Novel Toll/IL-1 Receptor Homologous Region Adaptors Act as Negative Regulators in Amphioxus TLR Signaling

Jian Peng, Xin Tao, Rui Li, Jingru Hu, Jie Ruan, Ruihu Wang, Manyi Yang, Rirong Yang, Xiangru Dong, Shangwu Chen, Anlong Xu and Shaochun Yuan

*J Immunol* published online 31 August 2015
http://www.jimmunol.org/content/early/2015/08/30/jimmunol.1403003

---

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/08/30/jimmunol.1403003.DCSupplemental

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Novel Toll/IL-1 Receptor Homologous Region Adaptors Act as Negative Regulators in Amphioxus TLR Signaling

Jian Peng,*† Xin Tao,* Rui Li,* Jingru Hu,* Jie Ruan,* Ruihua Wang,* Manyi Yang,* Rirong Yang,* Xiangru Dong,* Shangwu Chen,* Anlong Xu,*† and Shaochun Yuan*†

Studies have shown that the basal chordate amphioxus possesses an extraordinarily complex TLR system, including 39 TLRs and at least 40 Toll/IL-1R homologous region (TIR) adaptors. Besides homologs to MyD88 and TIR domain-containing adaptor molecule (TICAM), most amphioxus TIR adaptors exhibit domain architectures that are not observed in other species. To reveal how these novel TIR adaptors function in amphioxus Branchiostoma belcheri tsingtauense (bbt), four representatives, bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD, were selected for functional analyses. We found bbtTIRA to show a unique inhibitory role in amphioxus TICAM-mediated pathway by interacting with bbtTICAM and bbt receptor interacting protein 1b, whereas bbtTIRB specifically inhibits the amphioxus MyD88-dependent pathway by interacting with bbtMyD88 and depressing the polyubiquitination of bbt TNFR-associated factor 6. Although both bbtTIRB and bbtTIRD are located on endosomes, the TIR domain of bbtTIRB can interact with bbtMyD88 in the cytosol, whereas the TIR domain of bbtTIRD is enclosed in endosome, suggesting that bbtTIRD may be a redundant gene in amphioxus. This study indicated that most expanded TIR adaptors play nonredundant regulatory roles in amphioxus TLR signaling, adding a new layer to understanding the diversity and complexity of innate immunity at basal chordate. The Journal of Immunology, 2015, 195: 000–000.
cephalochordate amphioxus possesses at least 39 TLRs and >40 TIR adapters (17). Among these adapters, amphioxus MyD88, TRAM-like [also known as amphioxus TIR domain-containing adaptor molecule (TICAM)], and SARM have been well studied. Amphioxus MyD88 was shown to mediate the activation of NF-κB through its middle and death domains, whereas TICAM was shown to mediate the most primitive TRIF-dependent activation of NF-κB (18–20). Amphioxus SARM plays inhibitory roles in both MyD88- and TICAM-dependent pathways by interacting with MyD88, TRAF6, and TICAM (19, 21). Recently, ubiquitination was shown to be essential in regulating the activation of amphioxus NF-κB (22, 23). In addition to these TIR adapters having homologies with their vertebrate counterparts, most amphioxus TIR adapters exhibit novel protein domain architectures, such as the TIR domain linked with the kinase domain and the TIR domain linked with the caspase recruitment domain (CARD). To reveal how these novel TIR adapters function in amphioxus TLR signaling, we selected four representatives for functional analyses. The study will not only aid in defining the relationship among amphioxus TIR adapters, but also will provide further knowledge on the regulation of TLR signaling in vertebrates.

Materials and Methods

Animals and cells

Wild adult Chinese amphioxus Branchiostoma belcheri isingaenae (bft) were obtained from Qingdao, China. Human embryonic kidney (HEK) 293T and Hela cells were grown in DMEM supplemented with 10% FCS and antibiotics.

