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Molecular characterization, expression patterns, and functional analysis of toll-interacting protein (Tollip) in Japanese eel *Anguilla japonica*

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ABSTRACT

Toll-interacting protein (Tollip) is a key negative regulator of TLR-mediated innate immune responses. The structure and function of Tollip have been well identified in mammals, but the information about Tollip is still limited in teleost fishes. In the present study, the homologue of Tollip was cloned from Japanese eel. It contained an open reading frame encoding a polypeptide of 276 amino acids which shared high identities with other homologues from different species. Multiple alignment of the amino acid sequence showed that the *Aj*Tollip protein has the typical conserved domains including an N-terminal Target of Myb1 (Tom1) binding domain (TBD), a central conserved 2 (C2) domain, and a C-terminal coupling of ubiquitin to endoplasmic reticulum degradation (CUE) domain. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis revealed a broad expression for *Aj*Tollip in a wide range of tissues, with the highest expression in the liver, a relatively high expression in the spleen, kidney, gills, skin and intestine, and a low expression in the heart and muscle. The *Aj*Tollip expressions in the liver and kidney were significantly induced following injection with the bacterial mimic LPS, the viral mimic poly I:C, and *Aeromonas hydrophila* infection. *In vitro*, the *Aj*Tollip transcripts of Japanese eel liver cells were significantly enhanced by the treatment of LPS, poly I:C, CpG-DNA, and PGN or the stimulation of high concentration of *Aeromonas hydrophila* (1×10^7 cfu/mL and 1×10^8 cfu/mL). Subcellular localization study showed that *Aj*Tollip was mainly distributed in the cytoplasm in a condensed state. When *Aj*Tollip was co-transfected with *Aj*MyD88 into HEK293 cells, the luciferase activities of NF-κB were significantly decreased compared with that of *Aj*MyD88 single-transfection groups in natural state or under the stimulation of LPS and poly I:C. These results collectively suggested that *Aj*Tollip functions as a negative regulator of MyD88-dependent TLR signaling and plays an important role in fish defense against viral and bacterial infections.

1. Introduction

The toll-like receptors (TLRs) are a family of evolutionarily conserved pattern recognition receptors (PRRs) that recognize the pathogen-associated molecular patterns (PAMPs) found in microbial pathogens, such as LPS, peptidoglycans (PGN), CpG-DNA, single strand RNA (ssRNA), and double strand RNA (dsRNA), or its analogs poly I:C. Hence TLRs play a critical role in innate immune responses, functioning as the first line of defense against invading pathogens through activating signaling cascades to induce the expression of immune and pro-inflammatory genes [1]. However, when activation of TLR signaling pathways is out of control, the exaggerated expression of pro-inflammatory cytokines and signaling components may have devastating effects on the host, resulting in excessive inflammation, pathogenesis of autoimmune, and infectious diseases [2,3]. Therefore, TLR signaling must be tightly regulated to maintain immune response balance.

To date, many intracellular negative regulators of TLR signaling pathway have been identified such as myeloid differentiation factor 88 short (MyD88s) [4], Tollip (Toll-interacting protein) [5], SOCS1 (Suppressors of cytokine signaling 1) [6], Neuregulin receptor degradation protein-1 (Nrdp1) [7], and A20 [8]. They function as the negative regulators principally on the MyD88-dependent pathways during the TLR signaling cascades. Among them, Toll-interacting protein (Tollip) serves as one of the critical negative regulators of Toll-like receptors (TLRs) and IL-1Rs mediated signaling pathways [5]. Tollip gene was originally described as a new component of the IL-1R pathway in mice [9] and then have been identified and reported from invertebrates to vertebrates [10–14]. Three conserved domains were found in Tollip protein, including an N-terminal Target of Myb1 (Tom1) binding domain (TBD), a central conserved 2 (C2) domain, and a C-terminal

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coupling of ubiquitin to endoplasmic reticulum degradation (CUE) domain [9]. In mammals, Tollip proteins principally function as a modulator to negatively regulate NF-κB and JNK signaling through shutting down MyD88-dependent signaling pathways by inactivating IRAK-1 or directly binding to TLR2 and TLR4 [5,9,15]. Tollip also acts as an adaptor protein involved in the sorting and trafficking of proteins or degradation of ubiquitin-conjugated proteins [16] and negatively regulates the IL-1β and TNF-α signaling pathways through binding with Tom1 [17]. In recent years, with the application of gene knockout technology, more potential functions of Tollip proteins have been revealed, such as inhibiting the activity of the ST2L/IL-33/IRAK1 axis and STAT6 to reduce the IL-13-mediated pulmonary eosinophilia [18], sustaining mice neuron health by enhancing the completion of autophagy [19], and suppressing HIV-1 infection and regulating viral latency [20]. Together, these data suggest that Tollip is a multifunctional protein involving in various regulatory mechanisms of the host immune responses.

In teleost, Tollip genes have been only cloned and identified in a few species including Atlantic salmon (*Salmo salar*) [21], grass carp (*Ctenopharyngodon idellus*) [22], grouper (*Epinephelus coioides*) [23] and rainbow trout (*Oncorhynchus mykiss*) [24]. Protein structure analysis shows that the TBD domain, C2 domain, and CUE domain are conserved in fish [9]. Following viral infection, Tollip expression was found to be up-regulated in the liver of Atlantic salmon [21] or most immune-related tissues of grass carp [22], and the elevated expression of Tollip was also present in the liver of rainbow trout after the *Aeromonas salmonicida* infection [24]. These findings indicated that Tollip might be involved in the regulation of both viral and bacterial immune responses in teleost. The study *in vitro* on Tollip gene expression pattern in response to the different PAMPs stimulation and pathogens infection is essential to clarify the network regulation mechanism of the TLR signaling cascade in immune-related fish cells. However, to our knowledge, this information is still unknown in teleost fishes. Compared with mammals, the functional study of Tollip on the negative regulation of TLRs signaling was limited and only reported in grouper, which demonstrated that the over-expression of Tollip inhibited NF-κB signals activated by MyD88 in HEK293 cells under natural state [23]. However, whether fish Tollip impacts NF-κB signal regulation by the TLR adapter protein MyD88 under stimulation with PAMPs such as LPS or synthetic dsRNA poly I:C remains unclear. Therefore, the role of Tollip on the regulation in antiviral and antibacterial innate immune responses waits for further study in more teleost fishes.

Japanese eel (*Anguilla japonica*) is the commercially important species for aquaculture in Asia [25]. The eel industry has suffered huge economic losses caused by virus, parasite, and especially by a number of bacterial pathogens, such as *Aeromonas hydrophila* and *Vibrio vulnificus* [26]. Better understanding of the antiviral and the antibacterial immune mechanisms may contribute to the development of management strategies for disease control and long-term sustainability of eel farming. The activation of TLRs signaling pathways leads to the expression of immune and pro-inflammatory genes and plays an important role in host defense against invading various pathogens [1]. Unfortunately, the negative regulators of TLR pathways have not been described in *Anguilla*. In the present study, a Tollip homologue, *Aj*-Tollip, was cloned from Japanese eel and its characteristics of expression in response to various PAMPs and *Aeromonas hydrophila* infection was investigated both *in vivo* and *in vitro*. Subcelluar localization of *Aj*Tollip was detected in HEK293 cells and its potential function on the negative regulation of MyD88-dependent TLR signaling pathway was revealed by dual luciferase reporter assay in natural state, as well as under the stimulation of LPS or poly I:C. The findings presented in this study will provide more information for further insight into the regulating mechanisms of TLR signaling in the innate immune responses of bony fish upon viral and bacterial infection.

2. Materials and methods

2.1. Fish collection and immune challenge

Healthy Japanese eels, weighing 45–50 g, were purchased from an eel farm (Fuqing, China). They were kept in a 1000-L tank at 25 °C with recirculated and aerated water for a week to acclimate to laboratory conditions. The fish were first anesthetized by immersion in water containing 100 ppm Eugenol (The Second Reagent Factory of Shanghai) and then liver, spleen, gills, kidney, intestine, heart, skin, and muscle were harvested and frozen in liquid nitrogen and then stored at −80 °C for RNA extraction.

A. hydrophila, isolated from diseased eels in Fuqing, China, were inoculated in Tryptone soya broth (TSB) and incubated on a shaker at 28 °C for 24 h [26]. The bacteria were collected and diluted to the concentration of 4×10^4 cfu/mL in 0.01 mmol/L PBS (pH = 7.4). Fish immune stimulation was performed by intraperitoneal injection of 250 μL 4 mg/mL LPS (Sigma, USA) in phosphate buffered saline (PBS), 250 μL 2 mg/mL poly I:C (Sigma, USA) in PBS, and 250 μL 4×10^4 cfu/mL *A. hydrophila* in PBS, respectively. Fish injected with 250 μL PBS were used as controls. Four fish were sacrificed for the control group and experimental group for each time point. Liver, spleen and kidney of each group were collected at 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h after injection and preserved for quantitative real-time polymerase chain reaction (qRT-PCR).

2.2. Cell culture and treatments

For *in vitro* studies, Japanese eel liver cell line was cultured as described in our previous study [27]. Cells were then treated with 30 μg/mL LPS (Sigma, USA), 50 μg/mL poly I:C (Sigma, USA), 30 μg/mL CpG-DNA (Sangon Biotech, Shanghai, China), 30 μg/mL peptidoglycan (PGN, Sigma, USA), three different concentration of *A. hydrophila* (1×10^6 cfu/mL, 1×10^7 cfu/mL, and 1×10^8 cfu/mL, respectively), and the untreated cells were served as control. Four parallel samples were included of each group at 0 h, 3 h, 6 h, 12 h, 24 h, and 48 h after treatment. Total RNA from cells was isolated using E.Z.N.A.™ Total RNA Kit II (Omega, USA) following manufacturer's instructions.

