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Transcriptome-wide identification, functional characterization, and expression analysis of two novel invertebrate-type Toll-like receptors from disk abalone (*Haliotis discus discus*)

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ABSTRACT

Toll-like receptors (TLRs) are well-known pattern recognition receptors that play key immunological roles in a diverse range of organisms. In this study, two novel invertebrate TLRs from disk abalone (designated as AbTLR-A and AbTLR-B) were identified and functionally characterized for the first time. AbTLR-A and AbTLR-B comprised the typical TLR domain architecture containing an extracellular leucine-rich repeat domain, transmembrane domain, and Toll/interleukin-1 receptor domain. Expressional analysis revealed that both TLRs were constitutively expressed at all the early embryonic stages of disk abalone analyzed, with the highest level of *AbTLR-A* found at the 16-cell stage and *AbTLR-B* at the trochophore stage. According to tissue distribution analysis, prominent mRNA expression of *AbTLR-A* and *AbTLR-B* was detected in the hemocytes and gills, respectively. *AbTLR-A* and *AbTLR-B* mRNAs were significantly up-regulated in response to Gram-negative *Vibrio parahemolyticus*, Gram-positive *Listeria monocytogenes*, and viral hemorrhagic septicemia virus injections in abalone hemocytes and gills. Overexpression of AbTLR-A and AbTLR-B in HEK293T cells directly activated nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) responsive reporters. Neither TLRs showed a high response to pathogen-associated molecular patterns *in vitro*. Co-expression of AbTLR-A and AbTLR-B with AbMyD88-2 and AbMyD88-X activated NF-κB-responsive reporters in a synergetic manner. These findings demonstrate the involvement of AbTLR-A and AbTLR-B in abalone innate immunity.

1. Introduction

Multicellular organisms are invariably threatened by various pathogenic invasions and have evolved defense systems to combat these invaders to ensure host protection. Innate immunity provides the first-line defense against invading microorganisms or foreign materials and plays a critical role in eliminating infective pathogens in the host. Pathogen recognition is an important step in innate immunity and is accomplished by various germline-encoded pattern recognition receptors (PRRs) on effector cells including macrophages, dendritic cells, and B cells [1,2]. PRRs recognize microbial components known as pathogen-associated molecular patterns (PAMPs) in a diverse range of pathogenic microbes [3]. PAMP detection by PRRs triggers numerous physiological events, such as phagocytosis, opsonization, apoptosis induction, complement activation, and proinflammatory signaling pathways [3]. Distinct classes of PRRs have been identified in mammals, including RIG-I-like receptors, nucleotide-binding oligomerization domain-like receptors, C-type lectin receptors, AIM2-like receptors, and Toll-like receptors (TLRs) [4]. Among these, TLRs are known to be the well characterized PRR class.

The TLR family was initially identified in fruit fly (*Drosophila melanogaster*) as a gene product involved in regulating embryonic dorsoventral axis development and antifungal responses [5]. To date, 13 TLRs have been identified in mammals, whereas various numbers of TLRs have been reported in non-mammals including fish [6] and birds [7]. Structurally, a typical TLR molecule comprised an extracellular leucine-rich repeat (LRR) domain, transmembrane domain, and Toll/interleukin-1 receptor (TIR) domain [3]. The extracellular LRR domain

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is composed of varying numbers of LRR motifs and is involved in PAMP recognition [3,8]. The cytoplasmic TIR domain has also been identified in a number of cytoplasmic and transmembrane proteins and is responsible for downstream signal transduction and receptor localization [9]. Depending on the primary sequence and ligand specificity, vertebrate TLRs are categorized into several subfamilies: TLR1/2/6/10, TLR3, TLR4, TLR5, TLR7/8/9, and TLR11/12/13/21/22/23 [2,10]. Upon ligand recognition by the appropriate TLR, the receptor undergoes dimerization and initiates two different downstream signaling pathways based on the nature of the recruited adaptor molecule into the TIR domain of the TLR [4,11]. The myeloid differentiation primary response protein 88 (MyD88)-dependent and MyD88-independent pathways are well-described TLR signal transduction pathways in mammals and both are responsible for activating transcription factors including NF-κB and AP-1 as well as interferon regulatory factors, ultimately leading to the expression regulation of cytokines and type I interferons [4,12].

Genomic studies of invertebrates revealed that most genes encoding innate immune receptors are homologous to those in vertebrates. However, massive expansion of these gene families including TLRs or TLR-like genes was observed within their (invertebrate) genomes. For instance, 222, 83, and 72 TLR-encoding genes were identified in the genomes of purple sea urchin (*Strongylocentrotus purpuratus*) [13], Pacific oyster (*Crassostrea gigas*) [14], and amphioxus [15], respectively. To date, various TLR homologs have been identified in marine invertebrates including Yesso scallop (*Patinopecten yessoensis*) [16], Pacific oyster [17], triangle-shell pearl mussel (*Hyriopsis cumingii*) [18,19], Zhikong scallop (*Chlamys farreri*) [20], giant tiger shrimp (*Penaeus monodon*) [21], *Litopenaeus vannamei* [22], noble scallop (*Chlamys nobilis*) [23], and Chinese venus (*Cyclina sinensis*) [24]. The mRNAs of invertebrate TLRs are widely expressed in different tissues and undergo significant modulations upon stimulation by Gram-negative bacteria [17,22], Gram-positive bacteria [24], viruses [18], and immune stimulators [20,23]. Functional studies focusing on the mechanism of action, structure-function relationship, and inflammatory responses of TLRs in invertebrates are relatively limited compared to mammalian studies. However, some studies have attempted to determine the function of invertebrate TLRs using mammalian systems. For example, TLRs from Pacific oyster were overexpressed in HEK293 cells and their subcellular localization and ability for NF-κB activation were determined [25]. Moreover, the functions of the TLR from Zhikong scallop were characterized *in vitro* using HEK293T cells [26]. Additionally, overexpression of triangle-shell pearl mussel TLRs or their specific domains in Drosophila S2 cells revealed their possible functions in the Toll signaling pathway [18,19].

Disk abalones are popular and key species in the aquaculture industry of eastern Asia including Korea. However, abalones are highly vulnerable to the spread of infectious diseases caused by bacteria [27], viruses [28], and parasites [29] because of the high stock densities and poor environmental conditions in abalone aquafarms, leading to mass mortalities and ultimately economical loss. Because they lack a well-developed acquired immune system, invertebrate species including abalones largely rely on their innate immune system. Thus, detailed studies of the abalone innate immune system will enable introduction of new and efficient disease prevention strategies. In this study, two novel TLR homologs were identified and characterized at the molecular level. The mRNA expression of these two TLRs was examined at different early embryonic stages of abalones. To investigate their roles in post-innate immune responses, the transcriptional levels of both TLRs were determined under immune stimulation by bacteria and virus *in vivo*. Furthermore, both TLR homologs were overexpressed in HEK293T cells for functional characterization *in vitro*.

2. Materials and methods

2.1. Identification of coding sequences of abalone TLRs

The coding sequences of abalone TLRs were obtained from a previously constructed disk abalone transcriptome database [30] using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information (NCBI).

2.2. Bioinformatics analysis

Open reading frames (ORFs) and deduced amino acid (aa) sequences of AbTLR-A and AbTLR-B were determined using UGENE software [31]. Physiochemical properties and availability of signal peptides of deduced AbTLR-A and AbTLR-B were determined with the ExPaSy ProtParam [32] and signalP 4.1 [33] servers, respectively. The domain architectures of AbTLR-A and AbTLR-B were predicted by the simple modular architecture research tool (SMART) [34] and NCBI conserved domain servers. The ClustalW [35] and EMBOSS needle [36] tools were used to perform multiple sequence and pairwise sequence alignments of entire or TIR domains of abalone TLRs with other TLR homologs. The phylogenetic tree was constructed by the neighbor joining method using MEGA 6.06 software [37]. Three-dimensional (3D) structures were predicted by the ITASSER online server [38] and then visualized using BIOVIA Discovery Studio software 2016.

2.3. Experimental animals, collection of different developmental stages, challenge experiment and tissue isolation

Disk abalones (average weight of 50 g) were purchased from the Youngsoo abalone farm in Jeju Island, Republic of Korea. Abalones were acclimatized in circulating water tanks with sand-filtered aerated seawater at a temperature of 20 ± 1 °C and salinity of 34 ± 0.6‰. During the rearing period, abalones were fed daily with marine seaweed, *Undaria pinnatifida*.

In order to examine the expression of *AbTLR-A* and *AbTLR-B* during abalone development, different embryonic stages including egg, sixteen cell stage at 3 h post fertilization (pf), morula (4 h 30 min pf), gastrula (6 hpf), trochophore (16 hpf), early veliger (24 hpf), middle veliger (36 hpf) and late veliger (48 hpf) stages were obtained. Samples of each stage collected were washed with ice cold PBS (phosphate-buffered saline) and stored at -80 °C.

The hemocytes, gills, muscles, digestive tract, hepatopancreas, and mantle were collected from five un-challenged animals to analyze the tissue-specific mRNA expression of *AbTLR-A* and *AbTLR-B*.

To investigate immune responses, the mRNA expression profiles of *AbTLR-A* and *AbTLR-B* were determined after immune stimulation with live bacteria and virus *in vivo*. Briefly, for the bacterial challenge, 100 μL of Gram-negative *Vibrio parahemolyticus* and Gram-positive *Listeria monocytogenes* (1×10^4 colony-forming units/mL) suspensions were intramuscularly injected into the abalones. For the viral challenge, animals were intramuscularly injected with 100 μL of viral hemorrhagic septicemia virus (VHSV) (1×10^8 plaque-forming units/mL) suspension. Additionally, 100 μL of sterile saline was intramuscularly injected into a separate abalone group as an injected control. Another abalone group was maintained as an un-injected control. Hemocytes and gills were collected from four animals at 3, 6, 12, 24, 48, 72, and 120 h post-injection (p.i.) of each challenge group and tissues were immediately snap-frozen in liquid nitrogen prior to storage at −80 °C until RNA extraction.

