nano-Drop (2000) spectrophotometer (Thermo, USA) and agarose gel electrophoresis, respectively. For the cDNA synthesis, PrimeScripy RT reagent kit with gDNA Eraser (Takara Bio, Co. Ltd., Dalian, China) was used to perform the reverse transcription reaction. All the operations strictly followed the manufacturer's instruction.

2.4. Primer design for gene cloning and plasmid construction

To clone the complete CDS region of *spTLR22-1*, *spTLR22-2* and *spTLR22-3*, the specific primers were designed based on the transcripts information from previous transcriptome database [24]. All the *spTLR22s* were cloned and inserted into TA clone vector. After acquiring the right sequences, all the *spTLR22s* were subcloned into pcDNA3.1 and pCMV-C-EGFP plasmids by the method of homologous recombination. All the primers were shown in Supplementary Table 1.

2.5. Sequence analysis and phylogenetic analysis

The similarity and conservatism of the amino acid sequences among spTLR22-1, spTLR22-2 and spTLR22-3 were analyzed by BLAST algorithm at NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). The multiple sequence alignments of spTLR22-1, spTLR22-2 and spTLR22-3 were performed by ClustalW (https://www.genome.jp/tools-bin/clustalw). Furthermore, the structural domains of spTLR22-1, spTLR22-2 and spTLR22-3 were predicted by the SMART program (http://smart.embl-heidelberg.de/), and the LRR domains were identified according to previous methods [26–28]. Subsequently, the phylogenetic tree was established by MEGA 7.0 software by using the Neighbor-joining method. The GenBank accession numbers of selected proteins were shown in Supplementary Table 2.

2.6. Real-time PCR assay

The expression levels of *spTLR22-1*, *spTLR22-2* and *spTLR22-3* in *S. prenanti* and the expression levels of *IFN*, *Mx*, *ISG15* and *IL-10* in EPC cells were evaluated by real-time PCR with using SYBR® green dye (Takara Bio, Co. Ltd., Dalian, China), following conditions: 95 °C for

3 min; 39 cycles of 95 °C for 5 s and several annealing temperature for 30 s; 95 °C for 10 s; melt curve detection of 65 °C for 5 s to 95 °C increment 0.5 °C. The specific primers were designed by Primer 5 software and shown in Supplementary Table 1. The relative expression levels of these genes were calculated by using the $2^{-\triangle\triangle Ct}$ method with *β-actin* as the reference gene.

2.7. Cell culture, transfection and luciferase activity assays

HEK293T is a common operating cell line that was culture in DMEM medium which contained 10% FBS (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were culture at 37 °C and 5% CO_2 in cell culture flasks. HKE239T cells were transfected with empty plasmid (150 ng/well), spTLR22-1 (150 ng/well), spTLR22-2 (150 ng/well) or spTLR22-3 (150 ng/well), together with NF-κB (200 ng/well), IFN-β (200 ng/well) or IFN-γ (200 ng/well) and renilla luciferase reporter plasmid (20ng/well) in 24-well plate by using the jetPRIME transfection reagent (Polyplus-transfection Inc., New York, USA). These luciferase reporter plasmids were friendly provided by Prof. J. Jin (Life Sciences Institute, Zhejiang University, China). Then, the medium was replaced by the fresh complete medium after 4 h incubation to maintain the dynamic status of the cells. After 24 h of transfection, the luciferase activity was measured by using the dual-luciferase reporter assay kit (Promega), and the results were presented as fold change by comparing to the control group after normalizing to renilla luciferase activity. To detect the relationship between spTLR22s and ligands, the cells were transfected with LPS (500 ng/well) or Poly(I:C) (500 ng/well) for 12 h by using jetPRIME transfection reagent after transfecting spTLR22s and NF-κB/IFN-β and renilla reporter gene plasmids for 24 h. Then, the cells were collected to detect the luciferase activity after intracellular stimulation.

EPC cells were cultured in DMEM medium which contained 10% FBS (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were cultured in cell culture flasks with 5% CO_2 at 28 °C. For the overexpression analysis of spTLR22s, EPC cells were transfected with spTLR22-1 (400 ng/well), spTLR22-2 (400 ng/well), spTLR22-3 (400 ng/well) or the equal amount of empty plasmid in 24-well plate. Then, the cells were collected after 24 h of transfection and stored in −80 °C until used for subsequent real-time PCR assay.

2.8. Confocal fluorescence microscopy

The spTLR22-1-GFP, spTLR22-2-GFP and spTLR22-3-GFP plasmids (400 ng/well) were transfected into HEK293T cells in 24-well culture plate, respectively. After 24 h of transfection, the cells were removed into 6 cm glass bottom cell culture dish. After overnight culture, the cells were washed by Hank's Balanced Salt Solution (HBSS, Solarbio Science & Technology Co., Ltd., Beijing, China), and then incubated with 1 μM ER-Tracker Red (Invitrogen) for 20 min to stain the endoplasmic reticulum or 5 μg/mL Wheat Germ Agglutinin (WGA) for 20 min to stain the cytomembrane. Subsequently, the nucleus was stained by using 5 μg/mL Hoechst 33342 dye for 30 min after the cells were washed three times with HBSS. Finally, after washing the cells twice with HBSS, the location of spTLR22-1, spTLR22-2 and spTLR22-3 in HEK293T cell was observed and recorded by the confocal fluorescence microscopy.

2.9. Statistical analysis

Date analysis and histogram were done by SPSS 22.0 and Graphpad Prism 5 software, respectively. The mRNA expression levels were analyzed by using One-way ANOVA method. The luciferase activities were analyzed by using *t*-test or One-way ANOVA. The *p* value less than 0.05 was considered as the statistical significance.

3. Results

3.1. Molecular characterization of spTLR22-1, spTLR22-2 and spTLR22-3

We cloned and identified the complete CDS of three *S. prenanti* TLR22 genes, named as *spTLR22-1*, *spTLR22-2* and *spTLR22-3* (GenBank accession number: MN082612, MN082613 and MN082614, respectively). The full-length coding sequences for *spTLR22-1*, *spTLR22-2* and *spTLR22-3* were 2841, 2805 and 2868 bp long, respectively (Supplementary Fig. 1A, B and C). They encoded 946, 934 and 955 aa-long proteins with the molecular weight of 108.5, 107.4 and 109.9 kDa, respectively. The functional domains of spTLR22s were predicted including a signal peptide of 22 aa and 26 LRR domains in spTLR22-1 and spTLR22-3, and a signal peptide of 32 aa and 25 LRR domains in spTLR22-2. All the spTLR22s contain a transmembrane (TM) domain and a Toll/IL-1-like receptor (TIR) region. In the TIR region, we identified three conservative regions: box1 (YHAF-SY), box2 (LCL-RD-PG) and box3 (a conserved W surrounded by basic residues).