2.3. Cloning of full-length cDNA of AjTollip

Total RNA from tissues was isolated using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol. Total RNA from cells was isolated using E.Z.N.A.™ Total RNA Kit II (Omega) following manufacturer's instructions. Total RNA from the liver of Japanese eel was used to synthesize the first-strand cDNA for the RACE reaction using the SMART RACE cDNA Amplification Kit (Takara, Japan) according to the manufacturer's instructions. Primers were designed according to the partial sequence of Tollip from the Japanese eel transcriptome database in our lab with a local version of the Primer Premier 5.0 design software tool (http://www.premierbiosoft.com/primerdesign/index.html) (Table 1) and PCR was performed to amplify the partial cDNA sequences of eel Tollip gene. The purified PCR product was inserted into the pMD19-T vector (Takara, Japan) and transformed into JM109 competent cells. The plasmids from positive clones were subjected to DNA sequencing by Sangon Biotech Corp (Shanghai, China). Based on the partial gene sequence of Tollip, the full-length cDNA sequence was pulled out using 5′and 3′ RACE System for Rapid Amplification of cDNA Ends (Takara, Japan) with gene-specific primers as listed in Table 1. The RACE PCR products were gel-purified, cloned, and sequenced as described above.

2.4. Bioinformatics analysis

Sequence similarity analysis was performed using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The full-length cDNA

Table 1
Primers used for *Aj*Tollip and *Aj*MyD88 gene cloning and expression analysis.

Target gene	Primer sequence
Primers for partial gene cloning	
5′-Tollip	5′- TGGCTACAAGGATCAGCACGCA -3′
3′-Tollip	5′- CAGCTCCTCGGTCATCTGAAGAAGA -3′
Specific primers for 5′ RACE and 3′ RACE	
Tollip-5′OUT	5′- CTCCCGCTCAGGCTGTACCACTCA -3′
Tollip-3′OUT	5′- CGACTGGGCTACGCCGTCTACGAAAC -3′
UPM	5′- CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT -3′
Tollip-5′IN	5′- TCGTAGACGGCGTAGCCCAGT -3′
Tollip-3′IN	5′- AATCAACTCTCTTCTTCAGATGACCGA -3′
NUP	5′- AAGCAGTGGTATCAACGCAGAGT -3′
Specific primers for head to toe PCR	
5′ORF-Tollip	5′- ATGGCTACAAGGATCAGCACGCA -3′
3′ORF-Tollip	5′- CAGCTCCTCGGTCATCTGAAGAAGA -3′
Specific primers for qRT-PCR	
5′real-Tollip	5′- TGGCGTGGATTCCTTCTACCT -3′
3′real-Tollip	5′- CCTTCGGGGATGGTGATGT -3′
5′-ßactin	5′- ATCGTGCGTGACATCAAGGA -3′
3′-ßactin	5′- GCTCGTTGCCGATGGTGAT -3′
Specific primers for pEGFP-N1 recombinant expression plasmid	
5′pEGFP-Tollip	5′- TTCGAATTCATGGCTACAAGGATCAGCACGCA -3′
3′pEGFP-Tollip	5′- GGTGGATCCCGCAGCTCCTCGGTCATCTGAAGAAGA -3′
5′pEGFP-MyD88	5′- GATCTCGAGATGGCTGACAGTTCGAGCATAGATTA -3′
3′pEGFP-MyD88	5′- TGGATCCCGGGAGAGACAGAGCTCTAGCCAGT -3′
Specific primers for pcDNA3.1 + recombinant expression plasmid	
5′pcDNA-Tollip	5′- GGATCCATGGCTACAAGGATCAGCACGCA -3′
3′pcDNA-Tollip	5′- GAATTCCTACAGCTCCTCGGTCATCTGAAGAAGA -3′
5′pcDNA-MyD88	5′- GGATCCATGGCTGACAGTTCGAGCATAGATTA -3′
3′pcDNA-MyD88	5′- GAATTCCTACGGGAGAGACAGAGCTCTAGCCA -3′

sequence of *Aj*Tollip was analyzed with ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The deduced amino acid sequence was analyzed using the ExPASy Molecular Biology server (http://www.us.expasy.org/tools/). Multiple sequence alignment was performed using the CLUSTALW program (http://www.ebi.ac.uk/clustaw/). The protein domain features were predicted using NCBI CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/docs/cdd_search.html). The phylogenetic tree was constructed using MEGA 5 software based on Neighbour-Joining method with the bootstrapping of 1000 repetitions.

2.5. Expression analysis of AjTollip by qRT-PCR

First strand cDNA was synthesized from total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Japan) following the manufacturer's instructions, and the genomic DNA was ruled out by the gDNA Eraser (with highly efficient DNAase activity for the gDNA degrading) provided by the Kit. Then, synthesized cDNA was diluted with nuclease-free water by 10-fold and stored at −20 °C until use. Primers for *Aj*Tollip and β-actin (endogenous control gene) were designed using Primer 5.0 software (Table 1) and tested to ensure the amplification of a single discrete band with no primer-dimers. The PCR product was also sequenced to verify the specificity of RT-PCR. The PCR reactions were set up in a total volume of 20 μL, containing 10 μL of 2 × AceQ® qPCR SYBR® Green Master Mix (Vazyme, China), 1 μL of the diluted cDNA, 0.5 μL of each primer (10 μM), and 8 μL of nuclease-free water. The amplification was performed on a Roche Light Cycler 480 machine (Roche, Sussex, UK), and the qRT-PCR conditions were as follows: an incubation at 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Dissociation analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The relative quantification of the target and reference genes was evaluated using standard curves. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to determine the relative mRNA expression level of *Aj*Tollip as described in our previous studies [27]. All data were given in terms of relative mRNA expression as arithmetic means ± standard error of the mean (SEM) of four separate individuals, each assayed in triplicate.

2.6. Subcellular localization

To examine the subcellular localization of *Aj*Tollip *in vitro*, the ORFs of *Aj*Tollip was cloned and inserted into pEGFP-N1 vector using corresponding primers (Table 1). The constructed recombinant plasmid was confirmed by sequencing as described above. HEK293 cells were seeded in 6-well plate and transfected with purified plasmids using Lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer's recommendations, with the pEGFP-N1 vector as a control. After 24 h, cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with 6-diamidino-2-pheny-lindole (DAPI) (1 mg/mL) as described in Ref. [28]. Samples were observed under confocal fluorescence microscopy (Leica).

2.7. NF-κB reporter gene activity

To study the effect of *Aj*Tollip overexpression on the regulation of NF-κB signaling pathway, the luciferase assay was performed using Dual-Glo luciferase assay system (Promega, USA) with pRL-TK vector (expressing Renilla luciferase under herpes simplex virus thymidine kinase promoter) employed as an internal control for normalization of transfection efficiency. The ORFs of *Aj*Tollip and *Aj*MyD88 were cloned and inserted into the plasmid vector pcDNA3.1+ (Invitrogen) using corresponding primers (Table 1). The HEK293 cells cultured in the 96-well plates were transfected with 20 ng pRL-TK reference plasmid, 80 ng NF-κB luciferase reporter plasmid (Genomeditech, China) and 300 ng of pcDNA-Tollip or pcDNA-MyD88 or both of 300 ng pcDNA-Tollip and pcDNA-MyD88 using Lipofectamine 2000 Reagent (Invitrogen, USA), and the pcDNA3.1 + empty vector untreated cells was served as control. After transfection for 24 h, cells were then treated 30 μg/mL LPS (Sigma, USA) and 50 μg/mL poly I:C (Sigma) for 12 h. Then the culture medium was aspirated off, and the transfected cells were washed once with PBS. Luciferase reporter gene activity was measured using the Dual-Luciferase Kit (Promega, USA). Relative luciferase activity was calculated as the ratio of firefly to Renilla