2.4. Total RNA extraction and cDNA synthesis

Total RNA was extracted from a pool of tissues collected from five un-challenged abalones and four immune challenged abalones from each experimental group corresponding to each p.i. using TRI Reagent®

Table 1
Primers used in this study.

Name	Target	Purpose	Primer sequence (5′-3′)
AbTLR-A-qF	*AbTLR-A*	qPCR	TCAACCGATGAGGTGCTTGAGGAA
AbTLR-A-qR	*AbTLR-A*	qPCR	AGGTCCAACCATGTAACGCCCATA
AbTLR-B-qF	*AbTLR-B*	qPCR	CATACCGATGGCGTTGGCACAT
AbTLR-B-qR	*AbTLR-B*	qPCR	GCAGGTTATTCCGTATCCACCCAGA
AbRib-F	*AbRib*	qPCR internal control	TCACCAACAAGGACATCATTTGTC
AbRib-R	*AbRib*	qPCR internal control	CAGGAGGAGTCCAGTGCAGTATG
AbTLR-A-cF	*AbTLR-A*	Cloning to pcDNA3.1	$(GA)_3$ggtacccaccATGGCTAATGGCTGTTGGATC
AbTLR-A-cR	*AbTLR-A*	Cloning to pcDNA3.1	(GA)3gaattcCTATCTCTGCAAACATTGTTTAAGTCGGATCCAG
AbTLR-B-cF	*AbTLR-B*	Cloning to pcDNA3.1	$(GA)_3$ggatcccaccATGGATATGATGCACATGTCCACTC
AbTLR-B-cR	*AbTLR-B*	Cloning to pcDNA3.1	$(GA)_3$gaattcTCATCGTTGCAAACAATGCTGGAGC
AbMyD88-2-cF	*AbMyD88-2*	Cloning to pcDNA3.1	$(GA)_3$gaattccaccATGGCGTCTAATTCGGACGACG
AbMyD88-2-cR	*AbMyD88-2*	Cloning to pcDNA3.1	$(GA)_3$ctcgagTCAACACTGACTGGATGGGAACAG
AbMyD88-X-cF	*AbMyD88-X*	Cloning to pcDNA3.1	$(GA)_3$gaattccaccATGGAAGCGCCACCTGAGATAAAG
AbMyD88-X-cR	*AbMyD88-X*	Cloning to pcDNA3.1	$(GA)_3$ctcgagTCATCTCTGGTTATTCTTTATGGCTTCCCT

(Sigma-Aldrich, St. Louis, MO, USA). The concentration and purity of extracted RNA were determined spectrophotometrically using a Multiskan GO microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA). Next, the first-strand cDNA was synthesized from each RNA sample (2.5 μg) using a PrimeScript™ First-Strand cDNA Synthesis Kit (TaKaRa, Japan) according to the manufacturer's instructions. The synthesized cDNA was diluted at a 1:40 ratio and stored at −20 °C until further experiments.

2.5. Construction of expression plasmids

In vitro functional analysis of AbTLR-A and AbTLR-B was conducted by overexpressing the proteins in mammalian cells. The ORFs of AbTLR-A and AbTLR-B were amplified from synthesized cDNA using TaKaRa Ex Taq™ DNA polymerase (TaKaRa) using gene-specific primers containing appropriate restriction recognition sites (AbTLR-A: *Kpn*I and *Eco*RI; AbTLR-B: *BamH*I and *Eco*RI) (Table 1) for ligation into pcDNA™3.1(+) (Invitrogen, Carlsbad, CA, USA) vectors, yielding the AbTLR-A/pcDNA™3.1(+) and AbTLR-B/pcDNA™3.1(+) constructs. The ORFs of abalone MyD88-2 (AbMyD88-2) and MyD88-X (AbMyD88-X) were amplified by PCR using forward and reverse primers with restriction recognition sites for *Eco*RI and *Xho*I, respectively (Table 1). The obtained products and pcDNA™3.1(+) vectors were digested with *Eco*RI and *Xho*I followed by ligation into pcDNA™3.1(+) to yield the AbMyD88-2/pcDNA™3.1(+) and AbMyD88-X/pcDNA™3.1(+) constructs. All plasmid constructs were verified by sequencing and isolated using the QIAfilter™ Plasmid Midi Kit (Qiagen, Hilden, Germany) prior to transfection.

2.6. Quantitative real-time PCR (qPCR)

The mRNA expression profiles of *AbTLR-A* and *AbTLR-B* at different developmental stages, tissues of un-challenged abalones, and hemocytes and gills of immune challenged abalones were analyzed by qPCR. To normalize *AbTLR-A* and *AbTLR-B* expression, abalone ribosomal protein L5 (*AbRib*) was selected as the qPCR internal control gene (GenBank accession: EF103443) [39]. Target-specific primers were designed using the PrimerQuest online tool (https://sg.idtdna.com/PrimerQuest/Home/Index). Following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines [40], the qPCR assay was carried out using the TP950 Thermal Cycler Dice™ real-time system (TaKaRa). The reaction was performed in a total volume of 10 μL containing 1.2 μL of sterilized PCR-grade water, 3 μL of synthesized cDNA as described in section 2.4, 5 μL of 2X SYBR® Premix Ex Taq™ (TaKaRa), and 0.4 μL of each primer (10 μM). Thermal cycler conditions were 5 min at 95 °C followed by 40 cycles of 5 s at 95 °C, 10 s at 58 °C, 20 s at 72 °C, and one cycle of 5 s at 95 °C, 30 s at 58 °C, and 15 s at 95 °C. Each assay was performed in triplicate. The specificity of the amplified products was determined by corresponding melting curve analysis. Expression levels of target genes were calculated according to the Livak method [41]. For the *in vivo* immune challenge experiment, relative expression levels of *AbTLRs* were expressed as fold-changes compared to the saline-injected control.

2.7. Cell culture

Human embryonic kidney 293T (HEK293T) cells were routinely cultured in Dulbecco's Modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and appropriate antibiotics. Cells were maintained at 37 °C in a CO_2 (5%) humidified incubator.

2.8. Transfection, ligand treatment, and luciferase assay

To evaluate the functional roles of AbTLR-A and AbTLR-B, both abalone TLRs were overexpressed in HEK293T cells and activation of transcription factors such as NF-κB and AP-1 was determined by a luciferase assay in the presence or absence of TLR ligands. Briefly, HEK293T cells were grown in 12-well plates at a density of 2×10^5 cells/well for 24 h at 37 °C. Next, the cells (80% confluence) were transiently transfected with 1 μg of total DNA/well consisting 0.7 μg of AbTLR-A/pcDNA™3.1(+) or AbTLR-B/pcDNA™3.1(+) or empty pcDNA™3.1(+), 0.25 μg of NF-κB (pNL3.2.NF-κB-RE; Promega, Madison, WI, USA) or AP-1 (pGL4.44, Promega) luciferase reporter vectors and 0.05 μg of normalization vector pRL-TK (Promega). Transfection was performed using the Mirus TransIT-X2® Dynamic Delivery System (Mirus Bio LLC, Madison, WI, USA) following the manufacturer's instructions. After 24 h of transfection, the cells were stimulated with 250 and 500 ng/mL of LPS (*Escherichia coli* 055:B5; Sigma), poly I:C (Sigma), peptidoglycan (PGN) from *Staphylococcus aureus* (Sigma) and CpG ODN2006 (0.25 and 0.5 μM) (InvivoGen, San Diego, CA, USA). The cells were incubated for 12 h at 37 °C and luciferase activity was measured via The GloMax® 96 Microplate Luminometer (Promega) using a luciferase assay kit (Biotium, Fremont, CA, USA) following the manufacturer's instructions. Each assay was performed in triplicate.

2.9. AbTLR-A and AbTLR-B association with AbMyD88-2 and AbMyD88-X to activate downstream transcription factors

TLRs are known to be involved in various inflammatory responses via transcription factor activation; to evaluate, this we overexpressed abalone TLRs together with cytoplasmic adaptor molecules, such as MyD88. To examine transcription factor activation by abalone TLRs and MyD88s, HEK293T cells were seeded into 12-well plates at a density of 2×10^5 cells/well. After 24 h, the cells (80% confluency) were transiently transfected with 1 μg of plasmid DNA containing,

0.3 μg of AbTLR-A/pcDNA™3.1(+) or AbTLR-B/pcDNA™3.1(+) or AbMyD88-2/pcDNA™3.1(+) or AbMyD88-X/pcDNA™3.1(+) or empty pcDNA™3.1(+) and of 0.15 μg of NF- κB luciferase reporter vectors and 0.05 μg of normalization vector pRL-TK. For co-expression, constant amounts (0.3 μg) of AbMyD88-2/pcDNA™3.1(+) or AbMyD88-X/pcDNA™3.1(+) were co-transfected with increasing amounts (0.3–0.5 μg) of AbTLR-A/pcDNA™3.1(+) or AbTLR-B/pcDNA™3.1(+) and 0.15 μg of NF-κB luciferase reporter vectors and 0.05 μg of normalization vector pRL-TK. The total DNA amount was adjusted to 1 μg using empty pcDNA™3.1(+) vectors. Transfection was performed using the Mirus TransIT-X2® Dynamic Delivery System (Mirus Bio LLC) following the manufacturer's instructions. Thirty-six hours after transfection, the cells were lysed and a luciferase assay was performed to evaluate NF-κB promoter activation.