Cloning of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD cDNAs

TIR adaptor orthologs were identified in the B. floridanae genome. Based on these sequences, partial sequences of bbtTIRA, bbtTIRB, and bbtTIRD were cloned from bft intestinal cDNA by specific primer pairs derived from bbtTIRA (gene ID in B. floridanae is 78250), bbtTIRB (gene ID in B. floridanae is 89683), bbtTIRC (gene ID in B. floridanae is 89399), and bbtTIRD (gene ID in B. floridanae is 69123). Subsequently, 5′-RACE and 3′-RACE were performed according to the manufacturer’s protocol using a GeneRACE Kit (Invitrogen) for full-length sequence cloning. These sequences have been submitted to the National Center for Biotechnology Information database [http://www.ncbi.nlm.nih.gov/genbank/] and accession numbers are KM288437 (bbtTIRA), KM288440 (bbtTIRB), KM288438 (bbtTIRC), and KM288439 (bbtTIRD), respectively.

Identification and sequence analysis of TIR adaptor-like genes in Chinese amphioxus (B. belcheri)

The draft genome of B. belcheri and the related analysis tools can be accessed at: http://mosas.sysu.edu.cn/genome. The following Pfam accession numbers were obtained from http://pfam.sanger.ac.uk/Software/Pfam: Ankyrin repeat, PF00023; Arm, PF00619; Death, PF00531; DED, PF01135; Glycogen_transf_1, PF00534; HEAT, PF02985; Helicase_C, PF00271; LRR_1, PF00560; MBT, PF02820; NAC_1, PF00931; OAS1_L, PF01421; Pkinase_Tyr, PF07714; Pro_isomerase, PF00160; Ras, PF00071; ResIII, PF04851; RIG-I_C-DR, PF11648; SAM_1, PF00536; SEFIR, PF08357; TIR, PF01582; TRP_1, PF00515; TSP_1, PF00090; WD40, PF00400. Domain and gene identification were mainly performed with the HMMER2.0 plus SMART database (http://smart.embbl-heldelberg.de). Previously unidentified protein architectures were validated by searching the expressed sequence tag (EST) and cDNA evidence from the EST database of B. belcheri, as well as the EST dataset and cDNA sets of B. floridanae (Supplemental Table 1).

Acute immune challenges of adult amphioxus and real-time PCR

A total of 15 μl per animal of LPS and lipopolysaccharide (LTA; 1 mg/ml) mixture in PBS was injected into the amphioxus coelom. The challenged and unchallenged amphioxus were cultured in separate tanks as described in our previous study (20). Intestines from five individuals were collected at 2, 4, 8, 12, 24, 36, 48, and 72 h postinjection as a single sample. Intestines from five PBS-injected animals (15 μl per animal) were collected concurrently as nonchallenged controls.

For the NF-κB–specific inhibitor helenalin-treated experiments, the adult amphioxus were separated into three groups and treated with: 1) DMSO as a negative control, 2) the LPS and LTA (1 mg/ml) mixtures, or 3) helenalin administered before inoculation with the LPS and LTA mixtures (15 μl per animal). The animals were cultured in separate tanks, and the intestines from five individuals were collected at 3 h postinjection as a single sample.

The following primer pairs were used: BbtTIRA-F: 5′-GGCTATGACGCTCAGGGACTTC-3′, BbtTIRA-R: 5′-CGCTTGACTGGTTGTCGTTGTTG-TGT-3′; BbtTIRB-F: 5′-TGCTTACCTCCGGTCTTGGC-3′, BbtTIRB-R: 5′-CGCTGACTCAGGTTGCTATCGTT-3′; BbtTIRC-F: 5′-GTCATCGGTTGCCAAGTT-3′, BbtTIRC-R: 5′-TCTCCTCCACGATCGTCAT-3′; BbtTIRD-F: 5′-AGCTTCTCAGGACATACCTGAG-3′, BbtTIRD-R: 5′-CA-CCGCCACGAGAACATAC-3′; Bβ-actin-F: 5′-CCCTCTGGCTCTGACTGAG-3′, Bβ-actin-R: 5′-ACACGGCCATCCGAGATCC-3′.