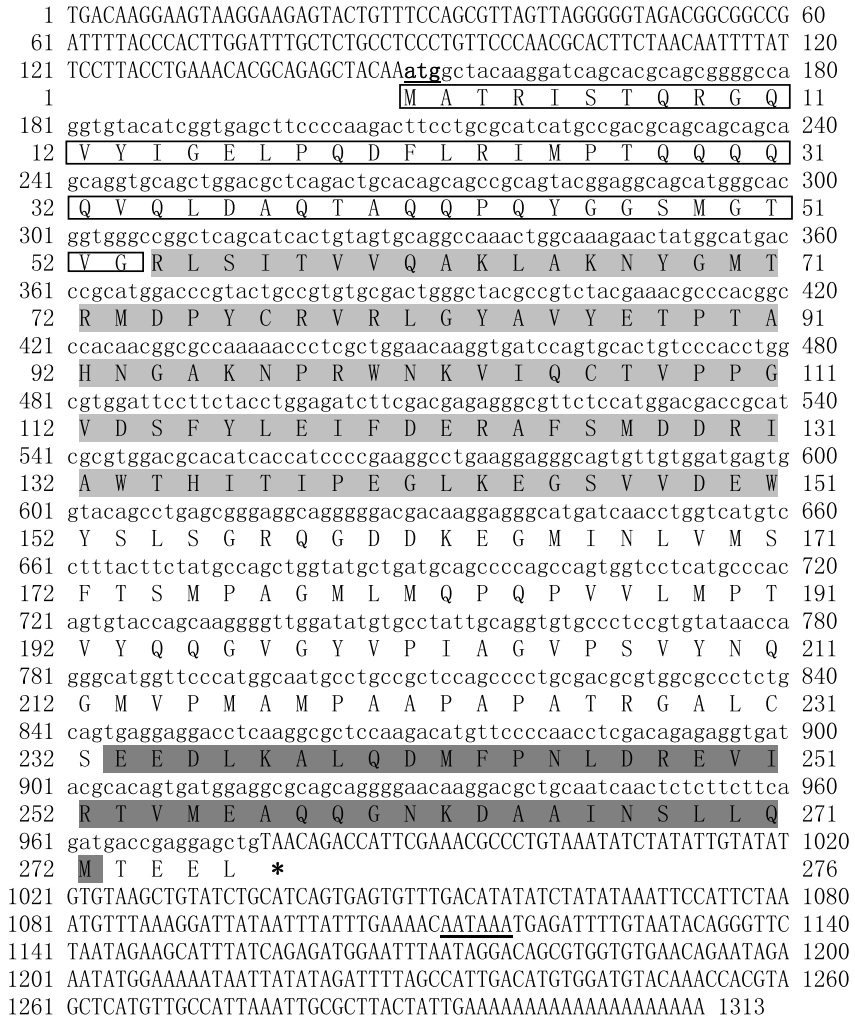

Fig. 1. Nucleotide and deduced amino acid sequences of *Aj*Tollip. The nucleotide and amino acid sequences were numbered on the left. The start codon (ATG) was in bold under line and stop codon (TAA) was marked with an asterisk. The polyadenylation signals AATAAA were under line. The N-terminal TBD domain was boxed (1–53 aa). The C2 domain was marked with light grey (54–151 aa). The C-terminal CUE domain was shaded in dark grey (233–272 aa).

luciferase activity and expressed as arithmetic means ± standard error of the mean (SEM). Each experiment was conducted for three independent samples in triplicate.

2.8. Statistical analysis

Data from all experiments were analyzed with SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). Significance of differences was determined by Student's *t*-test. *P* values smaller than 0.05 were considered as statistically significant.

3. Results

3.1. cDNA sequence of AjTollip

The full-length cDNA sequence of *Aj*Tollip (GenBank accession No: KF990164) was composed of 1313 bp with a 5′-untranslated region (UTR) of 148 bp, an open reading frame (ORF) of 831 bp encoding, and a 3′-UTR of 334 bp within a eukaryotic polyadenylation signal (AATAAA) (Fig. 1). The ORF encoded a 276 amino acids protein with a calculated molecular mass of 30.6 kDa and a theoretical isoelectric point of 5.04. *Aj*Tollip contained a typical Tollip structure including Tom1-binding domain (TBD) (1-53aa), the conserved 2 domain (C2) (54–151 aa) and the coupling of ubiquitin to ER degradation (CUE) domain (233–272 aa) (Fig. 1).

3.2. Structural analysis of AjTollip

Multiple alignments and amino acid sequence similarity comparison of Japanese eel *Aj*Tollip with Tollip in zebra fish, rainbow trout, medaka, African clawed frog, mouse, and human were shown in Fig. 2. Similar domain organization of Tollip proteins was as follows: The N-terminal TBD domain, the C2 domain, and the C-terminal CUE domain. The putative protein of *Aj*Tollip has an identity with that of rainbow trout (91%), zebrafish (89%), mouse (86%), medaka (84%), human (82%), and African clawed frog (79%). Using structure modeling with the template of Tollip from human (PDB code: 1wgl), the predicted three-dimensional structure of *Aj*Tollip is very similar to that of human Tollip and consists of five alpha helixes and 11 beta folds (Fig. 3).

3.3. Phylogenetic analysis

To determine the evolutionary position of *Aj*Tollip, phylogenetic trees were constructed using the NJ method (Fig. 4.). *Aj*Tollip was clustered with other bony fish Tollips to form one branch, and the mammals with the bird and reptile were clustered to another branch, whereas the amphibian formed the transition type.

3.4. Expression of AjTollip mRNAs in tissues

The constitutive expressions of *Aj*Tollip in the tissues of healthy

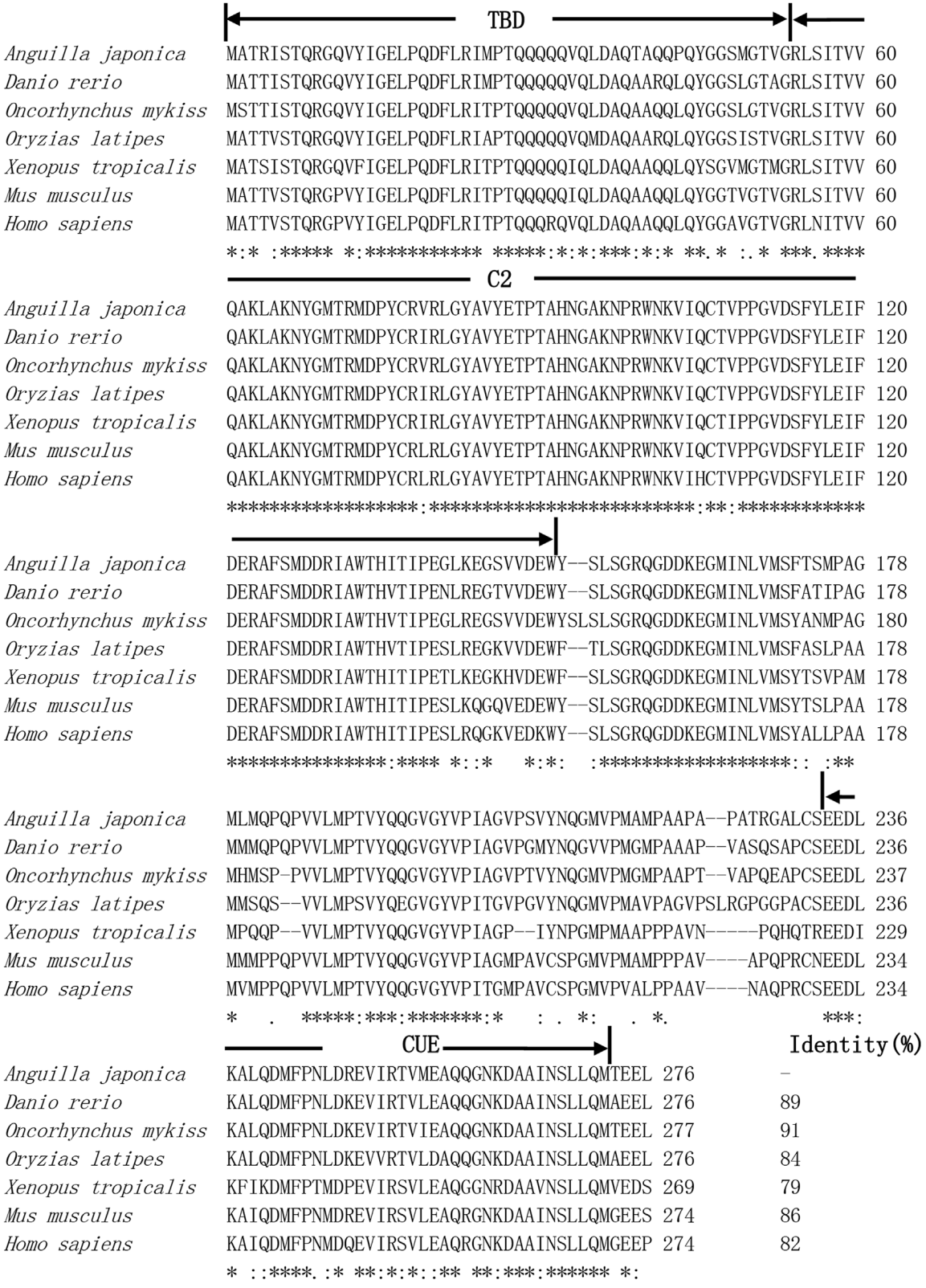

Fig. 2. Multiple alignment of the *Aj*Tollip amino acid sequences with other reported Tollips. Identical (*) and similar (: or.) residues identified by the ClustalW program were indicated. The amino acid sequences of Tollip from *Anguilla japonica* (GenBank accession n. KF990164), Danio rerio (GenBank accession n. Q7ZV43.1), Oncorhynchus mykiss (GenBank accession n. NP_001117892), Oryzias latipes (GenBank accession n. XP_004084776), Xenopus tropicalis (GenBank accession n. NP_001006923), Mus musculus (GenBank accession n. NP_076253), Homo sapiens (GenBank accession n. NP_061882). The N-terminal TBD domain, the C2 domain, and the C-terminal CUE domain was marked with arrow.

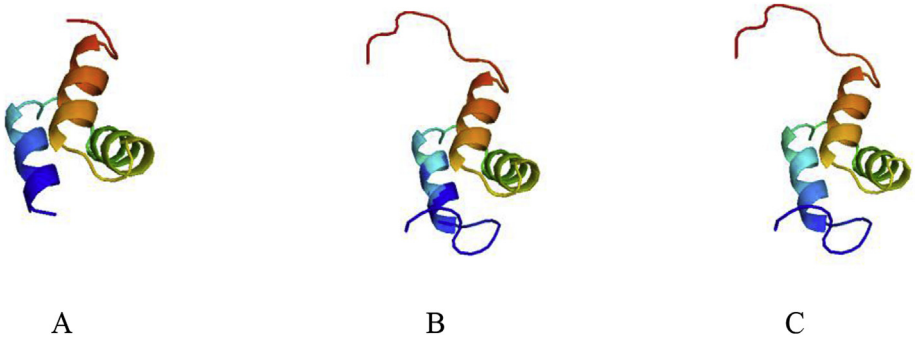

Fig. 3. Predicted tertiary structures of *Aj*Tollip and human Tollip, obtained using the SWISS MODEL website http://swissmodel.expasy.org/. (A) Ribbon diagrams of *Aj*Tollip. (B) Ribbon diagrams of *Aj*Tollip overlapped with human Tollip. (C) Ribbon diagrams of human Tollip.