3.2. Phylogenetic analysis of spTLR22-1, spTLR22-2 and spTLR22-3

To reveal the evolutionary relationship between spTLR22s and TLR22 of other fishes, the phylogenetic tree was constructed using the neighbor-joining (NJ) method based on their amino acid sequences. According to the phylogenetic tree (Fig. 1), spTLR22s belonged to TLR11 subfamily, and further clustered together with other TLR22s. Among the TLR22 subfamily, spTLR22s showed the closest relationship to the *Cyprinus carpio* TLR22s.

3.3. Tissue distribution of spTLR22-1, spTLR22-2 and spTLR22-3 in healthy S. prenanti

The mRNA expression of *spTLR22-1*, *spTLR22-2* and *spTLR22-3* was detected in all the tested tissues. But their expression patterns presented slight diversity. As shown in Fig. 2, the highest expression of *spTLR22-1* was observed in gill, while the highest expression of *spTLR22-2* and *spTLR22-3* was in spleen. The lowest expression of *spTLR22-1* was observed in brain, while the *spTLR22-2* and *spTLR22-3* was in midgut.

3.4. Expression profiles of spTLR22-1, spTLR22-2 and spTLR22-3 in S. prenanti after LPS and A. hydrophila challenge

In previous studies, the expression levels of the *TLR22* gene were significantly up-regulated in other fish species after LPS and bacteria stimulations [16,29]. In order to explore whether the up-regulation of *TLR22* gene expression exists in *S. prenanti*, the expression levels of *spTLR22s* in two important immune-related organs, spleen and head kidney, were detected after LPS and *A. hydrophila* stimulation. As shown in Fig. 3A and D, *spTLR22-1* positively responded to LPS and *A. hydrophila* stimulation in both tissues at the 12 h and 24 h time points, except for LPS stimulation in head kidney at 24 h. Like *spTLR22-1*, the expression levels of *spTLR22-3* were significantly up-regulated at 12 h both in head kidney and spleen, but the up-regulated degree was less than the *spTLR22-1* (Fig. 3C and F). Interestingly, *spTLR22-2* did not respond to the stimulations of LPS and *A. hydrophila* and was even down-regulated at 6 h and 12 h in spleen (Fig. 3B and E).

3.5. Expression profiles of spTLR22-1, spTLR22-2 and spTLR22-3 in S. prenanti after Poly(I:C) challenge

Lots of reports have showed that TLR22 participates in the anti-viral immune response [17,30]. To determine whether spTLR22s involves in the anti-viral immune response, the spTLR22s mRNA expression levels in head kidney and spleen were detected in *S. prenanti* after stimulation with Poly(I:C). As shown in Fig. 4A and D, *spTLR22-1* presented high expression levels at 12 h in both head kidney and spleen, but *spTLR22-2*

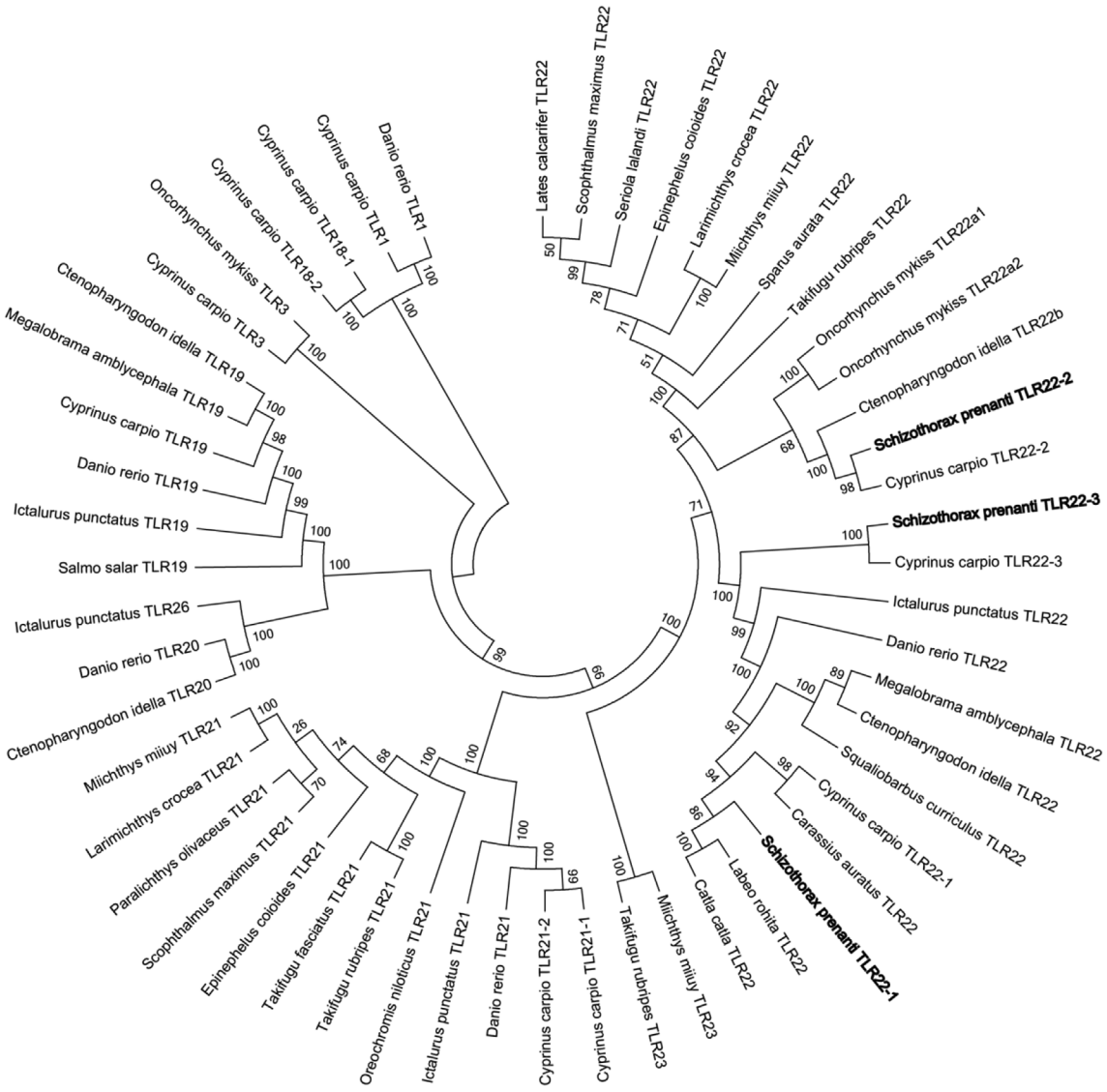

Fig. 1. Phylogenetic tree illustrating the relationship between spTLR22s and other TLRs. The phylogenetic tree was generated by using the method of Neighbor-Joining in MEGA 6.0 software.