Expression plasmids

For the expression of bbtTIRA in HEK293T cells, PCR fragments encoding for aa 1–160, 161–620, 891–899, 890–1142, and 1142–1249 of bbtTIRA were inserted into pcCMV-Flag, pcCMV-Myc, and pcCMV-HA (Clontech) and designated bbtTIRA1, bbtTIRA4, bbtTIRA7, and bbtTIRA-FL, respectively (Fig. 3B). For the expression of bbtTIRB in HEK293T cells, PCR fragments encoding for aa 1–161, 162–302, and 303–409 of bbtTIRB were inserted into pcCMV-Flag, pcCMV-Myc, and pcCMV-HA (Clontech) and designated bbtTIRB1, bbtTIRB2, and bbtTIRB-FL, respectively (Fig. 4D). For the expression of bbtTIRB and bbtTIRD in HEK293T cells, PCR fragments encoding for aa 1–493 of bbtTIRB and 1–429 of bbtTIRD were inserted into pcCMV-Flag, pcCMV-Myc, and pcCMV-HA (Clontech) and designated bbtTIRB-FL and bbtTIRD-FL, respectively. For the study of subcellular localization, full-length bbtMyD88, bbtTICAM, bbt receptor interacting protein 1b (bbtRIP1b), and bbtTRAFs were inserted into pEGFP-N1 (Clontech). To determine topologies of membrane proteins, we inserted full-length bbtTIRB into pEGFP-N1 and inserted full-length bbtTIRB into pEGFP-C1 (Clontech) (Fig. 2B). PCR fragment encoding for mCherry was inserted into pcDNA3.1 (Invitrogen). PCR fragments encoding for aa 1–964 of bbtTICAM fused with 3′ Flag tag was inserted into a pcDNA3.0 vector (Invitrogen) and designated bbtTICAM-FL. Full-length bbtMyD88, bbtTRAF6, and bbtRIP1b were inserted into a pcDNA3.0 vector (Invitrogen) fused with 3′ Flag and 3′ HA tag for the endosomal and mitochondrial markers, the PCR fragment encoding for the endosomal marker protein CD63 and the mitochondrial marker PHB1 were inserted into pEGFP-N1 vectors fused with GFP-tag, respectively. For the endoplasmic reticulum (ER), the PCR fragment encoding for the ER marker ERM was inserted into a pEGFP-N1 vector fused with GFP-tag.

Fluorescence protease protection

Protein localization could be determined by fluorescence protease protection (FPP) assay (24) and passage of the Hela cells into cell chambers (Lab-Tek chambered cover glass). At 16 h postpassage, cells were transfected with plasmid coding for the bbtTIRB or bbtTIRD tagged with GFP and soluble fluorescent protein mcherry (Red) expression plasmid. After 20 h posttransfection, cell culture medium was removed and cells were washed three times for 1 min each in KHNN (110 mM potassium acetate, 2 mM MgCl2, 20 mM HEPES, pH 7.2) buffer at 25°C. Then 20 μM digitonin (Sigma-Aldrich) in KHNN buffer was added to the cells to permeabilize the plasma membrane. To determine the permeabilization situation after digitonin application, the red fluorescent of mcherry (Red) was tested with microscopy. When the red fluorescent disappeared, the KHNN buffer with digitonin was removed and cells were washed quickly but thoroughly with KHNN buffer. Then 4 mM of the protease trypsin (in KHNN buffer) was added directly onto the cells, and images were immediately taken in the fluorescence microscope to record whether GFP fluorescent signals persist or disappear.

Coinmunoprecipitation (Co-IP), immunofluorescence imaging, transient transfection, and luciferase reporter assay were performed as previously described (21, 25).

Results

Sequences and structure analyses of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD

Annotation of immune-related molecules in amphioxus B. floridanae has identified >40 TIR adapters and ongoing domain shuffling among these adapters (17). Using another recently completed genome of amphioxus B. belcheri, we conducted a genomic survey of the TIR adaptor in B. belcheri and compared with
B. floridae. We found a similar number of TIR adaptors in the two species. In addition to several TIR adaptors that showed homologies with MyD88, TICAM, and SARM, some adaptors were shown to be similar to orphan vertebrate TIR genes, and most of

![Image](https://example.com/image1.png)