Japanese eels were determined by qRT-PCR. The *Aj*Tollip transcript was broadly expressed in the liver, spleen, kidney, gills, skin, intestine, heart, and muscle. The highest expression was observed in the liver, followed by the spleen, kidney, gills, skin, intestine, and relatively lower expression in the heart and muscle (Fig. 5).

3.5. The temporal expression pattern of AjTollip in liver, spleen and kidney after immune activation

To determine the effects of *Aj*Tollip on the antibacterial and antiviral responses, mRNA expression levels of *Aj*Tollip in the liver, spleen and kidney of Japanese eels were quantified by qRT-PCR at 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h after LPS, poly I:C, and *A. hydrophila* challenge.

The expression change of *Aj*Tollip was presented in Fig. 6. After injection with LPS, the *Aj*Tollip expression levels were shown to increase significantly in the liver and kidney and no obvious change was found in the spleen (Fig. 6A). For the liver tissue, the expression was significantly up-regulated at 12 h (2.4 folds, $P < 0.01$) and 72 h (1.8 folds, $P < 0.01$) after treatment with LPS. For the kidney tissue, the expression was significantly up-regulated at 12 h (1.4 folds, $P < 0.05$) and 72 h post-injection (1.5 folds, $P < 0.01$). With the infection of *A. hydrophila*, the mRNA levels of *Aj*Tollip were found to increase in the liver and kidney (Fig. 6B). For the liver tissue, significant up-regulation

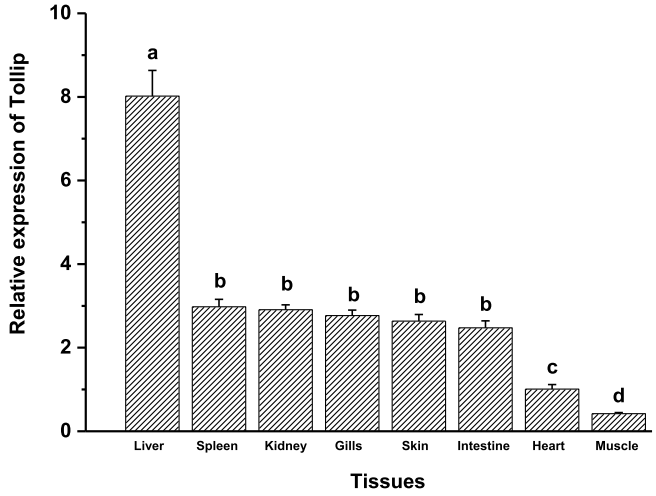

Fig. 5. Relative expression of *Aj*Tollip in different tissues of healthy Japanese eel, include liver, spleen, kidney, gills, skin, intestine, heart, and muscle, respectively. The mean values marked by the different letter indicated significant difference ($P < 0.05$).

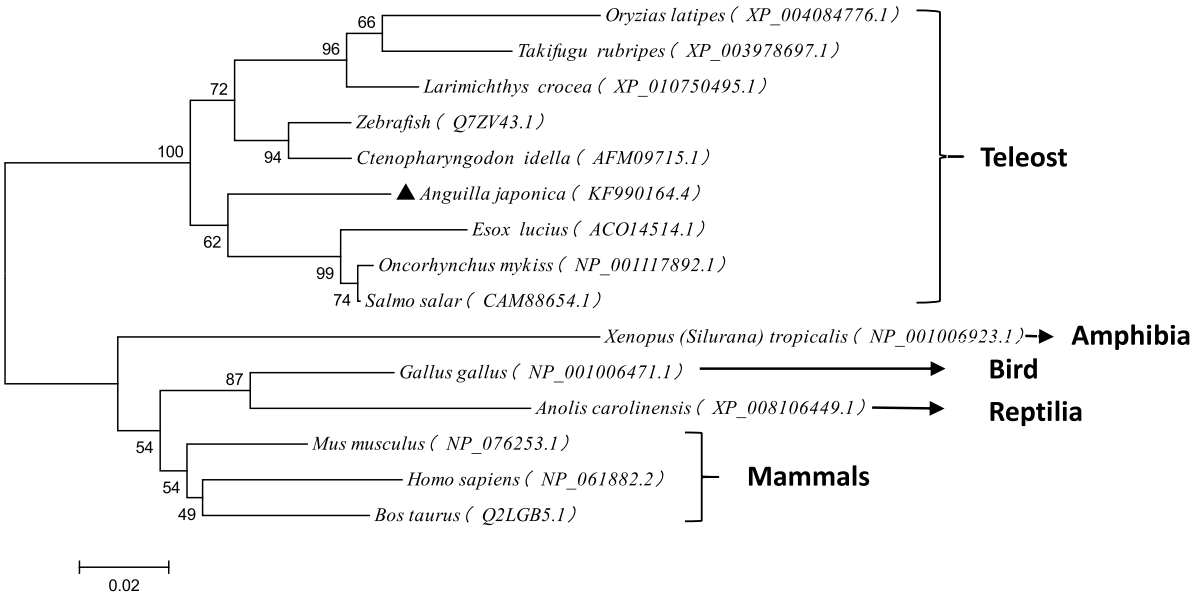

Fig. 4. Phylogenetic tree of *Aj*Tollip and other vertebrates Tollips. The phylogram was constructed with the MEGA5 program for the entire amino acid sequence by using the neighbour-joining (NJ) method. The numbers at the relevant branches refer to bootstrap values from 1000 replications. The accession numbers of the Tollip sequences were indicated in parentheses after the species names. The *Aj*Tollip was marked with a solid triangle.

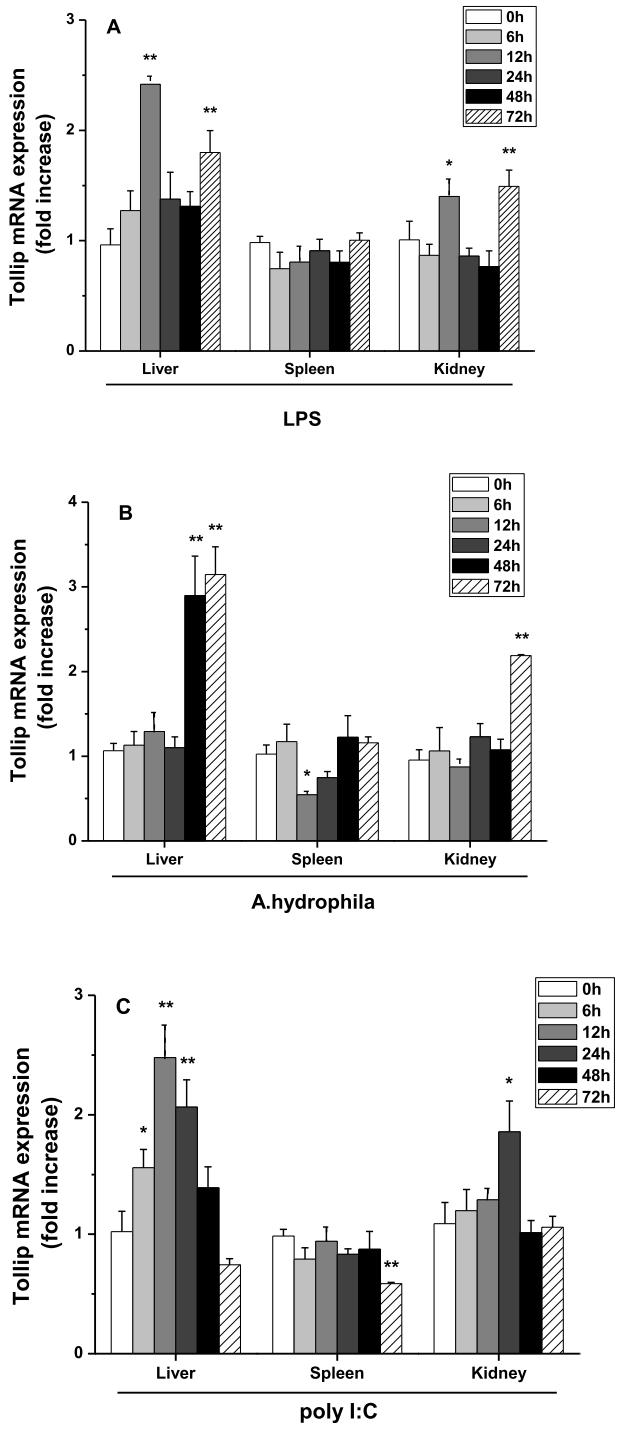

Fig. 6. Temporal expression profile of the *Aj*Tollip transcripts in the liver, spleen and kidney of Japanese eel at 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h after LPS (A), *A. hydrophila* (B), and poly I:C (C) challenge. The *Aj*Tollip expression level in LPS, *A. hydrophila*, and poly I:C treated samples was normalized to that in the PBS control group. Data are expressed as means ± SEM of four separate individuals, each assayed in triplicate. An asterisk (*) indicates a significant difference ($P < 0.05$) in expression level relative to the PBS treatment at the same sampling time and two asterisks (**) indicates ($P < 0.01$).

of *Aj*Tollip gene expression was shown at 48 h (2.9 folds, $P < 0.01$) and 72 h (3.1 folds, $P < 0.01$) post infection. For the kidney tissue, the expression was significantly up-regulated at 72 h (2.2 folds, $P < 0.01$) post infection. For the spleen tissue, the expression was significantly down-regulated only at 12 h (2.0 down folds, $P < 0.01$) and no significant changes in *Aj*Tollip mRNA expression in all other time points ($P > 0.05$). After stimulation with poly I:C, the up-regulation of *Aj*Tollip gene expression was observed in the liver and kidney (Fig. 6C). For the liver tissue, the expression was up regulated at 6 h by poly I:C (1.6 folds, $P < 0.05$), 12 h (2.5 folds, $P < 0.01$) and 24 h (2.1 folds, $P < 0.01$). For the kidney tissue, the expression was significantly up-regulated by poly I:C at 24 h post-injection (1.8 folds, $P < 0.05$). For the spleen tissue, a significant down-regulation of the gene expression was observed at 72 h (1.7 down folds, $P < 0.01$) and no obvious changes were found at other time points ($P > 0.05$) after treatment with poly I:C.