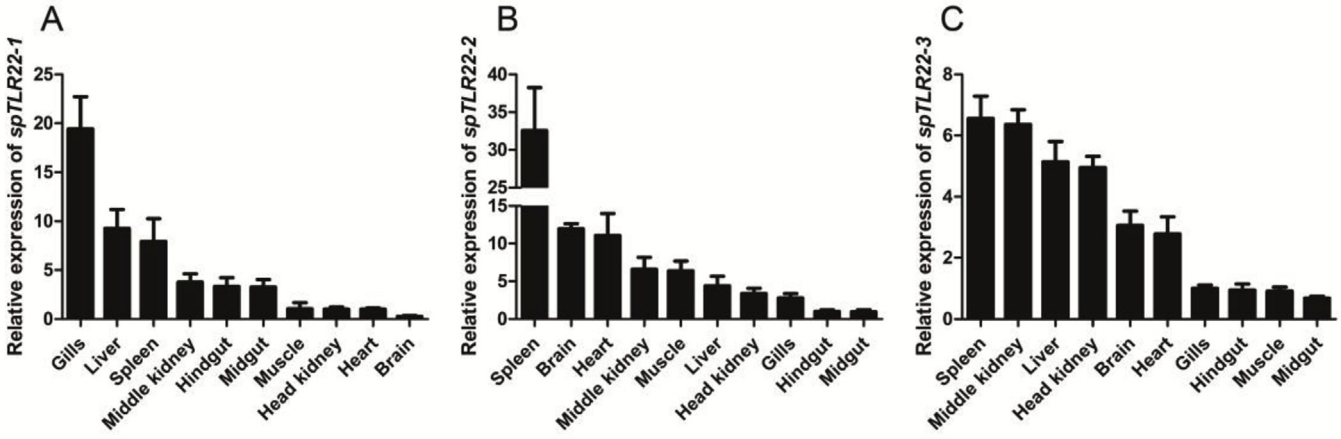

Fig. 2. The tissue expression of *spTLR22s* in healthy *Schizothorax prenanti*. The expression of *spTLR22-1* (A), *spTLR22-2* (B) and *spTLR22-3* (C) in head kidney, middle kidney, brain, spleen, liver, gill, heart, muscle, hindgut and midgut were analyzed by Real-time PCR with *β-actin* as the internal control. The expression of *spTLR22-1* in heart, *spTLR22-2* in hindgut and *spTLR22-3* in gill was chosen as calibrator (set as 1), respectively.

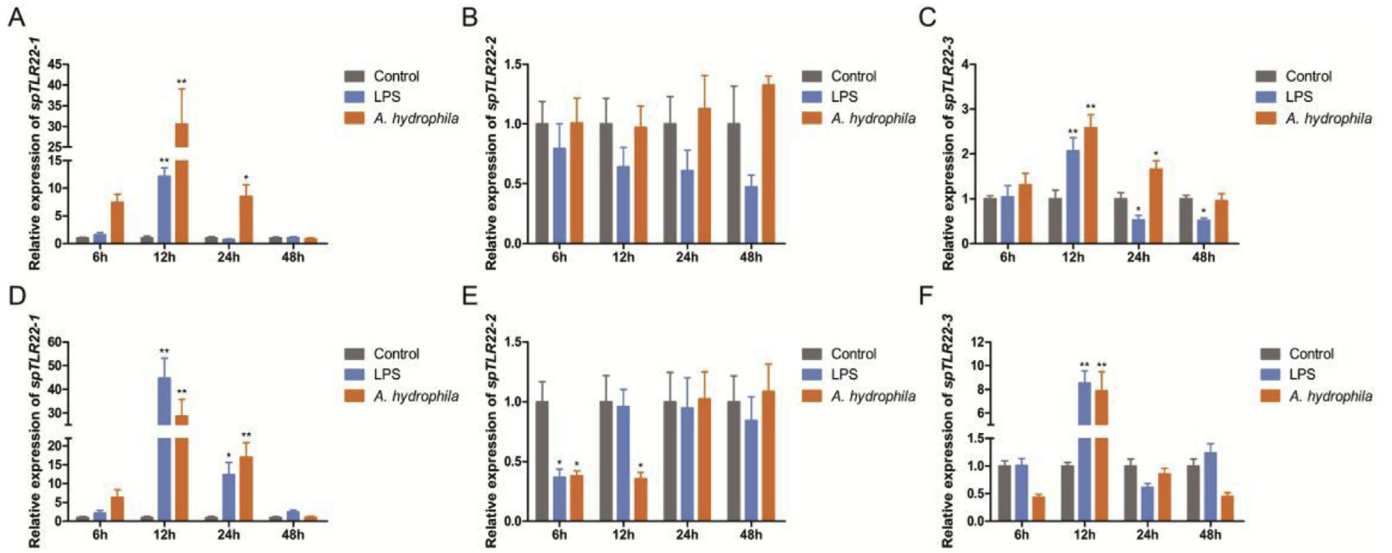

Fig. 3. The relative expression levels of *spTLR22s* in head kidney and spleen after stimulation with LPS and *A. hydrophila*. The relative expression levels of *spTLR22-1* (A and D), *spTLR22-2* (B and E) and *spTLR22-3* (C and F) in head kidney and spleen after LPS and *A. hydrophila* stimulation were examined at different time points by Real-time PCR assay with *β-actin* as the reference gene. The gene expression in control group at corresponding time point was chosen as calibrator (set as 1). Data are expressed as the mean ± SEM (n = 8), and the statistically significant differences between control and treatment groups at each time point are expressed with asterisks, $*P < 0.05$ and $**P < 0.01$.

might be insensitive to the Poly(I:C) stimulation (Fig. 4B and E). The expression levels of *spTLR22-3* were significantly enhanced only at 12 h and 24 h in head kidney (Fig. 4C and F).