**FIGURE 1.** Genomic sequence analysis of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD and their domain topology. (A–D) The TIR domain, STYKc domain, and transmembrane regions were predicted by the SMART Web site (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1). The TRAF6-binding motif [PxExx(Ac/Ar): Ar for aromatic residues, Ac for acidic residues, x for any residue]. Sequence analyses indicated that all bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD possess several conserved kB-binding motifs in the promoter region, and the reporter assays confirmed that the region containing some kB-binding sites upstream of the ATG of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD is essential for the binding to bbtRel. For the reporter assays, the increasing amounts of pGL3 basic vectors containing 2-kb genomic sequences upstream of the ATG of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD were transfected into HEK293T cells in the presence of 50 ng HsP65 or bbtRel expression vector. Hsp65 indicates *Homo sapiens* p65. Data are shown as mean ± SD of three samples per treatment, and values were considered significant when *p*, 0.05. Results were confirmed by at least three separate experiments. *p* < 0.05, **p** < 0.01.

![Image](https://example.com/image2.png)

**FIGURE 2.** Inhibitory efforts of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD on the NF-κB activation mediated by bbtMyD88 or bbtTICAM and their distributions. (A) bbtTIRB and bbtTIRD colocalized with the endosome marker CD63. Immunofluorescence microscopy images taken from HeLa cells cotransfected with GFP-fused CD63, together with HA-tagged pCMV-bbtTIRB, bbtTIRD, and stained with anti-HA and Alexa Fluor 532 Ab. (B) The FPP results showed that bbtTIRB anchored to the endosome with its TIR domain degraded by protease trypsin, whereas the TIR domain of bbtTIRD was not affected. The HeLa cells were transfected with mCherry (Red) inserted into pcDNA3.1 and bbtTIRB inserted into pEGFP-C1 or bbtTIRD inserted into pEGFP-N1. Fluorescence microscopy images were taken before or after digitonin and trypsin application. Fluorescence microscopy images are representative of at least three independent experiments with in which >80% of the Hela cells showed similar patterns. (C–E) HEK293T cells were cotransfected with NF-κB transcriptional luciferase reporter constructs, together with bbtMyD88 or bbtTICAM and (increasing amounts of) bbtTIRA, bbtTIRB, and (btbTIRC vectors as indicated. All reporter assays performed in HEK293T cells are shown as mean ± SD of three samples per treatment, and values were considered significant when *p*, 0.05. Results were confirmed by at least three separate experiments. *p* < 0.05.
them contain novel domain recombination, such as TIR+Pkinase-Tyr, CARD+TIR, and death effector domain (DED)+CARD+TIR (Supplemental Table I). To reveal how the novel TIR adaptors function in amphioxus TLR signaling, we chose eight TIR novel adaptors (CARD-TIR, B. floridae gene ID: 89399, 96757; TIR-HEAT, B. floridae gene ID: 93247; TIR-Pkinase_Tyr, B. floridae gene ID: 78250; TPR-TIR, B. floridae gene ID: 63858 and 68270; TIR, B. floridae gene ID: 69123 and 89683) with the following two characteristics for further cloning. First, they should have novel domain architectures. Second, they should exist in both *B. belcheri* and *B. floridae* simultaneously and not experience gene duplication. Adaptors with these two features should be more stabilized and have more specialized functions.

Unfortunately, just four of them with differential characteristics could be isolated from the *B. belcheri tsingtauense* intestine cDNA library and designated as bbtTIRA (corresponding to *B. floridae* gene ID: 78250), bbtTIRB (*B. floridae* gene ID: 89683), bbtTIRC (*B. floridae* gene ID: 89399), and bbtTIRD (*B. floridae* gene ID: 69123), respectively, suggesting that the other four TIR adaptors may be pseudogenes or genes with inducible transcription. BbtTIRA encodes a polypeptide of 1143 aa with two highly conserved protein structures: the TIR domain and the STYKc domain. The STYKc domain of bbtTIRA showed 30% amino acid identity with mammalian Tyrosine-protein kinase abelson murine leukemia viral oncogene homolog 2 (ABL2) (Fig. 1A, Supplemental Fig. 1A). BbtTIRB encodes a polypeptide of 493 aa and bbtTIRD encodes a polypeptide of 430 aa. Although both bbtTIRB and bbtTIRD contain two transmembrane regions and a TIR domain, the TIR domain of bbtTIRB is located at the N terminus, whereas that of bbtTIRD is located at the C terminus (Fig. 1B, 1D). Further sequence alignment showed that the characteristic sequence Box1, Box2, Box3, and the BB loop are well conserved in the TIR domains of these four adaptors (Supplemental Fig. 1B).

bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD are the target genes of NF-κB

RT-PCR was performed to determine the tissue distribution of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD, and results showed that transcripts of all the tested genes are abundant in amphioxus

---

**FIGURE 3.** bbtTIRA interacted with bbtTICAM and bbtRIP1b. (A) The full-length of bbtTIRA colocalized with bbtTICAM in Hela cells. (B) Truncated and site-directed mutants of bbtTIRA inserted into pCMV-Myc vector and named as indicated. Lys 635 was substituted for Arg 635. (C) bbtTIRA1, bbtTIRA4, and bbtTIRA7, but not bbtTIRA9, depressed the activation of the NF-κB by bbtTICAM. (D) Co-IP results showed that bbtTIRA1, bbtTIRA4, and bbtTIRA7, but not bbtTIRA9, could interact directly with bbtTICAM. (E) bbtTIRA inhibited NF-κB activation mediated by bbtRIP1b in HEK293T cells. (F) Confocal microscopy showed that bbtTIRA colocalized with bbtRIP1b. (G) The Co-IP results showed that bbtTIRA1, bbtTIRA4, and bbtTIRA7, but not bbtTIRA9, interacted directly with bbtRIP1b. (H) Compared with wild type bbtTIRA, the bbtTIRA-mutant could depress the activation of the NF-κB by bbtTICAM in a low level. Immunofluorescence microscopy images are representative of at least three independent experiments, in which >80% of the Hela cells showed similar staining patterns. All reporter assays performed in HEK293T cells are shown as mean ± SD of three samples per treatment, and values were considered significant when *p* < 0.05. Results were confirmed by at least three separate experiments. *p* < 0.05.
digestive system (Supplemental Fig. 1C), which is considered to be the first defense line of amphioxus (25, 28). To further study the immunological significance of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD in adults, we performed real-time PCR analyses, and results showed the transcriptions of these TIR adaptors in amphioxus intestines to be upregulated after challenge with LPS and LTA mixture (Supplemental Fig. 1D). In our previous study, we have demonstrated that hsp65 or bbtRel can recognize the sequence including NF-κB motif. Moreover, the interaction between κB motif and bbtRel can be blocked by the NF-κB–specific inhibitor helenalin (25, 29). In this study, we further showed that the upregulation of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD were inhibited in adult amphioxus, which are treated with helenalin before challenge with LPS and LTA mixture (Supplemental Fig. 1E). These results suggested that the transcription of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD are tightly regulated by amphioxus NF-κB.

To further investigate whether bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD are classical NF-κB target genes, we obtained the 2-kb genomic sequences upstream of the ATG of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD and subjected them to the Transcription Element Search System prediction program (www.chil.upenn.edu/cgi-bin/tess/tess) to determine whether these regions contain conserved NF-κB–binding motifs. All promoter regions of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD were found to contain several conserved κB-binding sites (Fig. 1).

Then the sequence including κB-binding sites of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD was inserted into pGL3 basic reporter vector and cotransfected with bbtRel or human p65 expression plasmid in HEK293T to reveal whether p65 can recognize these promoter sequences. Reporter assays showed that hsp65 or bbtRel can recognize the sequence including κB-binding sites of bbtTIRA, bbtTIRB, bbtTIRC, and bbtTIRD, suggesting that these TIR adaptors are classical targets of amphioxus NF-κB signaling (Fig. 1).