3.6. The temporal expression pattern of AjTollip in vitro after viral/bacterial PAMPs stimulation and different concentration of A. hydrophila infection

In order to understand the modulation of *Aj*Tollip expression in response to different pathogen associated molecular patterns (PAMPs), the transcriptional levels of *Aj*Tollip in Japanese eel liver cells were assessed by qRT-PCR after treatment with LPS, poly I:C, CpG-DNA, PGN (Fig. 7A) and different concentration of *A. hydrophila* infection (Fig. 3B) at 0 h, 3 h, 6 h, 12 h, 24 h and 48 h.

The expression change of *Aj*Tollip was presented in Fig. 7. As illustrated in Fig. 7A, with the treatment of LPS, the significant up-regulation of *Aj*Tollip expression was found at 3 h (1.2 folds, $P < 0.05$). Poly I:C was found to induce *Aj*Tollip gene expression at 6 h (1.6 folds, $P < 0.01$), 12 h (1.4 folds, $P < 0.01$), and 48 h (1.2 folds, $P < 0.01$). CpG-DNA was shown to significantly up-regulate the *Aj*Tollip expression at 3 h (1.7 folds, $P < 0.01$), 6 h (2.0 folds, $P < 0.01$), 12 h (1.4 folds, $P < 0.05$), and 24 h (1.3 folds, $P < 0.05$). PGN was shown to significantly enhance the *Aj*Tollip expression from 6 h to 48 h with the folds 2.3, 2.2, 1.3, 1.5 and 1.2, respectively ($P < 0.01$). As illustrated in Fig. 7B, the significant increase of *Aj*Tollip mRNA expression was found at 6 h under the 1×10^6 cfu/mL *A. hydrophila* infection (1.3 folds, $P < 0.01$). When the concentration of *A. hydrophila* was up to 1×10^7 cfu/mL, *Aj*Tollip expression was significantly induced from 6 h to 48 h with the folds 1.6, 1.5, 1.3 and 1.2, respectively ($P < 0.01$). Under the highest concentration of *A. hydrophila* (1×10^8 cfu/mL) infection, the expression was significantly up-regulated at 3 h (1.7 folds, $P < 0.01$), 6 h (2.5 folds, $P < 0.01$), and 12 h (1.6 folds, $P < 0.01$), and down-regulated expression was observed at 24 h (1.2 down folds, $P < 0.01$) and 48 h (1.3 down folds, $P < 0.01$).

3.7. The temporal expression pattern of AjMyD88 in vitro after viral/bacterial PAMPs stimulation and different concentration of A. hydrophila infection

In order to understand the modulation of *Aj*MyD88 expression in response to different pathogen associated molecular patterns (PAMPs), the transcriptional levels of *Aj*Tollip in Japanese eel liver cells were assessed by qRT-PCR after treatment with LPS, poly I:C, CpG-DNA, PGN (Fig. 8A) and different concentration of *A. hydrophila* infection (Fig. 8B) at 0 h, 3 h, 6 h, 12 h, 24 h and 48 h.

The expression change of *Aj*MyD88 was presented in Fig. 8. As illustrated in Fig. 8A, no significant change of *Aj*MyD88 expression was found with the treatment of LPS ($P > 0.05$). Poly I:C was found to induce *Aj*MyD88 gene expression at 12 h (1.4 folds, $P < 0.01$) and 24 h (1.2 folds, $P < 0.01$). CpG-DNA was shown to significantly up-regulate the *Aj*MyD88 expression at 6 h (1.3 folds, $P < 0.01$). PGN was shown to significantly enhance the *Aj*MyD88 expression at 3 h (1.2 folds, $P < 0.05$), 6 h (1.3 folds, $P < 0.01$), and 24 h (1.2 folds, $P < 0.05$). As illustrated in Fig. 8B, no significant change of *Aj*MyD88 mRNA expression was found in all time points under the 1×10^6 cfu/mL *A. hydrophila* infection ($P > 0.05$). When the concentration of *A. hydrophila* was up to 1×10^7 cfu/mL, *Aj*MyD88 expression was significantly induced at 24 h (1.3 folds, $P < 0.01$) and 48 h (1.2 folds, $P < 0.01$) whereas a significant decrease expression was found at 3 h (1.3 down

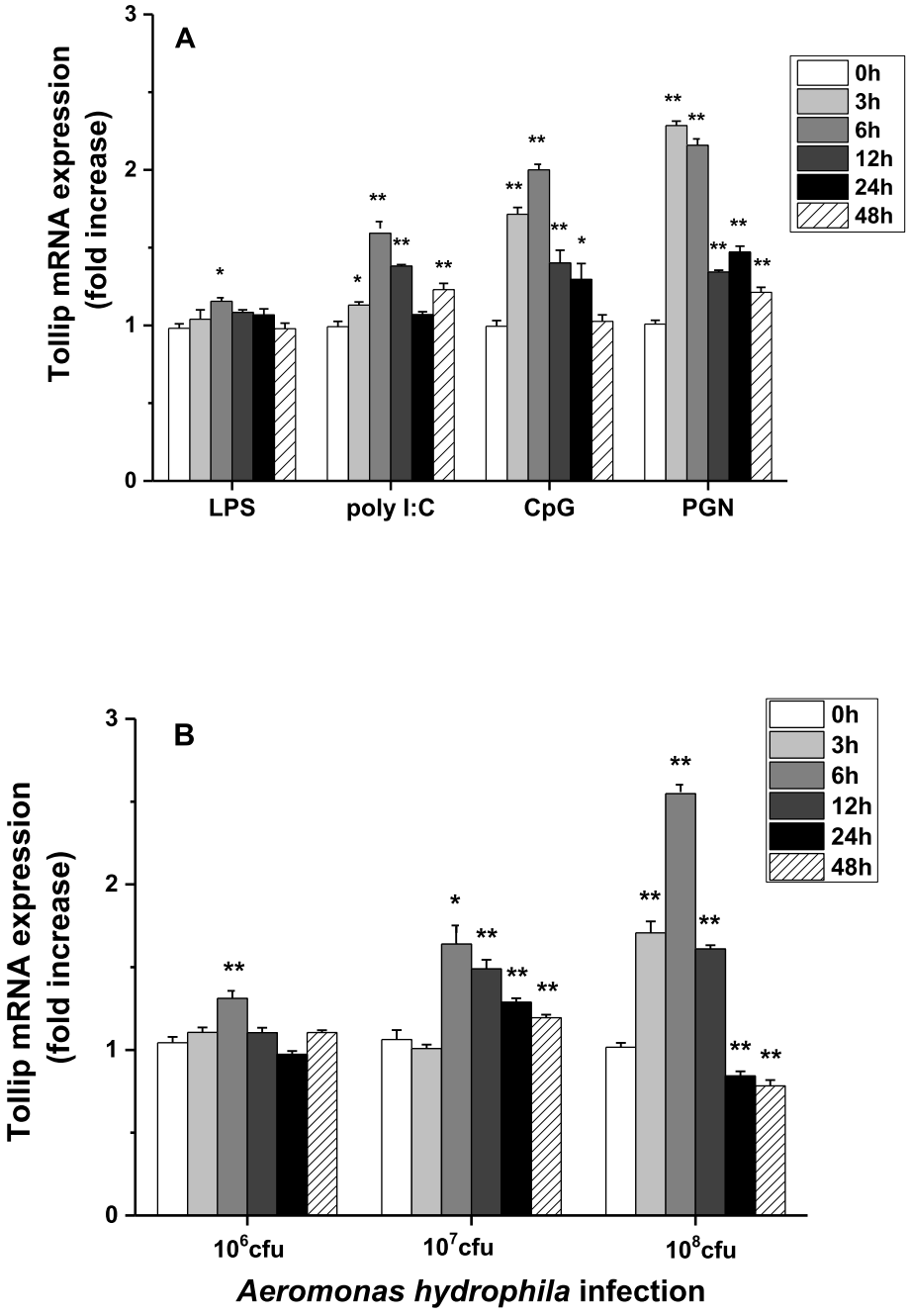

Fig. 7. Temporal expression profile of the *Aj*Tollip transcripts in Japanese eel liver cells at 0 h, 3 h, 6 h, 12 h, 24 h and 48 h after treatment with LPS, poly I:C, CpG-DNA and PGN (A), and infection with three different concentration of *A. hydrophila* (1×10^6 cfu/mL, 1×10^7 cfu/mL, and 1×10^8 cfu/mL) (B). The *Aj*Tollip expression level in LPS, poly I:C, CpG-DNA, PGN treated samples and the different concentration of *A. hydrophila* samples were normalized to that in the PBS control group, respectively. Data are expressed as means ± SEM of four separate individuals, each assayed in triplicate. An asterisk (*) indicates a significant difference ($P < 0.05$) in expression level relative to the PBS treatment at the same sampling time and two asterisks (**) indicates ($P < 0.01$).

folds, $P < 0.05$). Under the highest concentration of *A. hydrophila* (1×10^8 cfu/mL) treatment, the expression was observed to significantly decrease at 3 h (1.3 down folds, $P < 0.01$) but increase at 48 (1.2 folds, $P < 0.01$).