2.10. Statistical analysis

All data obtained from the qPCR and luciferase assays were subjected to one-way analysis of variance (ANOVA) or two-tailed unpaired Student's *t*-test to compare statistical significance between two groups or among multiple groups. $P < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Characterization of AbTLR-A and AbTLR-B

Coding sequences for *AbTLR-A* and *AbTLR-B* were identified from the disk abalone transcriptome database and the information were deposited in NCBI GenBank under accession numbers MH205666 and MH205667, respectively. ORFs of *AbTLR-A* and *AbTLR-B* were 2100 and 1965 base pairs in length encoding polypeptides of 700 and 655 amino acids (aa) with predicted molecular mass of 81.6 and 75.9 KDa respectively. Signal peptides were identified in the N-terminal regions of both abalone TLRs. AbTLR-A contained an extracellular domain with 7 LRRs (residues 89–437) flanked by LRR N-terminal (residues 36–70) and C-terminal (residues 453–506) domains, transmembrane region (residues 510–532), and cytoplasmic TIR (residues 560–700) domain (Fig. 1A). AbTLR-B was found to comprise an extracellular domain with 7 LRRs (residues 94–392), LRR C-terminal domain (residues 406–459), transmembrane region (residues 462–484), and TIR domain (residues 515–655) on the cytoplasmic side (Fig. 1A). In contrast to AbTLR-A, abalone TLR-B lacks LRR N-terminal domain in the extracellular region (Fig. 1A). Typically, LRR N-terminal domains are composed of disulfide-linked β-hairpins, whereas two helices are involved in maintaining the globular structure of LRR C-terminal domains [10]. The number of LRRs present in the extracellular domain and their arrangement vary based on type of TLR and host species. For instance, 4, 6, and 7 LRRs are found in TLR1 from human, mouse, and zebrafish, respectively. As in vertebrate TLRs, the number of LRRs in the extracellular domain of invertebrate TLRs ranges from 1 to 24. Interestingly, no LRRs were identified in the extracellular domains of TLR related molecules from primitive organisms including *Hydra magnipapillata* and *Acropora millepora* [42]. Thus, the pattern recognition ability of the LRR was gained through an evolutionary process after a duplication event in the bilateral TLR lineage [42]. The predicted 3D structures of LRRs of both abalone TLRs resembled a horseshoe shape structure similar to those in mammalian TLRs. The LRRs of AbTLR-A and AbTLR-B are composed of 4 and 5 α-helixes on their convex side and 14 and 13 β-sheets on their concave side, respectively (Fig. 1B and C). This typical spatial arrangement of LRRs of both abalone TLRs may provide maximum ligand recognition capacity and therefore is involved in efficient PAMP recognition. The cytoplasmic TIR domain of TLR is critically involved in interactions with cytoplasmic adaptor molecules including MyD88 to initiate downstream TLR-mediated signaling cascades [43]. Similar to other TIR domain-containing proteins, three conserved functionally important motifs, BOX1 ((F/Y)DA), BOX2 (RDXXPG), and BOX3 (FW), were identified in the TIR domains of AbTLR-A and AbTLR-B (Fig. 2) [44]. These motifs in the TIR domains of TLRs are involved in critical functions such as interacting with the TIR domain of MyD88 to initiate downstream signaling cascades (BOX1 and BOX2) [45] and with cytoplasmic components to maintain receptor localization (BOX3) [46]. Multiple sequence alignment of TIR domains revealed that functional motifs including BOX1, BOX2, and BOX3 of AbTLR-A and AbTLR-B shared significant higher conservation with those in mollusks, fish, birds, and mammals tested (Fig. 2). Analysis of the TIR domains of both abalone TLRs showed characteristic TIR features, such as centrally located 5-stranded parallel β sheets surrounded by 5 α helices (Fig. 1E and F). Interestingly, this arrangement of TIR domains in AbTLR-A and AbTLR-B is similar to that of human TLR5 (Fig. 1E, F and G). Typically, functional motifs including BOX1, 2, and 3 are located within 5-stranded parallel β sheets. Moreover, the BB-loop, which is known to be involved in the TIR-TIR interactions of TLRs and MyD88s, was identified in the TIR regions of both abalone TLRs and human TLR5 (Fig. 1E, F and G) [44]. The similar structural features of AbTLR-A and AbTLR-B suggest that these TLRs function in a similar manner as mammalian TLRs. However, further studies are required to clarify this.

The aa sequence of AbTLR-A was highly identical with Pacific oyster TLR2, whereas AbTLR-B showed the highest aa identity with TLR13 from Pacific oyster (Supplementary table 1). Compared to the overall aa sequences, somewhat higher aa identities were observed within the TIR domains of AbTLRs and other TLR homologs (Supplementary Table 1). Overall, the lower aa identities of the identified AbTLRs with other TLR homologs may be associated with more rapid evolutionary changes in extracellular LRR regions compared to that in TIR domains [47]. According to the phylogenetic tree, vertebrate and invertebrate TLRs were separately clustered and abalone TLRs were placed with mollusks (Supplementary Figure 1). AbTLR-A and AbTLR-B were not closely related to other TLR members in clade 2 (Supplementary Figure 1). Hence, TLR-A and TLR-B identified in disk abalone in the present study may represent a novel class of mollusk TLRs. Moreover, AbTLR-A and AbTLR-B exhibited the same evolutionary status, suggesting that both abalone TLRs were derived from a gene duplication event. Duplications can lead to the generation of novel TLR copies. For instance, a large number of TLR duplications was identified in Yesso scallop, suggesting that these TLRs functions in a specific and cooperative manner in the innate immune system [16]. Therefore, studying the evolution and duplication of novel TLRs can improve the understanding of their functions, particularly their ligand binding mechanisms.

3.2. Expression of AbTLR-A and AbTLR-B mRNA at different abalone early embryonic developmental stages

Studies of fruit fly (Drosophila) revealed that Tolls are actively involved in developmental processes apart from their immune functions [49]. Therefore, the mRNA expression of *AbTLR-A* and *AbTLR-B* was determined by qPCR in order to elucidate their putative involvement in abalone development. Specifically amplified PCR products were detected by qPCR melting curve analysis of *AbRib*, *AbTLR-A*, and *AbTLR-B*. Constitutively expressed mRNA transcripts of *AbTLR-A* and *AbTLR-B* were observed at every abalone early developmental stage analyzed (Fig. 3). In the *AbTLR-A* expression profile, the highest mRNA transcript level was detected at the 16-cell stage, followed by the gastrula and trochophore stages (Fig. 3). However, relatively low levels of *AbTLR-A* mRNA were detected at the egg, middle, and late veliger and morula stages (Fig. 3). *AbTLR-B* mRNA was prominently expressed at the trochophore stage, whereas the lowest expression was observed at the morula stage (Fig. 3). Studies focused on fruit fly documented dual functions of insect Toll family members in immunity and embryogenesis [50]. Moreover, the Toll protein in fruit fly prominently is expressed zygotically and is important for the development of muscles and motoneurons [51]. Differential mRNA expression patterns in