3.6. Expression profiles of spTLR22s in HKLs after LPS and Poly(I:C) stimulation

We also analyzed the expression pattern of *spTLR22s* in HKLs after LPS and Poly(I:C) stimulation. After the stimulation, the expression levels of *spTLR22-1* were significantly increased at 3, 6, 12 and 48 h (Fig. 5A), and the expression levels of *spTLR22-3* were obviously up-regulated at 3, 6, 12 and 24 h (Fig. 5B). But unfortunately, due to the low expression abundance of *spTLR22-2* in HKLs, its transcripts failed to be detected in the real-time PCR assay.

3.7. spTLR22s may activate NF-κB and IFN-β signaling pathway

To investigate which signaling pathway spTLR22s can activate, three luciferase reporter gene plasmids were used to detect the function of spTLR22s. As shown in Fig. 6A, spTLR22-1 significantly increased the NF-κB-luc and IFN-β-luc activity, but not IFN-γ-luc activity. The similar result was observed in spTLR22-2 (Fig. 6B), indicating that both spTLR22-1 and spTLR22–2 may activate the NF-κB and IFN-β signaling pathway. But the signal activation of spTLR22-3 was only characterized

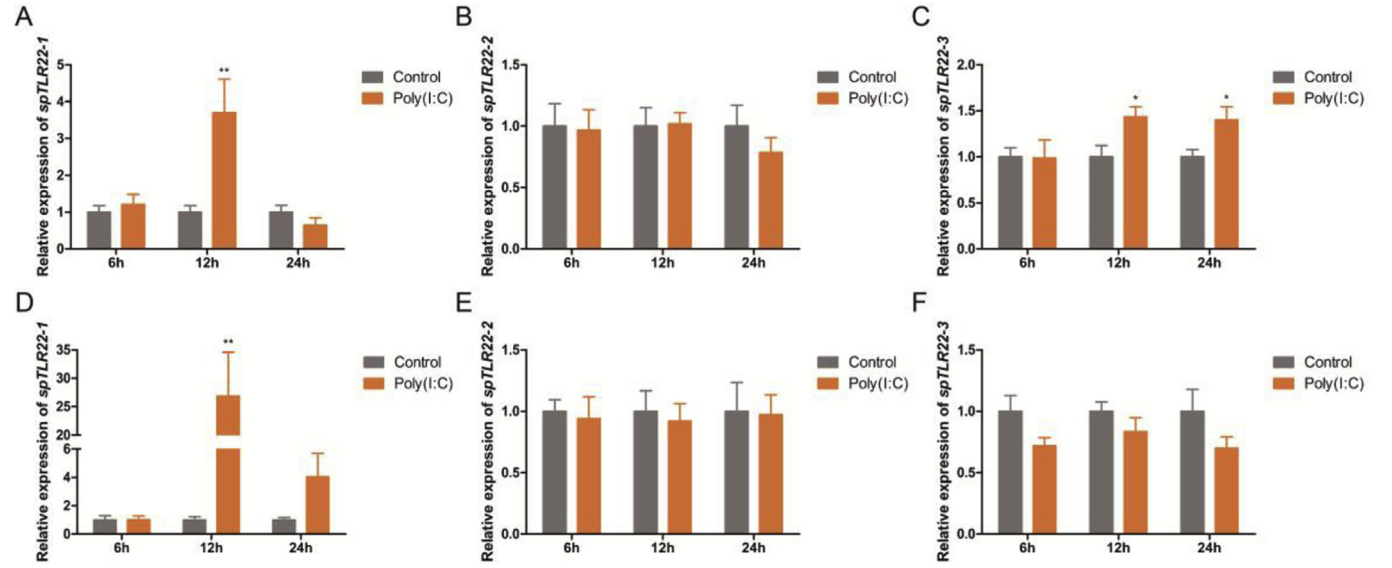

Fig. 4. The relative expression levels of *spTLR22s* in head kidney and spleen after stimulation with Poly(I:C). The relative expression levels of *spTLR22-1* (A and D), *spTLR22-2* (B and E) and *spTLR22-3* (C and F) in head kidney and spleen after Poly(I:C) stimulation were examined at different time points by Real-time PCR assay with *β-actin* as the reference gene. The gene expression in control group at corresponding time point was chosen as calibrator (set as 1). Data are expressed as the mean ± SEM (n = 8), and the statistically significant differences between control and treatment groups at each time point are expressed with asterisks, $*P < 0.05$ and $**P < 0.01$.

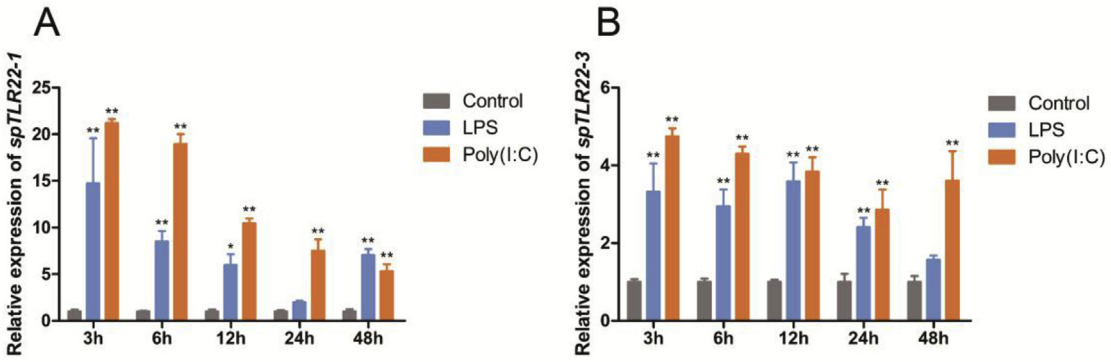

Fig. 5. The relative expression levels of *spTLR22-1* (A) and *spTLR22-3* (B) in head kidney leucocytes after LPS and Poly(I:C) stimulation. After stimulation with LPS and Poly(I:C), the expression levels of *spTLR22-1* and *spTLR22-3* were evaluated at different time points by Real-time PCR assay with *β-actin* as the reference gene. The gene expression in control group at corresponding time point was chosen as calibrator (set as 1). Data are expressed as the mean ± SEM (n = 6), and the statistically significant differences between control and treatment groups at each time point are expressed with asterisks, $*P < 0.05$ and $**P < 0.01$.