3.8. Subcelluar localization of AjTollip in HEK293 cells

Protein subcellular localization is a key functional characteristic and tightly concerned with its function. To determine the localization of *Aj*Tollip, the recombinant plasmid pEGFP-*Aj*Tollip was constructed and transfected in HEK293 cells. As shown in Fig. 9, *Aj*Tollip was distributed throughout the whole cytoplasm in a condensed form while a few of *Aj*Tollip protein were expressed in the nucleus with a condensed state. *Aj*MyD88 was only distributed in cytoplasm in a condensed form. As a contrast, GFP alone was uniformly distributed in both the cytoplasm and nucleus.

3.9. AjTollip inhibited NF-κB luciferase reporters in HEK293 cells

Mammalian Tollips are negative regulators of the NF-κB signaling pathway and MyD88 is one of the important molecules in NF-κB signaling pathway. As shown in Fig. 10, in natural state, the basic NF-κB activity of the control group was significantly inhibited by *Aj*Tollip with 79.6% ($P < 0.01$) and enhanced by *Aj*MyD88 with 1.97 folds ($P < 0.05$). When co-transfected with MyD88, *Aj*Tollip significantly inhibited 81.4% of the MyD88-induced NF-κB activity and 63.6% of the NF-κB activity of control group ($P < 0.01$); After stimulation with LPS, the basic NF-κB activity of the control group was significantly inhibited by *Aj*Tollip with 87.9% ($P < 0.01$) and enhanced by *Aj*MyD88 with 1.56 folds ($P < 0.05$). When co-transfected with MyD88, *Aj*Tollip significantly inhibited 92.8% of the MyD88-induced NF-κB activity and 89.1% of the basic NF-κB activity of control group ($P < 0.01$); When treatment with poly I:C, the NF-κB activity of the control group was

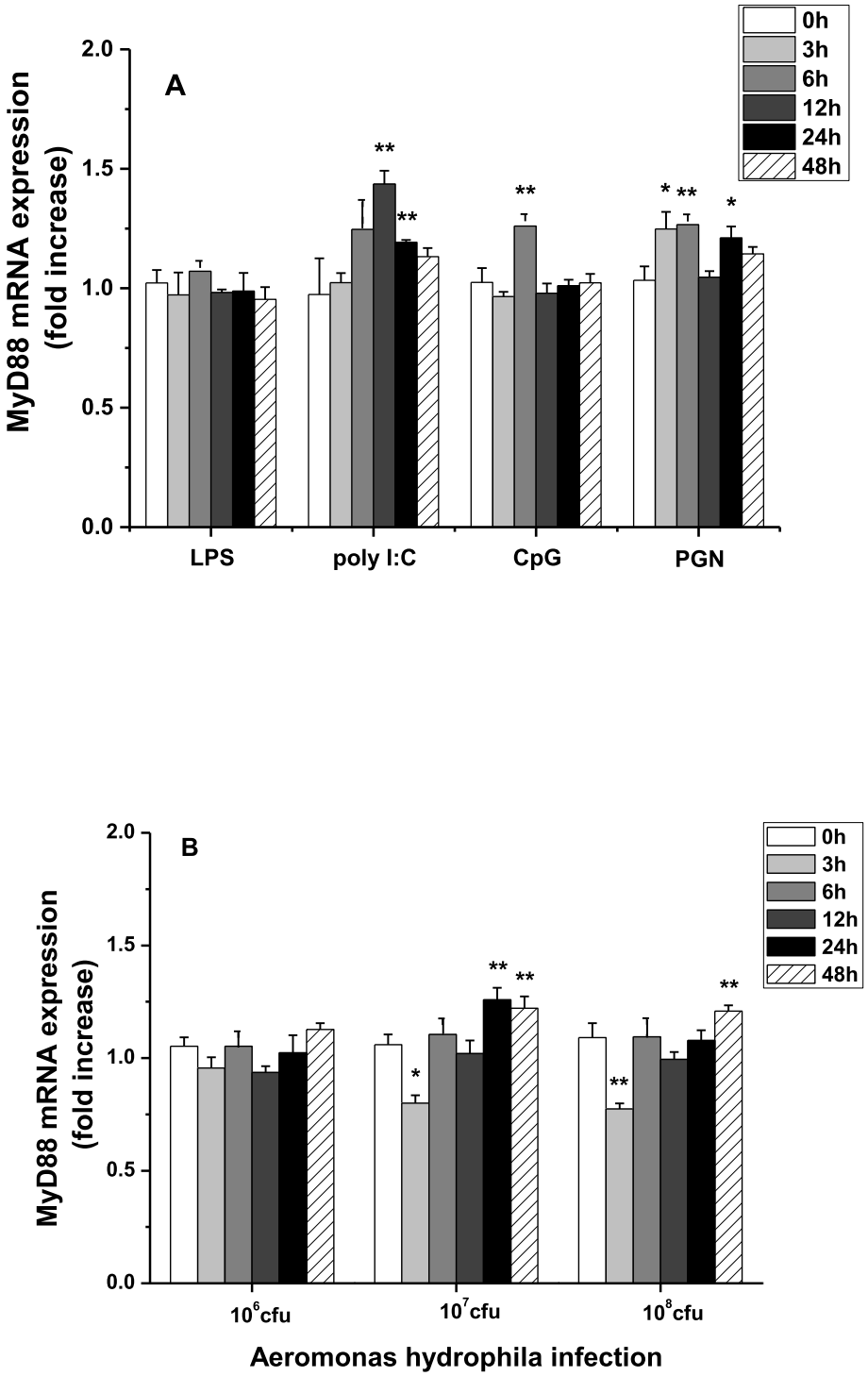

Fig. 8. Temporal expression profile of the *Aj*MyD88 transcripts in Japanese eel liver cells at 0, 3, 6, 12, 24 and 48 h after treatment with LPS, poly I:C, CpG-DNA and PGN (A), and infection with three different concentration of *A. hydrophila* (1×10^6 cfu/mL, 1×10^7 cfu/mL, and 1×10^8 cfu/mL) (B). The *Aj*MyD88 expression level in LPS, poly I:C, CpG-DNA, PGN treated samples and the different concentration of *A. hydrophila* samples were normalized to that in the PBS control group, respectively. Data are expressed as means ± SEM of four separate individuals, each assayed in triplicate. An asterisk (*) indicates a significant difference ($P < 0.05$) in expression level relative to the PBS treatment at the same sampling time and two asterisks (**) indicates ($P < 0.01$).

significantly inhibited by *Aj*Tollip with 89.6% ($P < 0.01$) and enhanced by *Aj*MyD88 with 1.93 folds ($P < 0.01$). When co-transfected with MyD88, *Aj*Tollip significantly inhibited 93.8% of the MyD88-induced NF-κB activity and 88.0% of the basic NF-κB activity of control group ($P < 0.01$).

4. Discussion

Toll-like receptors (TLRs) recognize distinct pathogen-associated molecular patterns (PAMPs) and play a critical role in innate immune responses, functioning as the first line of defense against invading pathogens through activating signaling cascade to induce the expression of immune and pro-inflammatory genes [1]. In order to avoid detrimental and inappropriate inflammatory response, many intracellular negative regulators have been identified, such as Tollip, MyD88s, SOCS1, and A20, which mediated the negative regulation at various stages of the TLR signaling cascade [4–6,8]. However, the immunosuppressive regulators of TLRs signaling pathway in *Anguilla* still remain unknown. In the present study, the homologue of Tollip was cloned from Japanese eel and its gene expression patterns and regulating functions were investigated.

The ORF of *Aj*Tollip contained 831 bp and encoded a polypeptide of 276 amino acids with the predicted molecular mass of 30.6 kDa. The deduced amino acids sequence of *Aj*Tollip shared high identities

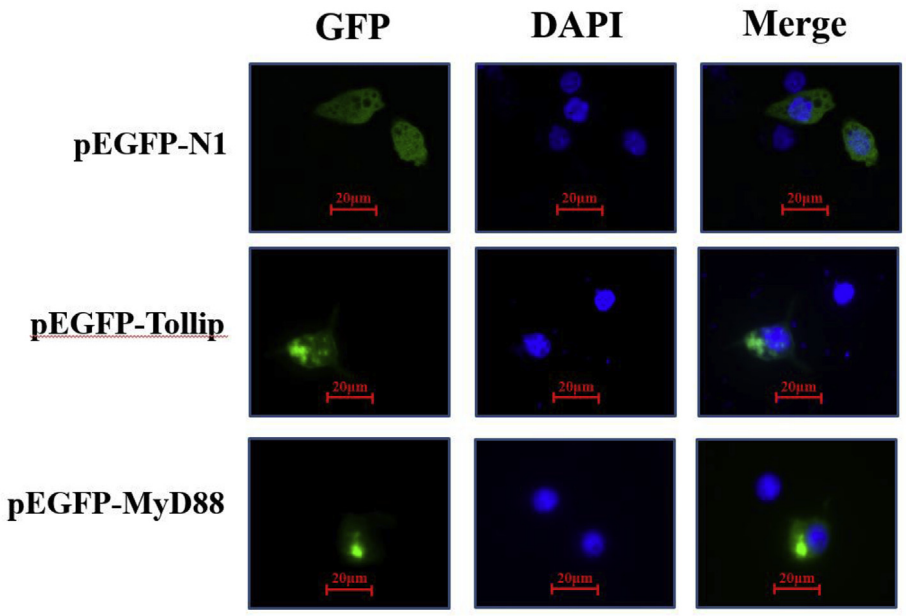

Fig. 9. Intracellular localization of Japanese eel *Aj*Tollip and *Aj*MyD88 by fluorescence microscopy. HEK293 cells was transfected with pEGFP-N1 (upper row), pEGFP-Tollip (middle row), or pEGFP-MyD88 (lower row) and the nucleus was stained with DAPI.