_bbtTIRA inhibits the bbtTICAM-dependent pathway, whereas bbtTIRB and bbtTIRC affect the bbtMyD88-dependent pathway_

Because bbtTIRB and bbtTIRD are transmembrane proteins with their TIR domain located at a distinct terminus, to investigate whether they have a specific subcellular location, we cotransfected them with three organelle markers. Results showed bbtTIRB and bbtTIRD to be colocalized with endosomes, but not with mitochondrial and ER when overexpressed in HeLa cells (Fig. 2A, Supplemental Fig. 2A, 2B). To investigate whether TIR domains of bbtTIRB and bbtTIRD faced to the cytosol, or enclosed in the endosome, we used further FPP experiment. In the FPP procedures, the bbtTIRB or bbtTIRD expressed cells were first treated with cholesterol-binding digitonin and then with trypsin. Because the plasma membrane contains more cholesterol than intracellular organelles, the low concentration of cholesterol-binding digitonin can increase the permeability of cell membrane and allow trypsin get into cytosol, but not intracellular organelles. Results showed that, when treated with trypsin, the fluorescence of GFP disappeared in GFP-tagged bbtTIRB-expressed cells, but was retained in GFP-tagged bbtTIRD-expressed cells. These results suggested that bbtTIRB anchored to the endosome with its TIR domain faced to the cytosol, whereas the TIR domain of bbtTIRD enclosed in the endosome or ER. When the TIR domain enclosed in the endosome or ER, it may have no chance to interact with other TIR-containing molecules, suggesting that bbtTIRD may be a redundant protein without signaling transduction activities in amphioxus TLR signaling (Fig. 2B).

To reveal whether bbtTIRA, bbtTIRB, and bbtTIRC are critical for the activation of NF-κB or other immune-related transcription factors, we conducted luciferase assays. Because of the lack of amphioxus cell lines at present, mammal cell lines were chosen.
Results showed none to have an effect on the induction of type I IFN or on the activation of API and NF-κB in HEK293T cells (Supplemental Fig. 2C–E). Because MyD88-dependent and TICAM-dependent pathways have been demonstrated in amphioxus (18, 19), we further used luciferase assays to test whether bbtTIRA, bbtTIRB, and bbtTIRC are involved in amphioxus MyD88- and TICAM-dependent signaling. Results showed that bbtTIRA specifically inhibited the bbtTICAM-dependent activation of NF-κB (Fig. 2C), whereas bbtTIRB and bbtTIRC could attenuate the bbtMyD88-mediated activation of NF-κB in a dose-dependent manner (Fig. 2D, 2E).

**BbtTIRA interacts with bbtTICAM and bbtRIP1b to inhibit the bbtTICAM-mediated NF-κB activation**

To further assess how bbtTIRA affects the bbtTICAM-mediated activation of NF-κB, we first performed confocal microscopy to show the colocalization of bbtTIRA with bbtTICAM, but not with bbtMyD88 in Hela cells (Fig. 3A, Supplemental Fig. 2F). Then four truncated mutants of bbtTIRA were constructed (Fig. 3B) and luciferase assays showed that the TIR domain, middle region, and STYKc domains of bbtTIRA attenuated the activation of NF-κB mediated by bbtTICAM (Fig. 3C). Co-IP assays confirmed that TIR domain, middle region, and STYKc domain of bbtTIRA, but not the C-terminal region, can interact with bbtTICAM (Fig. 3D). Because our previous study demonstrated that the association of bbtRIP1b with bbtTICAM is a crucial step in the bbtTICAM-mediated activation of NF-κB (19), to investigate whether bbtTIRA would interrupt the interaction between bbtTICAM and bbtRIP1b, we performed luciferase assays. Results showed that bbtTIRA attenuated the activation of NF-κB mediated by bbtRIP1b (Fig. 3E). Signal analysis by confocal microscopy indicated that bbtRIP1b colocalized with bbtTIRA when overexpressed in Hela cells (Fig. 3F). Furthermore, Co-IP assays showed that bbtTIRA could interact with bbtRIP1b through its N-terminal, middle region, and STYKc domain (Fig. 3G).