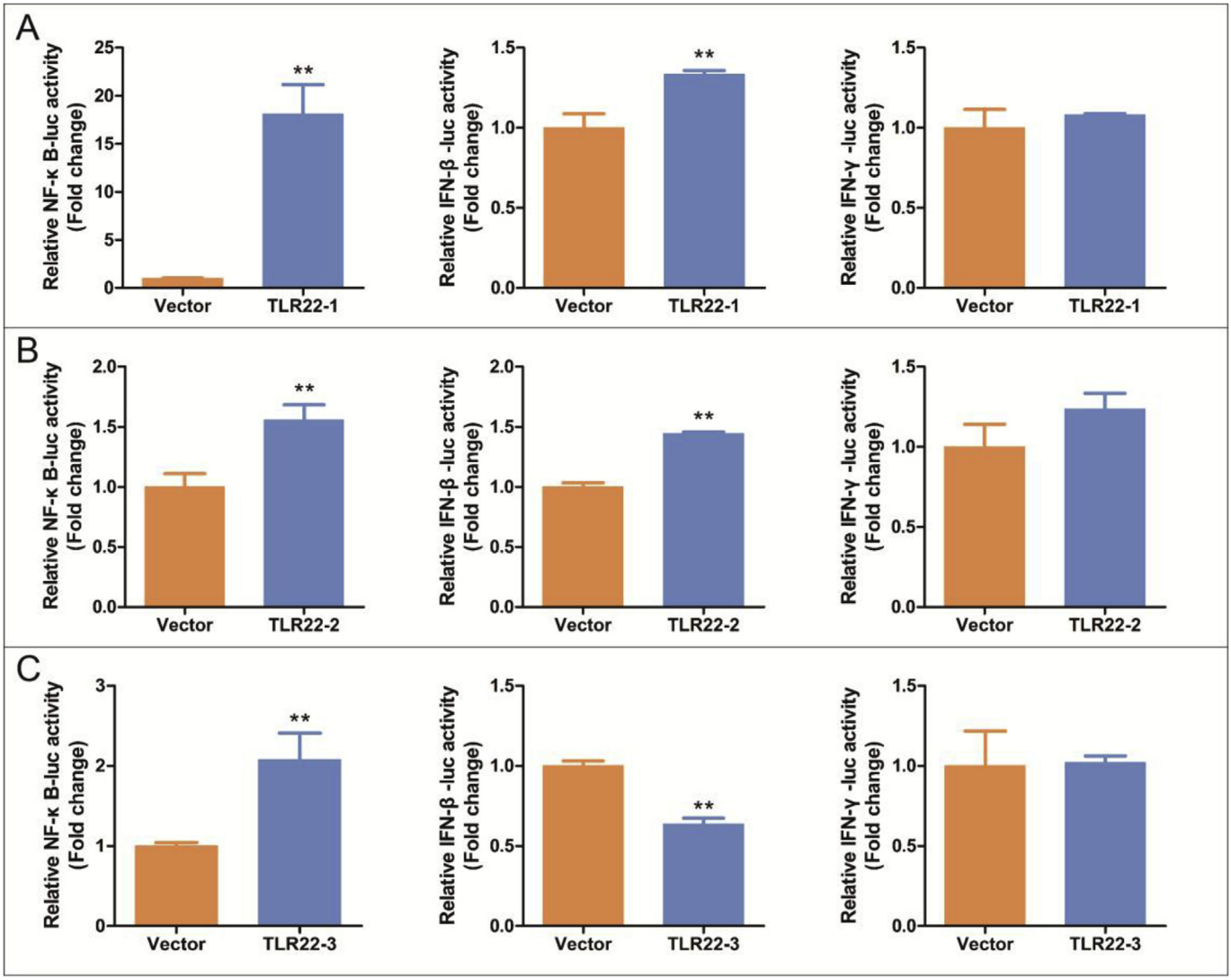

Fig. 6. The function of spTLR22s in the activation of NF-κB, IFN-β and IFN-γ signaling pathway. HEK293T cells were transfected with spTLR22-1 (A), spTLR22-2 (B) and spTLR22-3 (C) expression plasmids or empty control vectors together with NF-κB, IFN-β or IFN-γ and Renilla luciferase reporter plasmids. After 24 h of transfection, the relative luciferase activity was detected and calculated by normalizing to Renilla luciferase activity. The relative luciferase activity levels in control group were chosen as calibrator (set as 1). Data are expressed as the mean ± SD (n = 3), and the statistically significant differences are expressed with asterisks, $*P < 0.05$ and $**P < 0.01$.