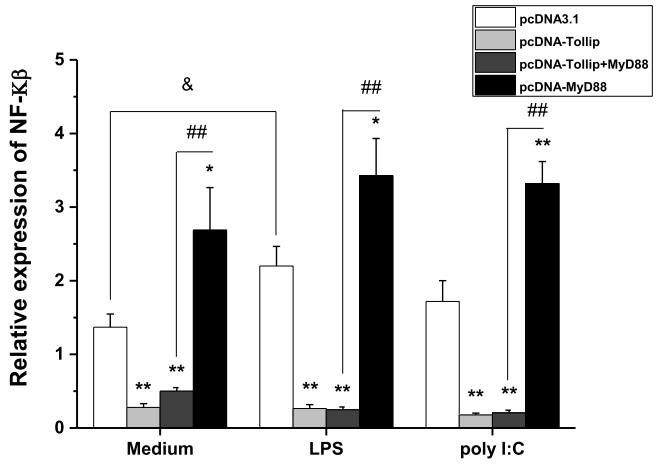

Fig. 10. Regulation of *Aj*Tollip on the NF-κB signaling pathway under natural state, LPS stimulation, and poly I:C stimulation. Cells were transfected with pcDNA3.1 + (300 ng), pcDNA-Tollip (300 ng), pcDNA-Tollip (300 ng) and pcDNA-MyD88(300 ng), and pcDNA-MyD88(300 ng), respectively. An asterisk (*) indicates a significant difference ($P < 0.05$) in expression level relative to the pcDNA3.1 + group and two asterisks (**) indicates ($P < 0.01$). "##" indicates highly significant ($P < 0.01$) differences between pcDNA-MyD88 (300 ng) group and pcDNA-Tollip (300 ng) and pcDNA-MyD88(300 ng) co-transfection group. "&" indicates significant ($P < 0.05$) difference between natural pcDNA3.1 + group and LPS stimulated pcDNA3.1 + group.

(79%–91%) with other reported Tollips. According to the phylogenetic tree, *Aj*Tollip was clustered with other bony fish Tollips to form one branch, indicating its relatively conserved evolutionary status. NCBI CDD analysis showed that *Aj*Tollip contained TBD domain, C2 domain and the C-terminal CUE domain and the multiple alignments suggested that these three domains were an evolutionary conserved class from bony fish to mammals. It has been confirmed that Tollip interacts with Tom1, endosomes, and ubiquitinated proteins through the N-terminal TBD including the Gln-rich stretch, the central region including the C2 domain, and the C-terminal CUE domain, respectively [16]. The CUE domain of Tollip was found to inactivate IRAK signaling by targeting it for ubiquitination [5]. The highly consistent three-dimensional structure between *Aj*Tollip and human Tollip further supported our speculation that *Aj*Tollip may have similar function on the ubiquitination of IRAK or other related signaling molecules [5,17,29].

As an important endocytic adaptor protein involved in protein ubiquitination, *Aj*Tollip gene expression was found in various tissues (Fig. 5). This universal distribution was similar to the Tollip in mouse [9] and other Tollip homologues from bony fish, such as Atlantic salmon [21], grass carp [22] and rainbow trout [24], suggesting that the constitutive expression pattern of Tollip is prevalent in teleost fishes. The highest abundance of *Aj*Tollip expression was observed in the liver which conformed to the results from grass carp [22]. In rainbow trout, the highest expression of Tollip was present in spleen and very low expression in the liver, and this might be concerned with the species specificity [24].

A. hydrophila is a prevalent pathogen to the European eel (*Anguilla anguilla*), Japanese eel, and American eel (*Anguilla rostrata*) and infections from this pathogen have resulted in hemorrhagic septicemia, red body disease, and liver hepatomegaly and bleeding [30]. In the present study, the *Aj*Tollip expression was strongly enhanced in liver post infection of *A. hydrophila* (Fig. 6B), which is consistent with the report in rainbow trout infected by *A. salmonicida* [24]. The high *Aj*Tollip expression in the liver might be related to the control of the inflammation reaction to avoid detrimental and inappropriate inflammatory responses. As the primary structure on the surface of bacteria, LPS induced elevated expression of *Aj*Tollip in liver (Fig. 6A), which further supported that the liver serves as a critical role in the innate immune response against the bacterial infection. Compared with the liver and the kidney, no increase of *Aj*Tollip expression was found in the spleen under *A. hydrophila* infection or LPS stimulation (Fig. 6A and B). Our previous studies demonstrated that a large number of Ig^+ cells were observed in the spleen and some in the kidney of eel, and no Ig^+ cells were present in the liver [31]. Hence, the spleen might be mainly associated with the specific immune response in Japanese eel. *In vitro*, the up-regulation of *Aj*Tollip expression (Fig. 7B) as well as the *Aj*MyD88 (Fig. 8B) was observed in Japanese eel liver cells under high concentration (1×10^7 cfu/mL, and 1×10^8 cfu/mL) of *A. hydrophila* infection, which indicated that the antibacterial immune response was evoked in the cells. One explanation of the correlation expression changes of *Aj*Tollip and *Aj*MyD88 might be that these two adaptors are jointly involved in the regulation of immune-related signal cascades

[23]. However, the study from human primary alveolar epithelial and nasal cells shows that no obvious change of the Tollip expression appeared following infection with *Staphylococcus aureus* (1.1×10^5 cfu/mL and 1.6×10^5 cfu/mL), which could partly be explained by differential cell types and pathogens resulting in the diverse regulatory mechanism of the host immune responses.

LPS is an outer-membrane component of gram-negative bacteria that can be recognized by TLR4 to trigger the activation of nuclear factor kappa B (NF-κB) signaling resulting in induction of genes involved in inflammatory responses [32]. In this study, the *Aj*Tollip expression was significantly enhanced in the liver and kidney by LPS stimulation (Fig. 6A) as the first evidence *in vivo* for fish in response to LPS. *In vitro*, the inductive expression of *Aj*Tollip gene was noted after LPS stimulation (Fig. 7A). This data is consistent with the evidence in the THP-1 cells, bovine endometrial epithelial cells, and human primary blood mononuclear cells, in which an increase of Tollip protein abundance is induced after LPS challenge [33,34]. However, no change of the major Tollip transcript was detected in mouse macrophages after LPS treatment [14], which may be due to the cell types or the various concentration of LPS. Together the data of *in vivo* and *in vitro* in this study, *Aj*Tollip might be involved in the regulation of the signal cascade induced by LPS.

As a major element of gram-positive bacterial cell walls, PGN significantly induces the *Aj*Tollip expression (Fig. 7A) as well as the *Aj*MyD88 in Japanese eel liver cells (Fig. 8A), indicating that the PGN could be recognized by liver cells of Japanese eel and evoke its immune response. In mammalian vertebrates, TLR2-mediated recognition of PAMPs from Gram-positive bacteria such as LTA or peptidoglycan (PGN) results in recruitment of a set of TIR domain-containing adaptor proteins such as MyD88 and in turn activates the NF-κB signaling cascades [35]. Ribeiro et al. [36] demonstrated that carp TLR2 could bind with PGN from *Staphylococcus aureus*, and the PGN was capable to induce the TLR2 gene expression in carp macrophages and lead to the phosphorylation of MAPK-p38 as well as the production of radical. Therefore, the precise regulation mechanisms of *Aj*Tollip on the TRL2 signaling pathway induced by PGN stimulation in Japanese eel liver cells remains to be clarified.

CpG-containing oligodeoxynucleotides (ODNs) are known to trigger the TLR/IL-1R signaling pathway via the molecules myeloid differentiation marker 88 (MyD88) and tumor necrosis factor receptor–-associated factor 6 (TRAF6), leading to the activation of NF-κB and MAPKs signal cascades [37]. In mammals, TLR9 was characterized as the receptor for the recognition of CpG motifs in bacterial and viral genomes to mediate the subsequent activation of cellular immune responses [1]. Although the direct evidence of the interaction between CpG ODN and TLR9 in bony fish remains ambiguous [38], results from Japanese flounder show that TLR9 could recognize CpG ODN resulting in the induction of TNF-a gene expression and the up-regulation expression of TLR9 and MyD88 were detected in blood, gill, kidney and spleen after *Edwardsiella tarda* challenge [39]. In the present study, the gene expression of *Aj*Tollip and *Aj*MyD88 were quickly up-regulated to the peak at 6 h by CpG DNA (Figs. 7A and 8A), which suggested that *Aj*Tollip might be involved in the regulation of the MyD88-dependent signal cascade triggered by CpG DNA in Japanese eel liver cells.