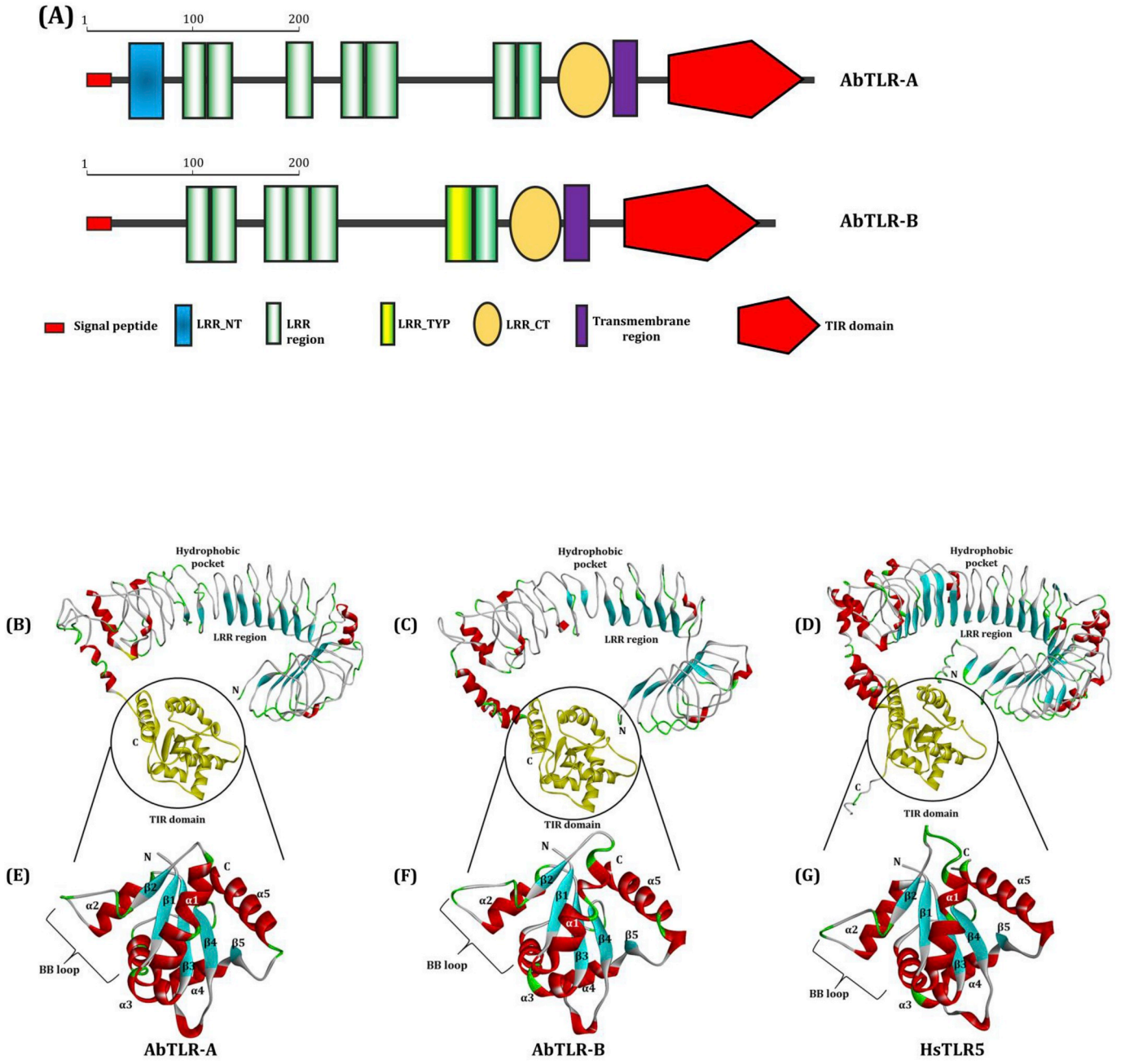

Fig. 1. Domain architecture of AbTLR-A and AbTLR-B (A). Predicted 3D homology models of entire proteins of AbTLR-A (B), AbTLR-B (C), human TLR5 (HsTLR5) (D), and TIR domain of AbTLR-A (E), AbTLR-B (F), and HsTLR5 (G). Helices are shown in red color, β sheets are depicted in sky blue, and loops are indicated in gray. In TIR domains, centrally located β sheets are marked as β1–β5 and surrounding helices are marked as α1–α5. N- and C-termini are indicated using N and C letters, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

amphioxus (*Branchiostoma belcheri tsingtauense*) TLR1 at embryonic development stages revealed the involvement of invertebrate TLRs in embryonic development [52]. Kannaki et al. also reported the mRNA expression profiles of chicken TLRs in embryonic tissues during embryonic development and suggested their potential roles in regulating this process [53]. Furthermore, the involvement of TLRs in mammalian development was investigated using mouse models [54]. In the teleost, number of TLRs were detected during the early developmental and embryonic stages [55]. Currently, there is no detailed information available regarding the expression profiles of TLRs during early embryonic development in mollusks. In the present study, the mRNA of *AbTLR-A* and *AbTLR-B* was detected starting at the egg stage, suggesting that both TLRs were derived from maternal origin, as mRNA transcription at the embryonic level starts from the morula stage in mollusks [56]. As reported previously, numerous maternally transferred immune components, including PPRs, lysozymes, antioxidant enzymes, and C1q domain-containing proteins, among others, were identified in mollusks [56]. Maternally transferred immune components are important for mollusks for several reasons. Fertilization occurs in an external environment and early embryos of mollusks tend to be exposed to pathogens or PAMPs in the water. Therefore, maternally transferred immunity may temporarily protect the embryos from pathogenic invasion until immune system development [56]. Additionally, Tolls of drosophila are necessary for dorsal–ventral axis development [57]. Thus, expression of *AbTLR-A* and *AbTLR-B* at early embryonic development stages in abalone is a sign of their putative

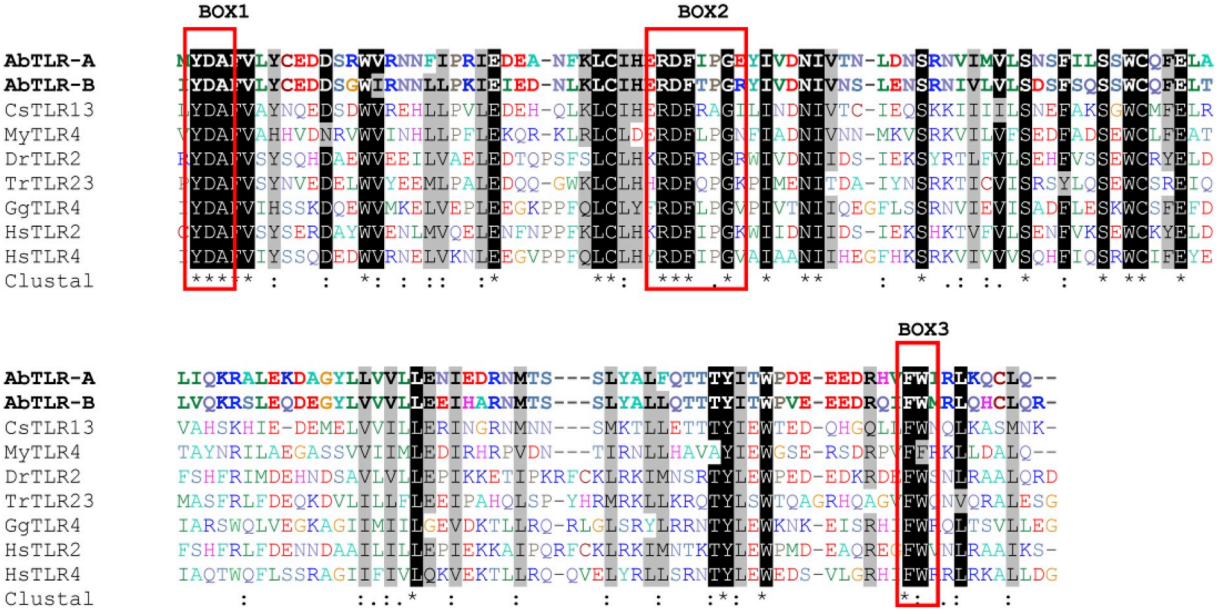

Fig. 2. Multiple sequence alignment of TIR domains of AbTLR-A and AbTLR-B with those in other invertebrates and vertebrates. Completely conserved amino acid residues are shaded in black and marked with asterisks (*) under the sequence. Conserved and partially conserved amino acid residues are shaded in gray and depicted using a colon (:) and dot (.) respectively. Three functionally important motifs, BOX1, BOX2, and BOX3, are indicated using red boxes. Alignment of TIR domains of TLR sequences include AbTLR-A and AbTLR-B as reference sequences and following TLR homologs: *Cyclina sinensis* TLR13 (CsTLR13), *M. yessoensis* TLR4 (MyTLR4), *D. rerio* TLR2 (DrTLR2), *Takifugu rubripes* TLR23 (TrTLR23), *Gallus gallus* TLR4 (GgTLR4), and *H. sapiens* TLR2 and TLR4 (HsTLR2 and HsTLR4). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

involvement in establishing the dorsal–ventral axis during embryogenesis in disk abalone. However, further experiments are required to confirm this. Altogether we suggest that the presence of *AbTLR-A* and *AbTLR-B* mRNA during different early embryonic developmental stages of disk abalone might be involved in immune related activities and developmental processes during abalone embryogenesis.

3.3. Expression of AbTLR-A and AbTLR-B mRNA in different abalone tissues

The tissue-specific mRNA distribution of *AbTLR-A* and *AbTLR-B* in un-challenged abalones was determined in a qPCR assay. Both abalone TLRs were ubiquitously detected in all tissues analyzed including the hemocytes, gills, muscles, digestive tract, hepatopancreas, and mantle (Fig. 4). *AbTLR-A* was prominently expressed in the hemocytes,

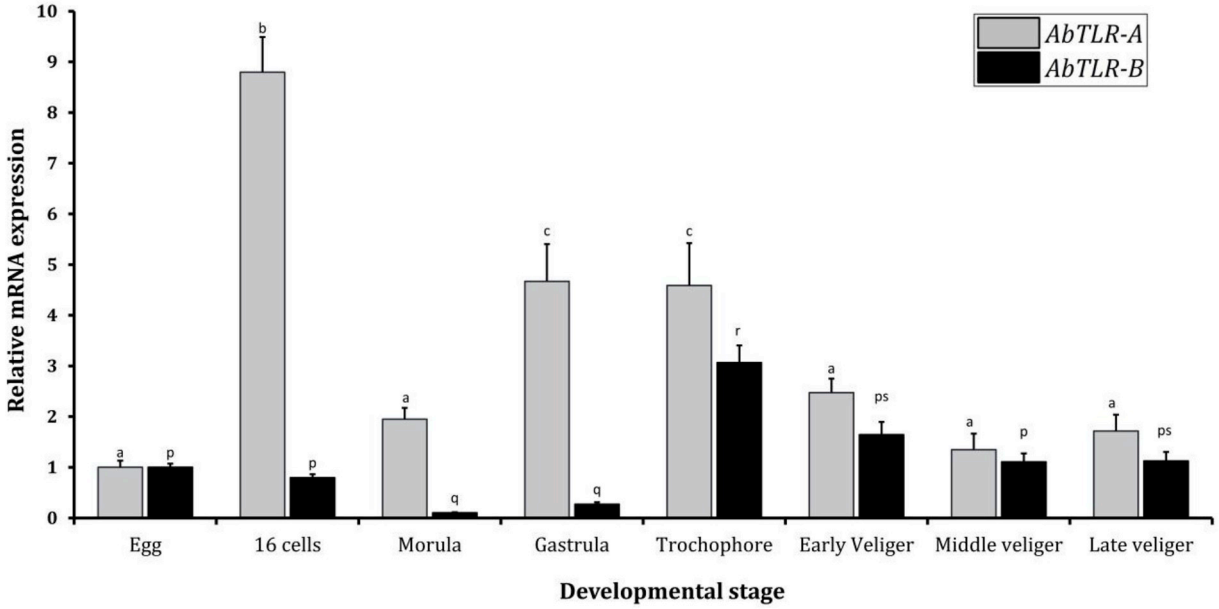

Fig. 3. Expression profiles of *AbTLR-A* and *AbTLR-B* mRNA in different abalone early embryonic stages. Relative mRNA expression was determined by qPCR. Abalone ribosomal protein L5 gene was used as an internal control gene. Expression levels of *AbTLR-A* and *AbTLR-B* in each development stage were calculated by the Livak method and reported as fold-changes relative to mRNA expression of the egg stage. Error bars represent standard deviation. One-way ANOVA followed by Tukey's range test was used to calculate significance ($p < 0.05$) among different early embryonic developmental stages and denoted using small case English letters above the bars.