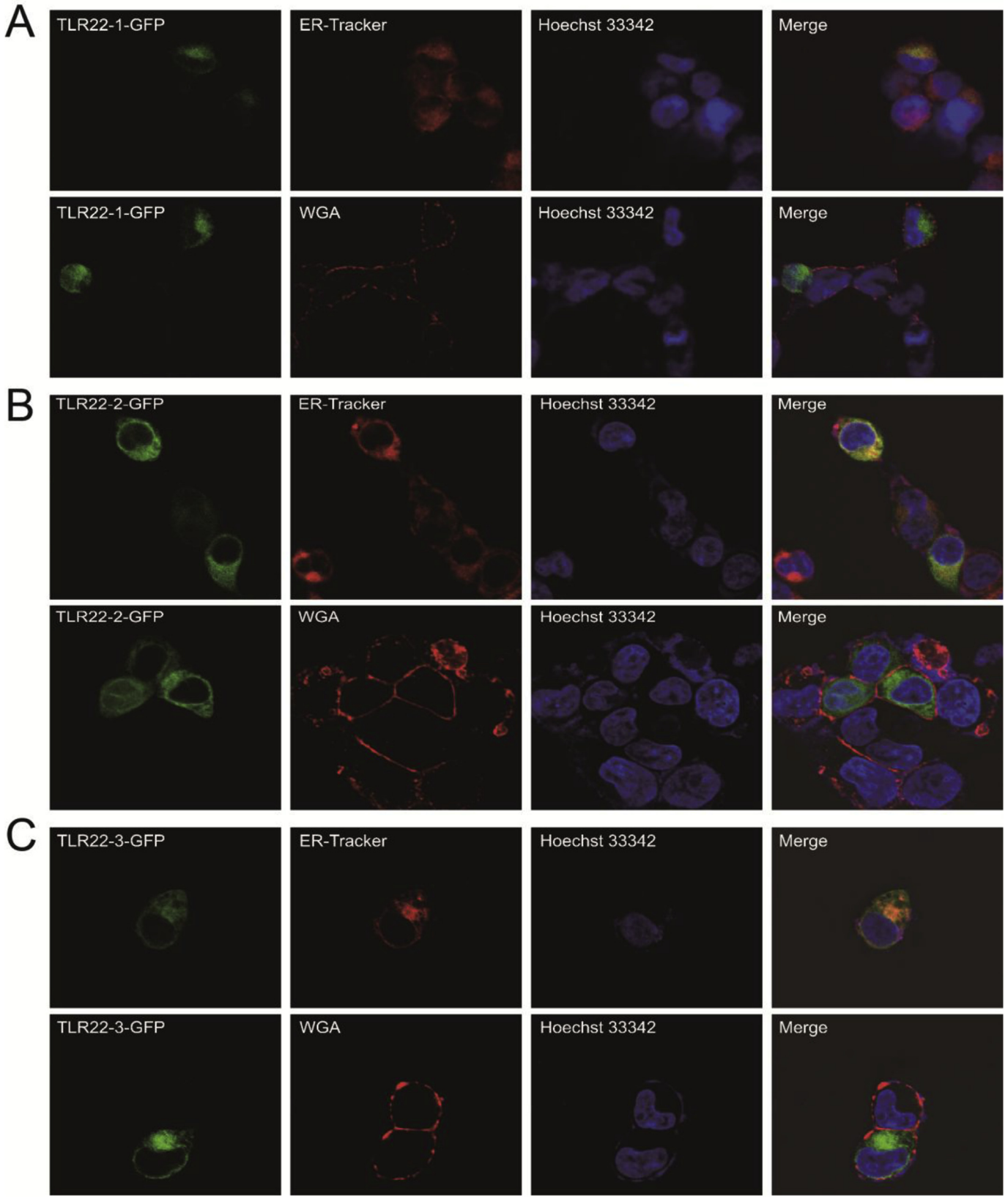

Fig. 7. spTLR22–1 (A), spTLR22–2 (B) and spTLR22–3 (C) locate to intracellular region. HEK293T cells were used to the confocal analysis. The cell membrane was dyed by WGA (5 μg/mL), while the endoplasmic reticulum was marked by ER-Tracker (1 mM), and the nucleus was labeled by Hoechst 33342 (5 μg/mL).

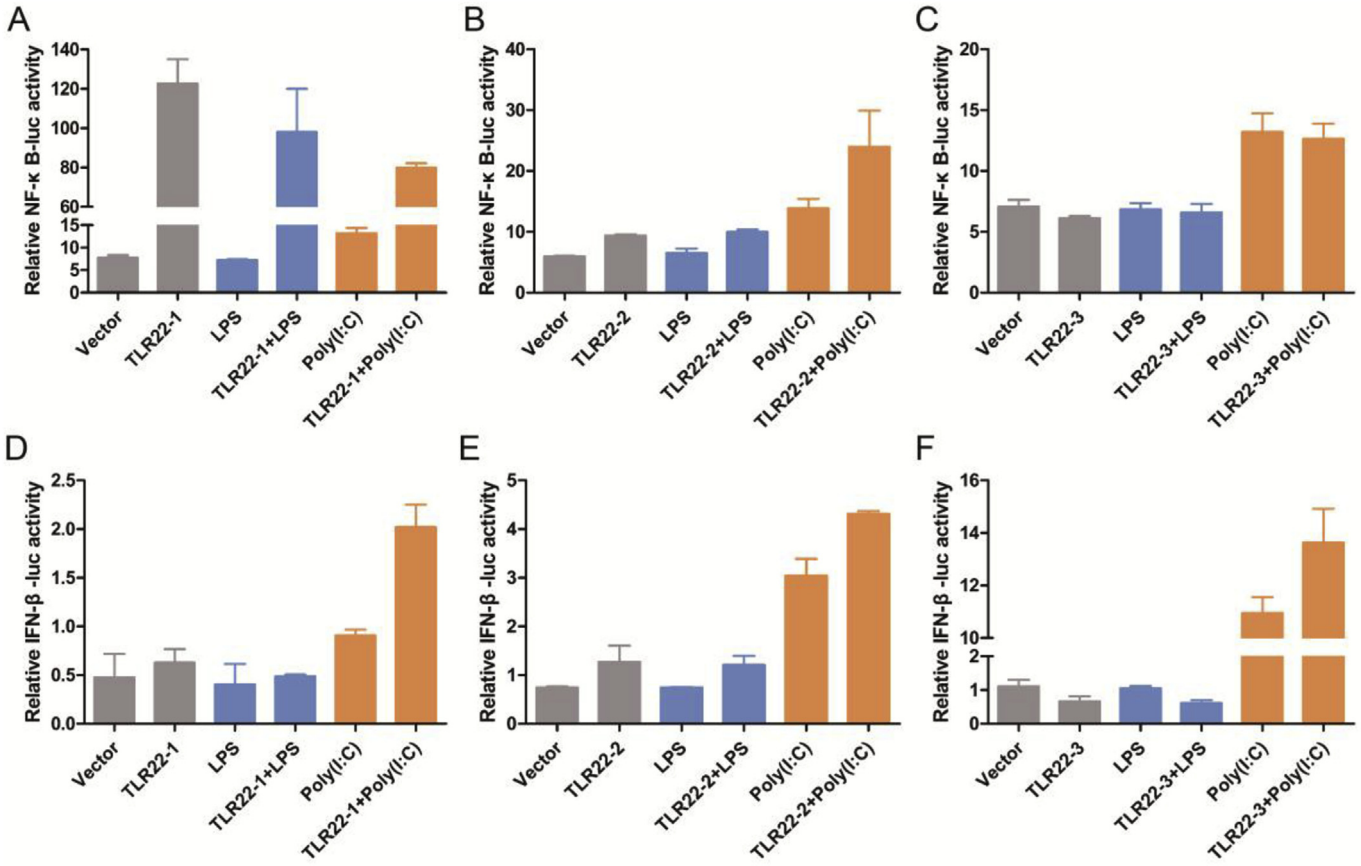

Fig. 8. spTLR22s may recognize Poly(I:C) and activate IFN-β signaling pathway. HEK293T cells were transfected with spTLR22-1 (A and D), spTLR22-2 (B and E) and spTLR22-3 (C and F) expression plasmids or empty control vectors together with NF-κB or IFN-β and Renilla luciferase reporter plasmid, and then transfected with LPS and Poly(I:C) to the cells. At 12 h post-transfection, the relative luciferase activity was detected and calculated by normalizing to Renilla luciferase activity. Data are expressed as the mean ± SD (n = 3).

by the NF-κB-luc activity analysis. Meanwhile, spTLR22-3 failed to increase the IFN-β-luc and IFN-γ-luc activity and even decreased the IFN-β-luc activity (Fig. 6C).