Viral PAMPs mainly include double strand RNA (dsRNA), or synthetic dsRNA (poly I:C), and single strand RNA (ssRNA), which are mainly recognized by TLR3 and TLR7/TLR8, respectively [40]. Rebl et al. [21] reported that Tollip expression was strongly induced in liver of rainbow trout (*Oncorhynchus mykiss*) after VHVS infection. Wei et al. found [10] that Singapore grouper iridovirus (SGIV) could induce the Tollip expression in the spleen and head kidney of grouper. In line with reports from rainbow trout and grouper, the significant up-regulation of *Aj*Tollip gene expression was observed in kidney and in liver with stimulation of poly I:C (Fig. 6C), indicating that *Aj*Tollip involved in the host immune response against viral infection. *In vitro*, poly I:C induced an increase expression of both *Aj*Tollip and *Aj*MyD88 in Japanese eel liver cells (Figs. 7A and 8A), this data is consistent with our previous study in which the gene expression of *Aj*TRL3 and *Aj*IFN were up-regulated by poly I:C stimulation [41,42]. These findings suggested that *Aj*Tollip might play a critical role in the regulation of antiviral immune response in Japanese eel. Wei et al. [10] observed that over-expressed Tollip of grouper inhibited SGIV replication during viral infection in grouper spleen cells. Hence, the function of *Aj*Tollip against viral infection in the Japanese eel liver cells needs to be confirmed.

Interestingly, the significant down-regulation of *Aj*Tollip transcription was found in the liver cells at 24 h an 48 h post its elevated expression from 3 h to 12 h under high concentration (1×10^8 cfu/mL) of *A. hydrophila* infection (Fig. 7B). The down-regulation of *Aj*Tollip expression was also detected in the spleen after stimulation by *A. hydrophila* and poly I:C (Fig. 6B and C). These findings suggested that Tollip expression should be under strict control so that the innate immune cells could retain the ability to be fully activated during infection. In mammals, the mechanism of transcription regulation of Tollip gene is still not very clear. Yutaka et al. [43] reported that Elf-1, as the special transcription factor of T lymphocytes, negatively regulated Tollip gene expression in the human monocytic cell line THP-1 cells but not in epithelial colonic adenocarcinoma cell line Caco-2. In mouse dendritic cells (DCs) and macrophages, Ezh1 was found to suppress the transcription of Tollip by directly targeting the proximal promoter of Tollip leading to an increase in the TLR-triggered production of type I IFN, IL-6, and TNF-a [44]. The recent study showed that the miR-31 located in the region of Tollip 3′-UTR (+1876/+2398) could suppress the translation of the Tollip gene in mice small IECs revealing that Tollip expression is strongly regulated at the post-transcriptional level [45]. To date, the regulation of Tollip gene expression in bony fish is still unknown. Whether the similar control strategies exist in fish or not remains to be further studied.

Protein subcellular localization is a key functional characteristic and tightly concerned with its function. In grass carp, Tollip was uniformly distributed and co-localized with IRAK-1 in the cytoplasm of CIK cells. When infected with viral, Tollip and IRAK-1 both trended toward the cell membrane partly in a condensed state, which indicated that IRAK-1 can interact with Tollip [22]. In this study, both *Aj*Tollip and *Aj*MyD88 were distributed in the cytoplasm in a condensed state (Fig. 9) in line with the report in grouper [23], which suggested that these two important adaptors perform their functions in cytoplasm for the regulation of TLRs signaling pathway.

In mammals, the activation of the TLR signaling pathway through ligand binding triggers a signaling cascade involving a variety of intracellular signaling adaptors including MyD88, IRAK, and TRAF6. MyD88 is critical for the signaling from all TLRs except TLR3. Upon stimulation with ligands, MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK-4) to TLRs through interaction of the death domains of both molecules. IRAK-1 recruited through IRAK-4 is activated by phosphorylation and associates with TRAF6, thereby activating the IKK complex and leading to activation of MAP kinases (JNK, p38 MAPK), NF-κB or interferon regulatory transcription factors, which in turn mediate inflammation through the production of inflammatory cytokines, type I IFN, chemokines and antimicrobial peptides [32]. Tollips are known to shut down MyD88-dependent signaling pathway by the inactivation of IRAK-1 [5]. In the present study, the *Aj*MyD88 transcripts of Japanese eel liver cells were significantly enhanced by poly I:C, CpG-DNA, and PNG (Fig. 8A) or the stimulation of the high concentration of *A. hydrophila* (1×10^7 cfu/mL and 1×10^8 cfu/mL) (Fig. 8B). These findings supported that *Aj*MyD88 plays a critical role in fish defense against viral and bacterial infection [46]. Interestingly, the expression pattern of *Aj*Tollip induced by I:C, LPS, CpG-DNA, and PGN or the different concentration of *A. hydrophila* infection (Fig. 7A and B) was found to be coincident with the expression modulation of *Aj*MyD88 in Japanese eel liver cells (Fig. 8A and B). This intriguing observation may possibly be concerned with the function of *Aj*Tollip in balancing the TLR signaling cascade induced by MyD88, thus protecting host

excessive immune response [5]. With this idea, the luciferase activity assay in HEK293 cells was performed to reveal the interaction between *Aj*Tollip and *Aj*MyD88 in NF-κB signal regulating network in natural state or under the stimulation of LPS and poly I:C.

In natural state, the overexpression of *Aj*Tollip in HEK293 cells significantly inhibited the activities of NF-κB luciferase reporter (Fig. 10). When *Aj*MyD88 and *Aj*Tollip were co-transfected into HEK293 cells, the luciferase activity of NF-κB was also significantly inhibited. This finding is in agreement with the report of the Tollip protein in Pacific white shrimp (*Litopenaeus vannamei*) [11] and in Grouper [23] and provides the evidence that *Aj*Tollip impairs MyD88-dependent NF-κB activation in mammalian cells.

LPS is an outer-membrane component of gram-negative bacteria that can be recognized by TLR4 on the surface of the host cell [47]. When LPS is recognized by cell surface receptor TLR4, two signal axes were involved in the activation of NF-κB. One is MyD88 dependent pathway by the association of IRAK1 with TRAF6, another is TRIF-dependent induction of TRAF6 signaling by RIP1 [1]. Bulut et al. [15] reported that when the human dermal endothelial cells (HMEC) were stimulated with LPS, the NF-κB activity was significantly enhanced, and the overexpression of Tollip was found to inhibit the TLR4-mediated NF-κB activation in a dose-dependent manner. In teleost, Rebl et al. [13] demonstrated that the overexpression of the trout Tollip was found to suppress TLR4-mediated NF-κB and SAA promoter activation in HEK293 under infection with *Escherichia coli*. In the present study, in contrast to the natural state, the NF-κB activity of HEK293 cells was enhanced with the stimulation of LPS (Fig. 10) which is in line with the observation in human dermal endothelial cells (HMEC) [15], indicating that NF-κB signal pathway was evoked by LPS. The induced NF-κB activity was down-regulated by the overexpression of *Aj*Tollip, which suggested that *Aj*TOLLIP was involved the negatively regulation of TLR4 signaling induced by LPS [48]. Although the overexpression of *Aj*MyD88 enhanced the NF-κB activity, the increased NF-κB activity was also significantly inhibited when co-transfected with *Aj*Tollip. This finding provided the direct evidence that the MyD88-dependent pathway of TLR4 activation was inhibited by the *Aj*Tollip. Even so, whether the *Aj*Tollip has the effect on the TRIF-dependent induction of TRAF6 signaling of TLR4 activation remains unclear. Furthermore, Zhang et al. [5] reported that Tollip could associate directly with TLR4 and plays an inhibitory role in TLR-mediated cell activation with the stimulation of LPS. Further study should be performed by the co-immunoprecipitation of co-transfected *Aj*Tollip and human TLR4 in HEK293 cells.

The present study showed that no elevated NF-κB activity was induced post poly I:C stimulation in HEK293 cell (Fig. 10), which is consistent with the reports from Matsumoto et al. [49]. Matsumoto et al. [49] reported that poly I:C failed to enhance the activity of NF-κB and IFN-β in HEK293 cells, but NF-κB and IFN-β were activated when transfected with a human TLR3 expression vector. In line with the results in natural state group and LPS simulation group, *Aj*Tollip was also shown to inhibit the NF-κB activity induced by the overexpression of *Aj*MyD88 post poly I:C stimulation (Fig. 10). These data collectively suggested that *Aj*Tollip was a negative regulator of MyD88-dependent TLR signaling cascade in mammalian cells and supported that Tollip functions as a key regulator of TLR signaling in an evolutionary conserved manner [12].

In conclusion, a homologue of Tollip was identified from Japanese eel. *Aj*Tollip transcripts were broadly expressed in all examined tissues with the highest level in liver and the lowest level in heart and muscle. *In vivo*, the expression of *Aj*Tollip in liver and kidney was significantly up-regulated in response to LPS, poly I:C, and *A. hydrophila* challenge. *In vitro*, its expression can be induced in liver cells infected with LPS, poly I:C, CpG-DNA, and PGN. Transfected into HEK293 cells, both *Aj*Tollip and *Aj*MyD88 were distributed in a condensed form in the cytoplasm. *Aj*Tollip could negatively regulate the *Aj*MyD88-dependent TLR signaling in HEK293 cells in natural state or under the stimulation of LPS and poly I:C. It is collectively suggested that *Aj*Tollip functions as a negative regulator of MyD88-dependent TLR signaling and plays an important role in fish defense against viral and bacterial infection.

Acknowledgments

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