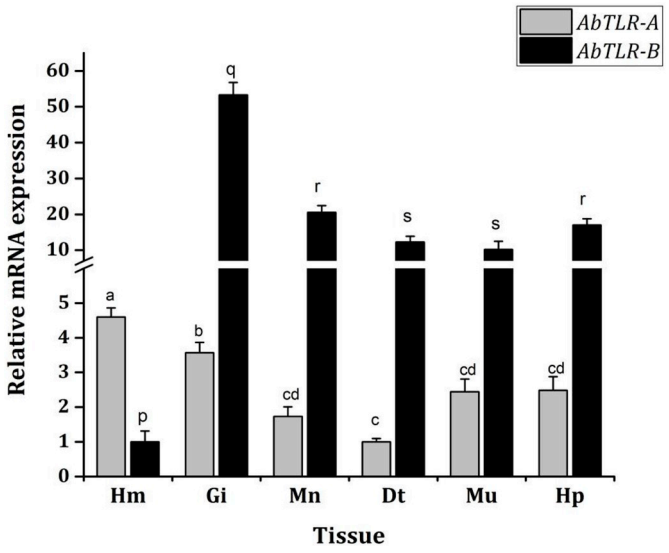

Fig. 4. Tissue distribution expression profiles of *AbTLR-A* and *AbTLR-B* mRNA in different tissues of un-challenged abalones. Relative mRNA expression was determined by qPCR. Abalone ribosomal protein L5 gene was used as an internal control gene. Expression levels of *AbTLR-A* and *AbTLR-B* in each tissue were calculated by the Livak method. Expression fold-differences in *AbTLR-A* and *AbTLR-B* were calculated relative to the expression level of digestive tract and hemocytes, respectively. For the consistency, each qPCR assay was performed for three-replicates from pooled tissues from five disk abalones. Error bars represent standard deviation (n = 3). One-way ANOVA followed by Tukey's range test was used to calculate significance ($p < 0.05$) among different tissues and denoted using lowercase English letters above the bars. Hm, hemocytes; Gl, gill; Mn, mantle; Dt, digestive tract; Mu, muscle; Hp, hepatopancreas.

followed by the gills, while the lowest expression was detected in the digestive tract (Fig. 4). As illustrated in Fig. 4, the highest mRNA expression of *AbTLR-B* was observed in the gills. Other tissues including the hepatopancreas, mantle, digestive tract, muscles, and hemocytes showed moderate or lower levels of *AbTLR-B* mRNA (Fig. 4). Some previous studies demonstrated the tissue-specific mRNA expression profiles of molluskan TLRs with the highest expression level in immunologically active tissues. For instance, TLR homologs of Pacific oyster [17,58], disk abalone [48], Zhikong Scallop [59], and Chinese venus [24] were abundantly expressed in hemocytes. The pathogen defense mechanism in invertebrates including mollusks primarily depends on innate immunity, as these organisms lack well-developed adaptive immunity to combat invading pathogens. When discussing mollusks innate immunity, circulating hemocytes or immunocytes are known as major immune effector cells and are thought to be involved in a wide array of physiological functions [60]. To accomplish the fundamental innate immune roles of hemocytes, the cells are equipped with secretable humoral factors including cytokine-like molecules, nitric oxide, antimicrobial peptides, lectins, and lysosomal enzymes [60]. Additionally, hemocytes are major sites for pathogen elimination by actively mediating phagocytosis or encapsulation of invading microbial pathogens [60]. Various PRRs including TLRs present in the membranes of hemocytes are involved in pathogen recognition, and thereby initiate phagocytosis or other relevant pathogen clearance machinery. Thus, the predominant transcript level of *AbTLR-A* in abalone hemocytes indicates its involvement in the pathogen recognition process. In contrast to *AbTLR-A*, abundant levels of *AbTLR-B* were found in abalone gills. Pallial organs including gills, mantles, and feet are potentially exposed to the external environment and are more vulnerable to direct contact with waterborne microbes compared to other tissues [61]. Therefore, these tissues may be equipped with a substantial pathogen recognition system to establish an efficient pathogen elimination mechanism. The highest mRNA transcription of TLRs/Tolls in gill tissues was also detected in amphioxus [52], freshwater crab (*Sinopotamon henanense*) [62], swamp crayfish (*Procambarus clarkia*) [63], marine crab (*Portunus trituberculatus*) [64], and the respiratory tree of sea cucumber (*Apostichopus japonicus*) [65]. These results are consistent with the mRNA expression profile of *AbTLR-B* in un-challenged abalones. Thus, based on previous results and those obtained in this study, the highest mRNA expression of *AbTLR-B* in abalone gills may be attributed to its involvement in host immunity.

3.4. Expression profiles of AbTLR-A and AbTLR-B after in vivo immune challenge

Increasing evidence has demonstrated that TLRs elicit potent post-immune responses upon pathogenic stress. Hence, an *in vivo* immune challenge experiment was conducted to determine the putative innate immune roles of AbTLR-A and AbTLR-B upon administration of two live bacteria and virus. As depicted in Fig. 5A, *AbTLR-A* mRNA was significantly up-regulated in hemocytes at 3 and 6 h p.i. of Gram-negative *V. parahemolyticus* and Gram-positive *L. monocytogenes* injection. Upon VHSV challenge, the mRNA transcription of *AbTLR-A* was significantly induced at 3, 6, 12, and 24 h p.i (Fig. 5A). Significantly up-regulated mRNA of *AbTLR-A* was also observed in abalone gills after the challenge experiment (Fig. 5B). In detail, *AbTLR-A* expression was significantly up-regulated at 12, 48, and 72 h p.i. of *V. parahemolyticus* and 48 h p.i. of *L. monocytogenes* injection (Fig. 5B). VHSV challenge also induced the expression of *AbTLR-A* mRNA in the gills; the highest level was reached at 6 h p.i. compared to in un-challenged animals (Fig. 5B). The mRNA expression profile of *AbTLR-B* in hemocytes is shown in Fig. 5C. Significantly up-regulated *AbTLR-B* was detected at 3, 24, 48, 72, and 120 h p.i. after *V. parahemolyticus* and 24, 48, 72, and 120 h p.i. after *L. monocytogenes* administration (Fig. 5C). Post-VHSV injection also induced *AbTLR-B* transcription in a later phase of experiment (24, 48, 72, 120 h) in hemocytes. In abalone gills, the mRNA expression of *AbTLR-B* was induced at 3, 6, 12, 24, 48, or 120 h p.i. after both bacterial and viral injections (Fig. 5D). Emerging evidence based on Toll signaling pathways of insects affirmed their involvement in host defense by regulating immune relevant gene expression [66,67]. Although there is no proper classification system for invertebrate TLRs and specific ligand recognition TLRs have not been identified, their post-immune responses were determined upon bacterial and viral infections. For instance, up-regulated mRNA expression of various TLRs from Pacific oyster [17,25], noble scallop [23], triangle-shell pearl mussels [19], Chinese venus [24], marine crab [64], earthworm (*Eisenia andrei*) [68], and shrimp [22] were detected after administration of bacteria or virus. In the present study, potent immune responses of *AbTLR-A* and *AbTLR-B* were observed following injection with either Gram-negative and Gram-positive bacteria or virus. Interestingly, both abalone TLRs in hemocytes showed a stronger response upon bacteria or virus challenge compared to those in the gills, as hemocytes are considered as key immune effector cells in invertebrates. As described above, hemocytes of invertebrates play important roles in pathogen clearance [69]. It is well-known that granulocytes are a key cell type present in the hemocytes of mollusks and are actively involved in eliminating invading microbes by phagocytosis, releasing granular contents (hydrolytic enzymes), and subsequently activating reactive oxygen species or reactive nitrogen species production [70]. These granulocytes can move through the blood or lymph stream to infection sites where they elicit inflammatory responses to eliminate invading pathogens [25]. Therefore, an efficient pathogen recognition mechanism is required for initiate aforementioned process, and abundant surface expression of TLRs in molluskan granulocytes provides positive evidence for prove this hypothesis [70]. Moreover, termination of TLR signaling by blocking peptide significantly inhibited degranulation of granulocytes and reduced TNF mRNA transcription upon *V. parahaemolyticus* infection in oyster hemocytes [25]. Thus strong responses of *AbTLR-A* and *AbTLR-B*