3.8. spTLR22s locate to the intracellular region

In order to determine the subcellular localization of spTLR22s, we used the endoplasmic reticulum and cell membrane dyes to analyze the localization of spTLR22s in HEK293T cells. As shown in Fig. 7, all the spTLR22s were located to the intracellular region.

3.9. spTLR22s may participate in the recognition of Poly(I:C) to activate the IFN-β signaling pathway

The potential of spTLR22s in ligand recognition was analyzed in current study, basing on the dual-luciferase reporter system and intracellular LPS/Poly(I:C) stimulation. After co-transfection with spTLR22-1 and Poly(I:C), the NF-κB-luc activity was significantly down-regulated ($P < 0.01$), but the IFN-β-luc activity was significantly increased ($P < 0.05$) (Fig. 8A and D). Of the spTLR22-3, the co-transfection with spTLR22-3 and Poly(I:C) did not interact to influence the NF-κB-luc activity, but it could significantly increase the IFN-β-luc activity ($P < 0.01$) (Fig. 8C and F). Moreover, the spTLR22-2-mediated NF-κB-luc and IFN-β-luc activity was not significantly changed by the intracellular Poly(I:C) stimulation (Fig. 8B and E). Apart from that, all the spTLR22s did not response to the intracellular LPS stimulation either in the NF-κB-luc or the IFN-β-luc activity analysis.

3.10. spTLR22s overexpression differently induced the gene expression of downstream cytokines in EPC cells

In order to explore the activated functions of spTLR22s for downstream signal molecules, we performed the overexpression analysis of spTLR22-1, spTLR22-2 and spTLR22-3 in EPC cells, and then the mRNA expression levels of downstream genes (*Mx*, *INF*, *IL-10* and *ISG15*) were analyzed by real-time PCR assay with *β-actin* as the reference gene. As shown in Fig. 9, the expression levels of *Mx*, *IFN* and *ISG15* genes were significantly increased in spTLR22-1 and spTLR22-3 overexpression groups, but not in spTLR22-2 overexpression group. The expression levels of *IL-10* gene were markedly decreased in spTLR22-1 and spTLR22-3 overexpression groups, but still not changed in spTLR22-2 overexpression group.

4. Discussion

With the discovery of toll-like receptors (TLRs) in mid-1990s, it is shown that the recognition of pathogen by immune system is specific. TLRs play an essential role in recognizing the conserved structures of pathogens, so called PAMPs [31]. TLR22, known as a typical member of fish-specific TLRs, belongs to TLR11 subfamily, but its functions remain controversial. In previous study of our lab, multiple TLR22 genes show different responses to bacterial invasion [24], which trigger our interest to further explore their functions in immune response.

Herein, we acquired the complete coding sequences of *spTLR22-1*, *spTLR22-2* and *spTLR22-3*, and analyzed their functional structures, including the LRR domains, the transmembrane structure and the TIR

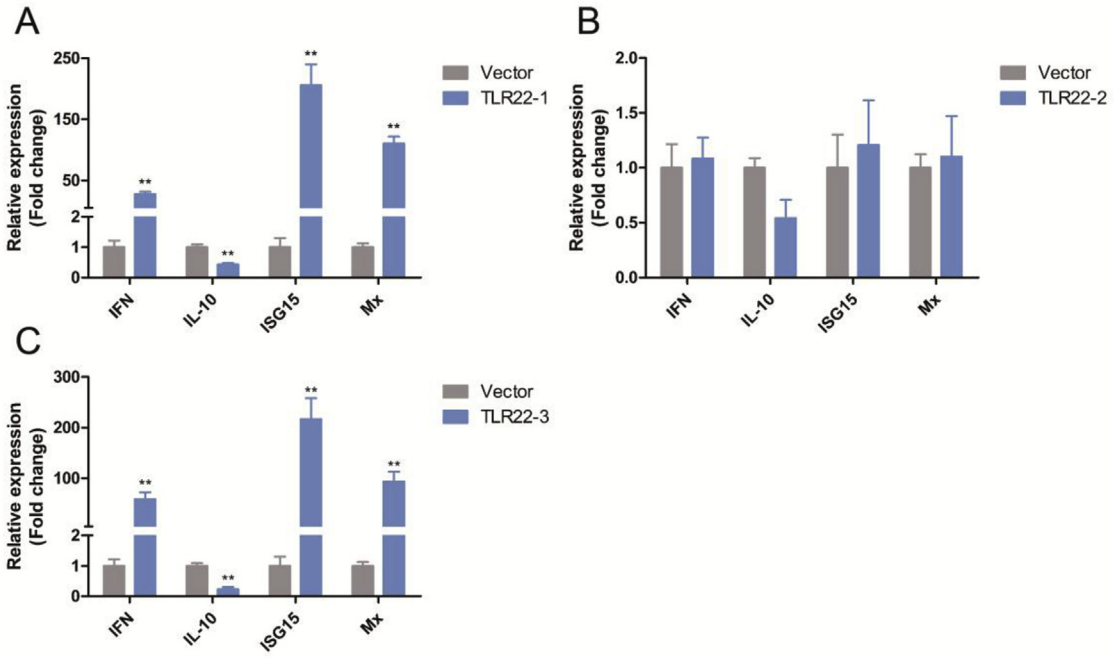

Fig. 9. The expression of cytokines in spTLR22s overexpressing EPC cells. The EPC cells were transfected with spTLR22-1 (A), spTLR22-2 (B), spTLR22-3 (C) or empty vector (pcDNA3.1). After 24 h of transfection, the expression levels of *Mx*, *IFN*, *ISG15* and *IL-10* were detected by real-time PCR assay with *β-actin* as the reference gene. The gene expression in control group was chosen as calibrator (set as 1). Data are expressed as the mean ± SEM (n = 3), and the statistically significant differences between control and treatment groups at each time point are expressed with asterisks, $*P < 0.05$ and $**P < 0.01$.