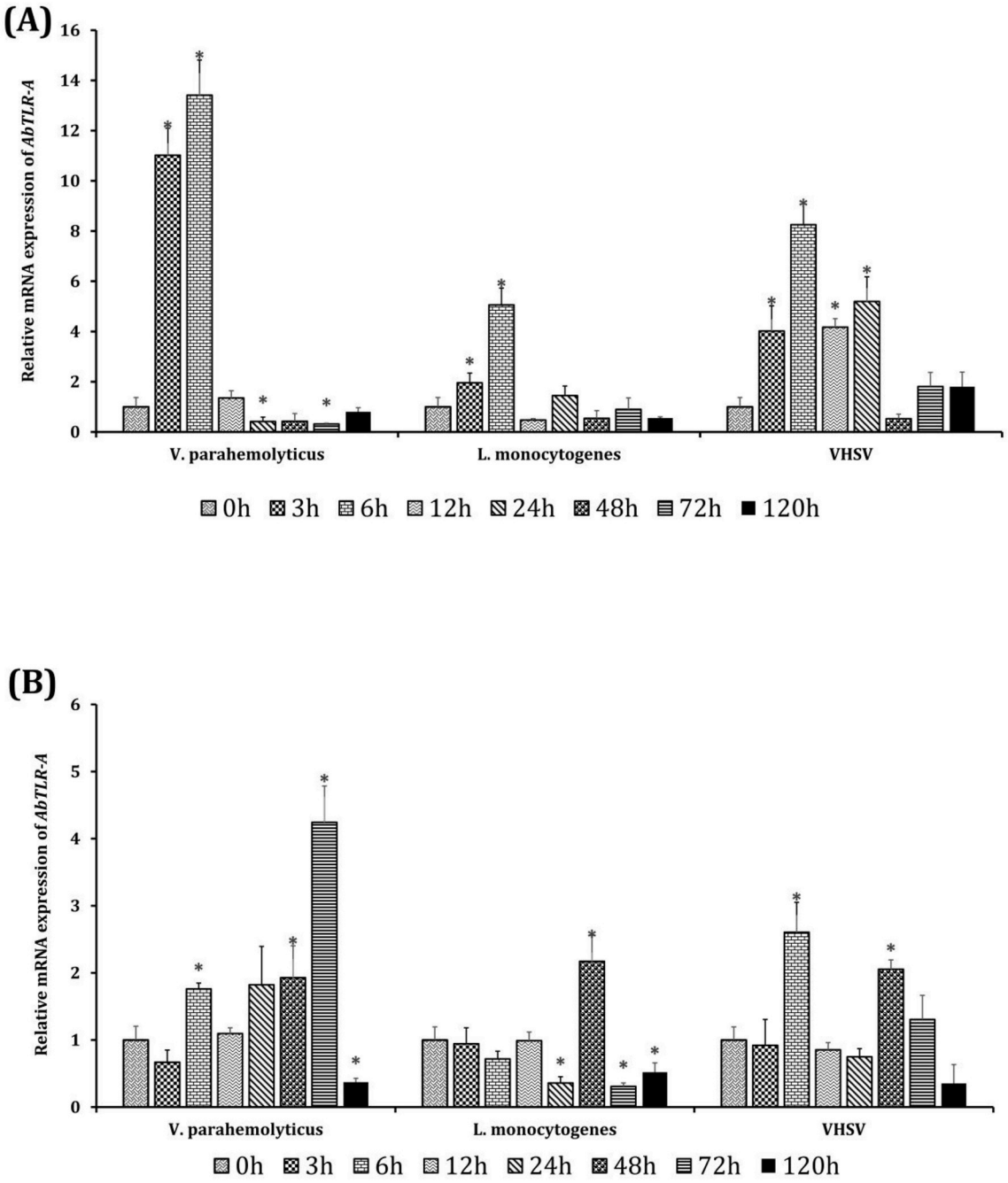

Fig. 5. mRNA expression profiles of *AbTLR-A* in hemocytes (A), gills (B) and *AbTLR-B* in hemocytes (C) and gills (D) after *V. parahaemolyticus*, *L. monocytogenes*, and VHSV challenge. Relative mRNA expression was determined by qPCR. Abalone ribosomal protein L5 gene was used as an internal control gene. Expression levels of *AbTLRs* in each experimental group were calculated by the Livak method. Fold-differences of *AbTLR-A* and *AbTLR-B* expressions in each challenge group were calculated relative to saline injected control group. For the consistency, each qPCR assay was performed for three-replicates from pooled tissues from four abalones. Error bars represent standard deviation (n = 3). Significance of differences ($p > 0.05$) between control and experimental groups were calculated by students' *t*-test and indicated using small asterisk (*) above the bars.

in hemocytes upon administration of bacteria or virus might be due to effectively initiate TLR signaling to regulate the further immune functions in abalones. Additionally, the responsive mediation of both abalone TLR mRNA detected upon immune challenge in the gills may be associated with their ability to recognize invaders. This observation was expected, as gills are continuously exposed to the exterior environment and are highly susceptible to pathogenic attack. Induced mRNA expression of TLRs/Tolls was also detected in the gills of marine crab [64], freshwater crayfish [71], and pacific white shrimp [22] against pathogenic infections. Unlike mammalian TLRs, invertebrate TLRs recognize multiple PAMPs or different pathogen types. For instance, recombinant TLR6 from Pacific oyster can bind with fungi, Gram-positive, and Gram-negative bacteria [17]. Moreover, Zhang et al. observed enhanced expression of Pacific oyster's TLRs in responses to various pathogenic ligands [25]. Thus, up-regulation of *AbTLR-A* and *AbTLR-B* mRNA transcripts in abalone hemocytes and gills after Gram-positive and Gram-negative bacteria and virus administration reflect their broader pattern recognition capacities as well as their involvement in anti-bacterial and anti-viral immunity in abalones.

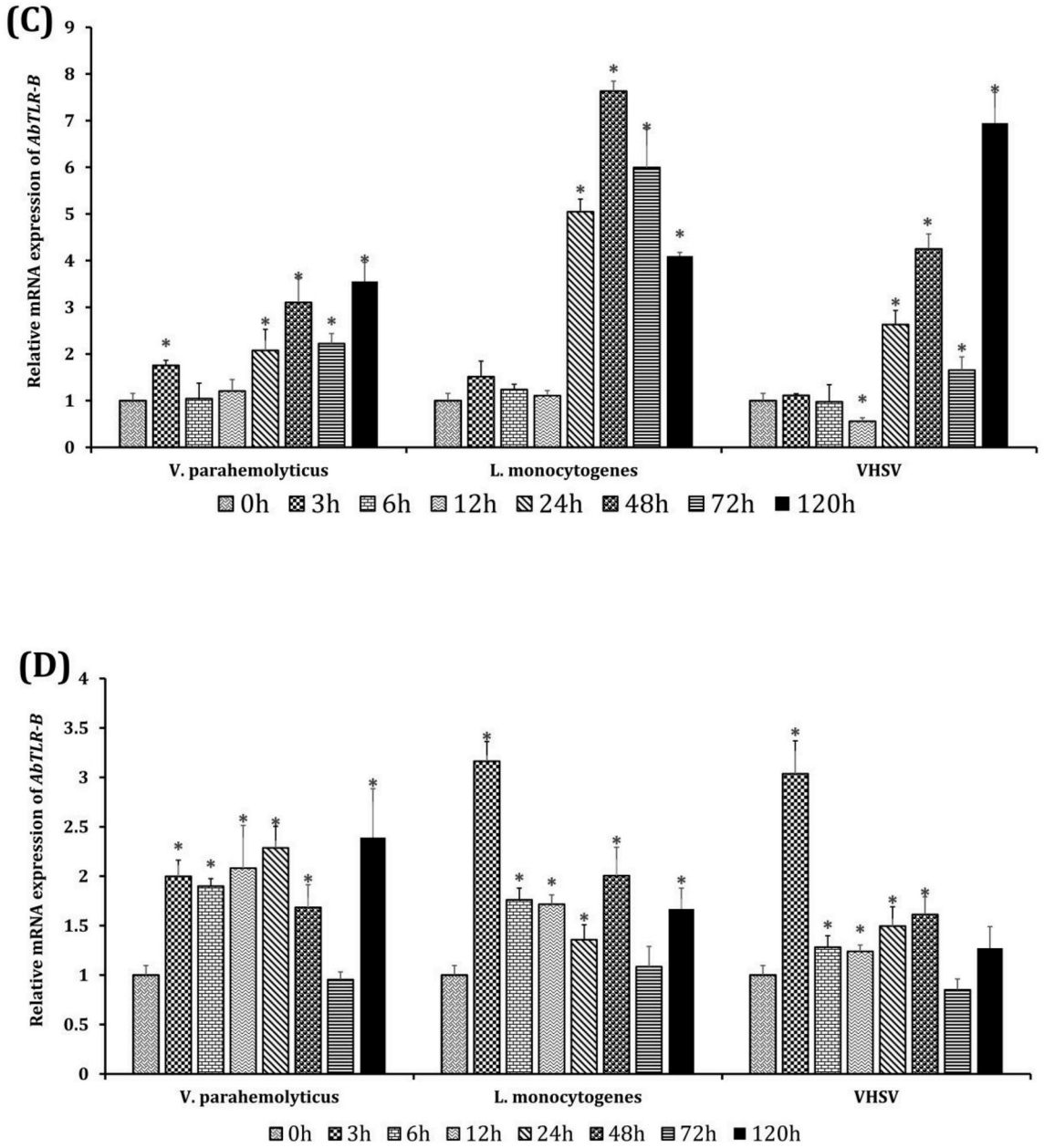

Fig. 5. (*continued*)

3.5. Activation of NF-κB and AP-1 reporters

It is well-known that stimulated TLRs can ultimately activate transcription factors including NF-κB and AP-1 [12]. Numerous studies showed that forced expression of mammalian TLRs leads to direct activation of NF-κB reporters. Therefore, NF-κB and AP-1 luciferase assays were employed to elucidate the functional aspects of AbTLR-A and AbTLR-B. The TIR domain is the fundamental domain of the TLR system, which plays a critical role in signal transduction upon association with cytoplasmic adaptors [12]. Some previous studies demonstrated the ability of invertebrate TLR members to use the mammalian TLR system to simulate their function *in vitro* [25,26,72]. In the present study, forced expression of AbTLR-A and AbTLR-B in HEK293T cells resulted in significantly higher NF-κB and AP-1 reporter activities compared to cells transfected with empty vectors (Fig. 6). Similar to the present study, functional investigation of four TLRs from Pacific oyster revealed that they can directly activate the NF-κB reporter in HEK293 cells [25]. However, overexpression of Zhikong scallop TLR did not directly activate the NF-κB reporter in HEK293T cells [26]. Moreover, up-regulated NF-κB luciferase activity was observed in rock bream (*Oplegnathus fasciatus*) membrane-anchored Toll-like receptor 5 (TLR5M) overexpressed HEK293T cells compared to in the mock control [73]. Transcription factor NF-κB activation is required for the regulation of proinflammatory cytokines such as IL6, IL1β, and TNFα expression [12]. Additionally, activation of MAPK family members upon TLR-mediated signaling allow phosphorylation and subsequent activation of transcription factor AP-1, thereby inducing expression of proinflammatory mediators [12,74]. Therefore, the findings of the present study together with those of previous studies suggest an ancient and conserved association among TLRs and AP-1 or NF-κB signaling in mollusks. Direct activation of AP-1 or NF-κB by abalone TLRs further revealed the potential involvement of AbTLR-A and AbTLR-B in abalone immunity by regulating proinflammatory cytokines through transcription factor activation.