region. Both spTLR22-1 and spTLR22-3 contain 26 LRRs, and spTLR22-2 contains 25 LRRs. The LRR domain of TLRs is an important structure for directly bind the corresponding ligands [32]. According to previous reports, the number of LRRs of TLR22 may be different in various fish species. For example, the TLR22 sequences of yellowtail, croaker and flounder contain 16, 19 and 19 LRR domains, respectively [20,29]. The diversity of the LRR number among three spTLR22s may relate to different modes of ligand binding. The TIR domain of TLR plays an important role in the signal transmission [33], which contains three highly conserved regions (box1, box2 and box3). The box1 and box2 can mediate the binding between receptors and signaling molecules, while the box3 directly controls the localization of receptors [34]. In this study, all the boxes were conservative in the TIR domains of spTLR22-1, spTLR22-2 and spTLR22-3, implying that spTLR22s may own conserved function for signal transmission as other fish TLR22s.

The spTLR22s exhibits ubiquitous expression in tissues. The *spTLR22-1* showed the skyscraping expression in gill that is similar to the results in common carp [20]. Both *spTLR22-2* and *spTLR22-3* were mainly expressed in spleen, suggesting that they may play an important role in immune response (Fig. 2). In fact, the tissue distribution of *TLR22* gene in different fish species exists diversity. In rohu, *TLR22* gene is highly expressed in liver, kidney and gill [35]. In turbot, the highly expression of *TLR22* gene is occurred in spleen and kidney [19]. In contrast, the *TLR22* gene in grass carp is mainly expressed in gill but lowly expressed in spleen [36]. Even so, the tissue distribution of *TLR22* gene is dominant in the immune-related tissue, such as spleen, head kidney and gill, indicating that TLR22 may be an important immune-related gene in the response of resisting pathogens invasion.

A. hydrophila, belongs to Gram-negative bacterium, and can cause bacterial hemorrhagic septicemia in teleosts [37]. LPS, a major component of Gram-negative bacterial cytoderm, is usually used to simulate the bacterial infection under experimental conditions [38–40]. In mammals, TLR4 can directly recognize LPS and activate NF-κB signaling pathway via MyD88-dependent pathway [41]. But in fish, TLR4 has not been reported as the LPS-directly receptor, because previous study reveals that it does not recognize LPS and even inhibits the activation of NF-κB signaling pathway [42]. Many TLRs have been reported to participate in the anti-LPS immune response [25,43,44], but the LPS recognition mediated by TLRs is still blurred in teleosts. In this study, *spTLR22-1* and *spTLR22-3* were shown the positive response to the process of *A. hydrophila* infection and LPS stimulation (Fig. 3). This is in agreement with the results in common carp [20], rohu [35] and orange-spotted grouper [29], suggesting that fish TLR22 may have a contribution to the anti-bacterial immune response. However, the expression levels of *spTLR22-2* did not present any significant up-regulation and even were inhibited in head kidney. This result proved that different genotypes of spTLR22 may have different immune functions. Given that the expression levels of spTLR22-2 are significantly down-regulated in the initial phase of the immune response, and some TLRs can protect the body from excessive inflammation [45,46], we speculate that spTLR22–2 may play a protective role in the excessive inflammation. After Poly(I:C) stimulation, the similar results of *spTLR22s* expression were detected (Fig. 4) in *S. prenanti*, suggesting that spTLR22-1 and spTLR22–3 may also play an important role in the anti-viral immune response. Consistently, LPS and Poly(I:C) also induced the expression of spTLR22-1 and spTLR22-3 in HKLs (Fig. 5). Taken together, these results indicate that spTLR22s may play different but important roles in the anti-bacterial and anti-viral immune response.

TLR-mediated signaling pathways mainly consist of two types, MyD88-dependent and MyD88-independent signaling pathway, which leads to the production of cytokines via NF-κB or IFN-mediated signaling pathway [47]. NF-κB protein acts as an intermediate bridge to trigger the production of inflammatory factors, while IFN mediates the expression of IFN stimulated genes (ISGs) to block viral invasion [48–50]. To explore which signaling pathway spTLR22s participate in, we detected the activity of signaling pathway by the luciferase reporter system (Fig. 6). The results reveal that spTLR22s can activate the NF-κB and IFN-β but not IFN-γ signaling pathway suggesting that spTLR22s may take part in the immune regulation by NF-κB and IFN-β signaling pathway. Then, we demonstrated that all spTLR22s were located in intracellular (Fig. 7) that was similar to the EcTLR22 [21], but that was different to the fgTLR22 which locates to the surface of the cell

membrane [51]. Besides, many reports reveal that teleost TLR22 can response to viral/Poly(I:C) or bacteria/LPS challenge [17–20], but whether TLR22 can recognize LPS/Poly(I:C) directly or three spTLR22s in *S. prenanti* perform the similar function in signaling pathway are still unclear. Thus, we explored the relationship between spTLR22s and two potential ligands, LPS and Poly(I:C). We observed that spTLR22-1 and spTLR22-3 were sensitive to Poly(I:C) stimulation but spTLR22-2 not. Interestingly, Poly(I:C) increased spTLR22-1-induced IFN-β-luc activity but down-regulated the spTLR22-1-induced NF-κB-luc activity (Fig. 8). In addition, all spTLR22s were insensitive to LPS stimulation. These results suggest that spTLR22-1 and spTLR22–3 may activate IFN-β signaling pathway by recognizing Poly(I:C) to participate in the anti-viral immune response. The increased expression of *Mx*, *IFN* and *ISG15* and down-regulated expression of *IL-10* gene in spTLR22-1 and spTLR22-3 overexpression experiments in EPC cells also further corroborated this speculation. In the overexpression experiments, the downstream signal molecules (*Mx*, *IFN* and *ISG15*) were induced without Poly(I:C) stimulation, suggesting that the overexpressed spTLR22-1 and spTLR22-3 can recruit their adaptor molecule and therefore to trigger the activation of IFN-β signaling pathway. The candidate adaptor molecule, including MyD88, TRIF and TIRAP, may be involved into the signal transduction of spTLR22s, but the difference of the adaptor molecule of spTLR22s during the signal cascades remains to be study in the future.

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Appendix A. Supplementary data

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