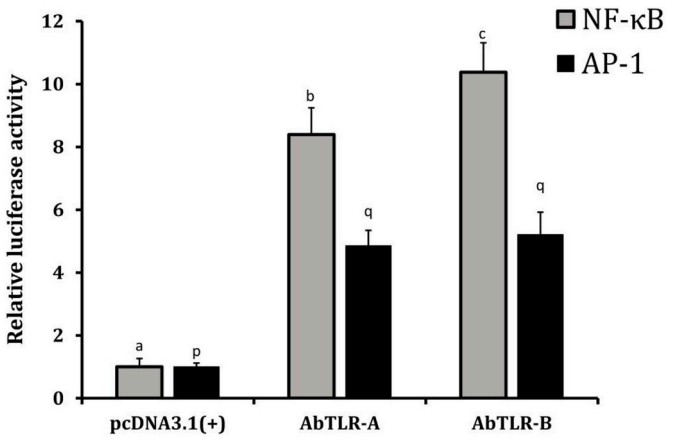

Fig. 6. Activation of NF-κB and AP-1 responsive reporters by AbTLR-A and AbTLR-B. HEK293T cells were transiently transfected with 0.7 μg of empty pcDNA3.1(+) vectors or AbTLR-A/pcDNA3.1(+) or AbTLR-B/pcDNA3.1(+) constructs with 0.25 μg of NF-κB or AP-1 responsive reporter vectors and 0.05 μg of pRL-TK internal control vectors per well in 12-well plate. After 36 h, the cells were lysed and a luciferase assay was performed. Results are represented as the mean value of triplicate (n = 3) experiments. Error bars represent standard deviation. One-way ANOVA followed by Tukey's range test was used to calculate significance ($p < 0.05$) among the experimental groups and denoted using small case English letters above the bars.

3.6. Responses of AbTLR-A and AbTLR-B to PAMPs stimulation

Although the proper classification of mammalian TLRs and information of their specific ligands are widely available, the ligand specificity of invertebrate TLRs remains unclear. Therefore, we evaluated the PAMP recognition ability of the newly identified AbTLR-A and AbTLR-B using NF-κB reporter assay. In this study, HEK293T cells which are known to be lack of expression of TLRs were transiently transfected with AbTLR-A-pcDNA3.1(+) or AbTLR-B-pcDNA3.1(+) or empty pcDNA3.1(+) and then stimulated with known mammalian TLR ligands. AbTLR-A-transfected cells showed no NF-κB reporter activity in response to LPS, poly I:C, and ODN2006 stimulation (Fig. 7A, B, and D). However, slight NF-κB activation was detected in AbTLR-A-transfected cells as a higher dose of PGN was used (Fig. 7C). In contrast to AbTLR-A, AbTLR-B-transfected cells exhibited a different PAMP recognition profile. Slight NF-κB activity was observed in AbTLR-B-transfected HEK293T cells at a higher dose of LPS (Fig. 7E). Treatment with poly I:C also contributed to transactivation of the NF-κB reporter in AbTLR-B-overexpressing cells (Fig. 7F). Interestingly, AbTLR-B-transfected cells did not show NF-κB activity in response to PGN and ODN2006 compared to the mock control (Fig. 7G and H). The lack of specific PAMP recognition by AbTLR-A was consistent with the results of previous studies examining Pacific oyster [25], Drosophila [50,51], and amphioxus [52]. Overexpression of four TLRs from Pacific oyster in HEK293 cells showed no response to 13 PAMPs tested [25]. However, TLRs from ascidian Cionaintestinalis exhibited low-level responses in the presence of high concentrations of PAMPs [75]. Collectively, these results reveal that the specific ligand recognition ability of TLRs developed during vertebrate evolution. However, unlike mammalian TLRs, endogenous assistant protein is required to activate some invertebrate TLRs. For instance, the cytokine-like molecule Spätzle is involved in assist PAMP recognition process of Tolls in *Drosophila* [5]. Interestingly, proteins similar to Spätzle were discovered in shrimps and thus the PAMP recognition mechanism of crustacean TLRs may be similar to that of *Drosophila* [22]. Moreover, TLR-related PAMP-binding proteins were identified in mollusks [76]. Surprisingly, scallop TLR-transfected HEK293T cells showed augmented activation of the NF-κB responsive reporter after adding PAMPs with scallop serum [26]. Therefore, the identified abalone TLRs may require an assistance protein to facilitate their PAMP recognition ability. However, further experiments are required to address this. Some studies identified TLRs with broader PAMP recognition ability in mollusks. For example, the Zhikong scallop TLR overexpressed in HEK293T cells responded to stimulation with various PAMPs, including, Pam3CSK4, GLU, Zymosan, poly I:C, PGN, LPS, and several types of CpGs [26]. Recombinant TLR6 from Pacific oyster was shown to bind fungi, Gram-positive and Gram-negative bacteria, LPS, and PGN [17]. The multiple PAMP recognition ability of AbTLR-B and some previously identified mollusk TLRs were quite different from those in the mammalian TLR system. Mammalian TLRs recognize specific ligands, such as, TLR1 and TLR2 for triacyl lipopeptides, TLR2 for PGN, phenol-soluble modulin, and glycolipids, TLR3 for viral double-stranded RNA, synthetic double-stranded RNA (poly I:C), TLR4 for LPS, TLR5 for flagellin, TLR7/TLR8 for single-stranded RNA, and TLR9 for CpG DNA [77]. As described above, invertebrates lack adaptive immunity, and therefore may contain more PRRs with border pattern recognition capabilities to recognize a diverse range of PAMPs or pathogens to elicit a stronger immune defense against invaders.

3.7. AbTLR-A and AbTLR-B association with AbMyD88-2 or AbMyD88-X to activate NF-κB reporter

Two MyD88 homologs were previously identified in disk abalone and designated as AbMyD88-2 and AbMyD88-X according to their homologies to known MyD88s [78]. To determine whether AbMyD88-2 and AbMyD88-X are conserved functional adaptor molecules in the abalone TLR signal transduction pathway, both MyD88s were cloned into pcDNA3.1(+) vectors and co-expressed with AbTLR-A or AbTLR-B in HEK293T cells. The results revealed that overexpression of AbMyD88-2 and AbMyD88-X significantly enhanced NF-κB luciferase activities compared to in their corresponding mock controls (Fig. 8). Interestingly, co-expression of AbMyD88-2 or AbMyD88-X with AbTLR-A or AbTLR-B increased TLR-dependent NF-κB activation as the amounts of AbTLR-A/pcDNA3.1(+) or AbTLR-B/pcDNA3.1(+) constructs were increased from 300 to 500 ng per well (Fig. 8). MyD88 is a conserved adaptor molecule in insects to mammals and can regulate canonical TLR dependent transcription factor activation [11,79]. The C-terminal TIR domain of MyD88 is responsible for associating with TLRs and an N-terminal DD allows for interactions with IRAK molecules to form the myddosome complex [11]. Functional analyses of TLR1 in Pacific oyster revealed its ability to activate the NF-κB reporter in a dose-dependent manner when overexpressed with oyster MyD88 in HEK293 cells [25]. Moreover, co-expression of rock bream TLR5M and MyD88 in HEK293T cells showed the highest NF-κB activity compared to TLR5M-transfected HEK293T cells, suggesting the synergistic functions of these molecules [73]. Furthermore, TIR domain-deleted MyD88 of Pacific oyster strongly blocked TLR-dependent NF-κB activation *in vitro* [25]. Therefore, the outcomes of this study and those of previous studies suggest that AbMyD88-2 and AbMyD88-X are functional adaptors in the TLR signal transduction system of disk abalone and may play a critical role in abalone immunity.

4. Conclusion

In the present study, two novel invertebrate TLRs, designated as AbTLR-A and AbTLR-B, were identified in disk abalone and molecularly and functionally characterized. AbTLR-A and AbTLR-B are composed of the typical structural features of TLRs. Both abalone TLR-A and TLR-B were found to be constitutively expressed in all abalone early embryonic development stages analyzed. The mRNA transcription of AbTLR-A and AbTLR-B was significantly modulated in response to bacteria and virus challenges in abalone hemocytes and gills. Overexpression of AbTLR-A and AbTLR-B in a mammalian system led to activation of the NF-κB and AP-1 reporters. Neither abalone TLR showed a strong response to PAMPs *in vitro*. Higher NF-κB reporter

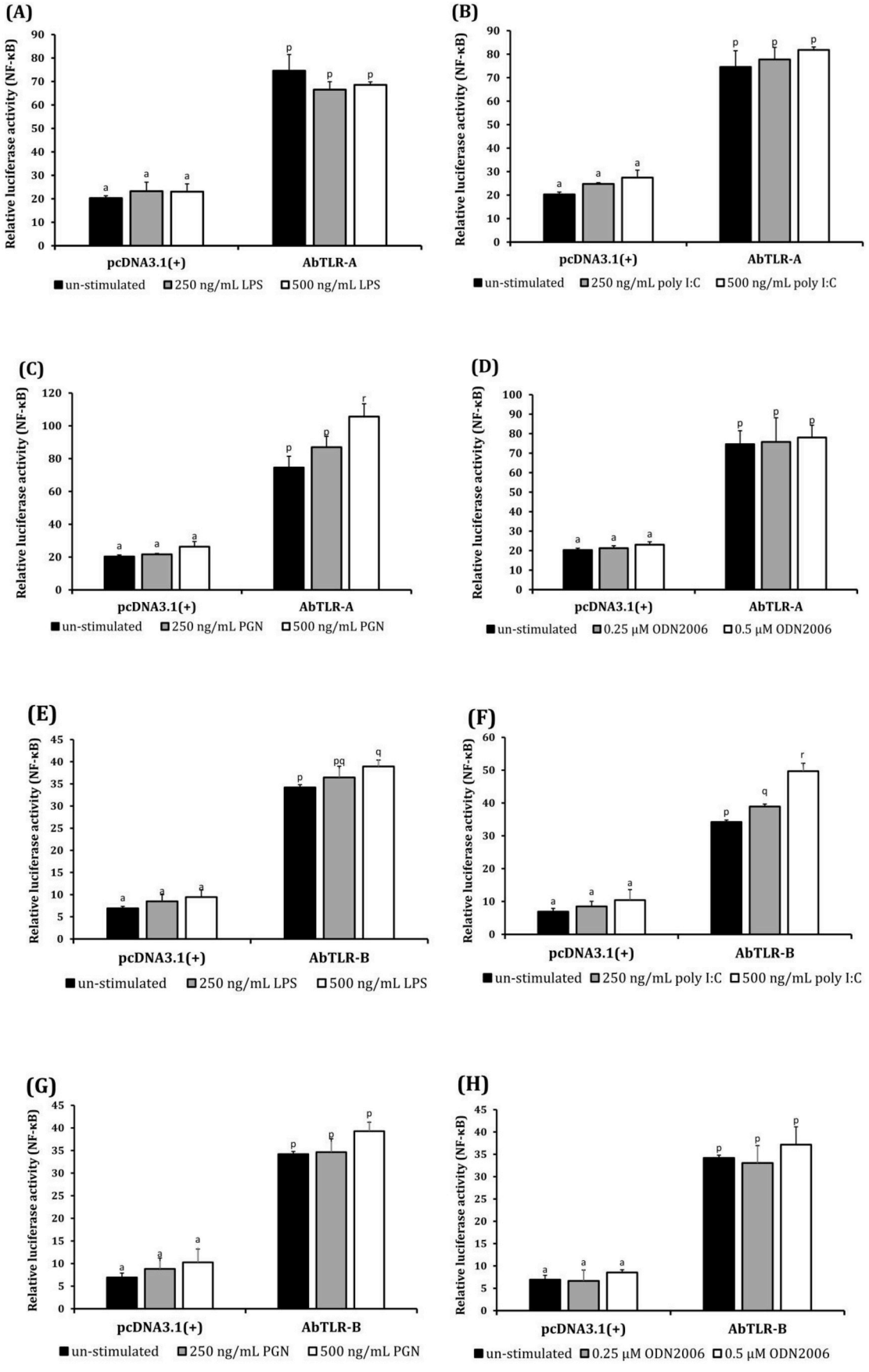

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Fig. 7. Activation of NF-κB reporter in AbTLR-A (A–D) and AbTLR-B (E–H) overexpressed cells in response to mammalian TLR ligands. HEK293T cells were transiently transfected with 0.7 μg of empty pcDNA3.1(+) vectors or AbTLR-A/pcDNA3.1(+) or AbTLR-B/pcDNA3.1(+) constructs with 0.25 μg of NF-κB responsive reporter vectors and 0.05 μg of pRL-TK internal control vectors per well in 12-well plate. Twenty-four hours after transfection, the cells were stimulated with two different doses of LPS, poly I:C, PGN, and CpG ODN2006. The cells were lysed after 12 h of post-stimulation and NF-κB luciferase activity was measured. The results are represented as the mean value of triplicate (n = 3) experiments. Error bars represent standard deviation. One-way ANOVA followed by Tukey's range test was used to calculate significance ($p < 0.05$) among experimental groups and denoted using small case English letters above the bars.

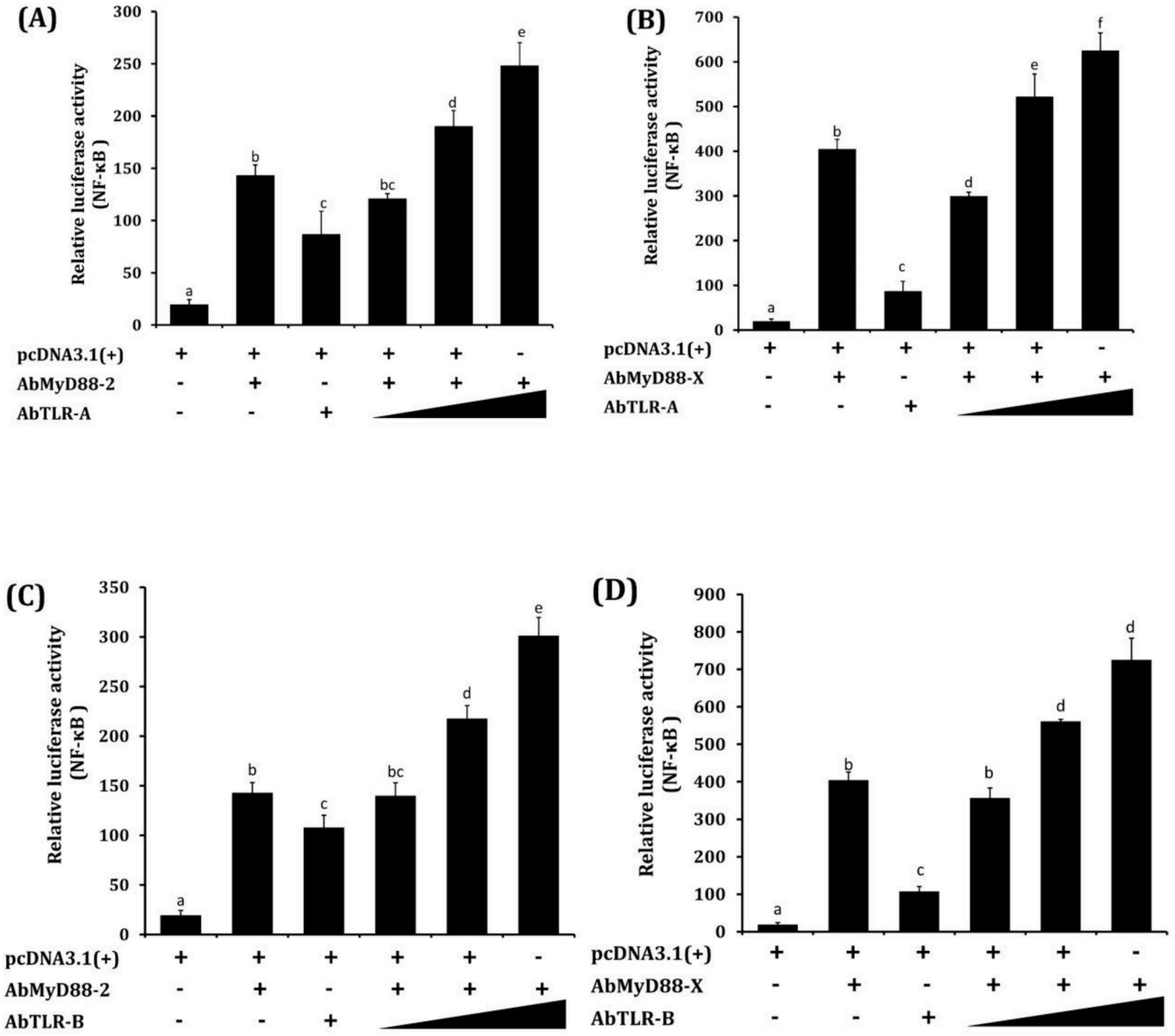

Fig. 8. AbMyD88-2 or AbMyD88-X induced NF-κB activation is AbTLR-A- (A and B) and AbTLR-B- (C and D) dependent. HEK293T cells were transiently transfected with a combination of 0.3 μg of AbMyD88-2/pcDNA3.1(+) or AbMyD88-X/pcDNA3.1(+) and AbTLR-A/pcDNA3.1(+) or AbTLR-B/pcDNA3.1(+) constructs with 0.25 μg of NF-κB responsive reporter vectors and 0.05 μg of pRL-TK vectors in 12-well plates. The triangle increasing sign depicts the increasing amount of AbTLR-A/pcDNA3.1(+) or AbTLR-B/pcDNA3.1(+) dose transfected into each well (300–500 ng). After 36 h, the cells were lysed and a luciferase assay was performed. The results are represented as the mean value of triplicate (n = 3) experiments. Error bars represent standard deviation. One-way ANOVA followed by Tukey's range test was used to calculate significance ($p < 0.05$) among the experimental groups and denoted using small case English letters above the bars.

activities were observed when AbTLR-A and AbTLR-B were overexpressed with abalone MyD88-2 and MyD88-X. Overall, these TLRs in disk abalone are likely involved in immune responses against pathogenic attack.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.062.

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