**FIGURE 5.** bbtTIRC inhibited the NF-κB activation induced by bbtTRAF6. (A) HEK293T cells were cotransfected with NF-κB transcriptional luciferase reporter constructs, together with bbtTRAF6 and increasing amounts of bbtTIRC vectors as indicated. (B) The full length of bbtTIRC colocalized with bbtTRAF6. Immunofluorescence microscopy images are representative of at least three independent experiments, in which >80% of the Hela cells showed similar staining patterns. (C) The Co-IP results showed bbtTIRC to interact directly with bbtTRAF6. (D) bbtTIRC2 inhibited NF-κB activation to the same extent as seen with the full length, but mutant bbtTIRC1 had no effect. (E) Co-IP results showed that bbtTIRC2 interacted directly with bbtTRAF6. Site-directed mutants used. The TRAF6-binding motif (PxExx(Ac/Ar); Ar for aromatic residues, Ac for acidic residues, x for any residue). (G) bbtTIRC-mutant did not interact with bbtTRAF6. (H) Compared with the wild-type bbtTIRC, activity of bbtTIRC-mutant was attenuated to 40%. (I) Compared with the wild-type bbtTIRC, mutant almost did not depress the polyubiquitination of bbtTRAF6. All reporter assays performed in HEK293T cells, and data are shown as mean ± SD of three samples per treatment. Values were considered significant when \( p < 0.05 \). Results were confirmed by at least three separate experiments. *\( p < 0.05 \).
Sequence analysis of the STYKc domain of bbtTIRA and mammalian ABL2 showed ~30% identity (SupplementalFig.1A), leading to the question of whether this domain can mediate tyrosine phosphorylation to affect NF-κB activation. The inactive mutation of Abl2-like kinase into c-Abl1, in which the Lys290 critical for ATP binding was mutated to arginine, resulted in a complete loss of kinase effect (30). Thus, site-directed mutagenesis of bbtTIRA was conducted by replacing the conserved residues 635K within the STYKc domain with 635R (Fig. 3B). Compared with wild type bbtTIRA, reporter assay showed that the K635R mutant can suppress the NF-κB activation mediated by bbtTIRC in a low level (Fig. 3H). Thus, it appears that the TIR domain, middle region, and STYKc domain of bbtTIRA participated in inhibiting the activation of NF-κB in the MyD88-independent signal pathway.

BbtTIRC interacts with bbtMyD88 and bbtTRAF6 and depresses polyubiquitination of bbtTRAF6

Because bbtTIRB and bbtTIRC were shown to attenuate the NF-κB activation mediated by bbtMyD88, confocal microscopy was performed. Results showed both bbtTIRB and bbtTIRC to colocalize with bbtMyD88, but not with bbtTICAM when over-expressed in Hela cells (Fig. 4A, Supplemental Fig. 2G). Since bbtTIRB contains two transmembrane regions, it is easily gathered and shows significant organelle localization (Fig. 2A, Supplemental Fig. 2A, 2B). However, bbtTIRC may be widely distributed in the cytosol. Thus, when bbtMyD88 was co-transfected with bbtTIRB or bbtTIRC, it showed distinct distribution. Co-IP assay further confirmed the direct interaction of bbtMyD88 with both bbtTIRB and bbtTIRC (Fig. 4B, 4C). To investigate which domain of bbtTIRC is responsible for its function, two truncated mutants, bbtTIRC1 (the N-terminal) and bbtTIRC2 (the C-terminal TIR domain with TRAF6-binding motifs), were constructed for further analysis (Fig. 4D). Reporter and Co-IP assays showed that although bbtTIRC1 and bbtTIRC2 were both shown to interact with bbtMyD88, only bbtTIRC2 inhibited NF-κB activation mediated by bbtMyD88 in a dose-dependent manner (Fig. 4E, 4F). Because recruitment of TRAF6 to MyD88 complexes is a key step in the activation of NF-κB (31, 32), we performed reporter assay to investigate the effect of bbtTIRC on bbtTRAF6 activity. Results showed that bbtTIRC inhibits TRAF6-mediated NF-κB activation in a dose-dependent manner (Fig. 5A). Moreover, bbtTIRC can interact and colocalize with bbtTRAF6 (Fig. 5B, 5C). As mentioned earlier, the analysis of the bbtTIRC sequence revealed two TRAF6-binding motifs (PExExx) in the C terminus. To verify whether the activity of bbtTIRC depends on these two motifs, two truncated mutants (bbtTIRC1 and bbtTIRC2) were coexpressed with bbtTRAF6. Results showed that only bbtTIRC2, which contains two TRAF6 binding motifs, can interact with bbtTRAF6 and inhibit the TRAF6-mediated activation of NF-κB in a dose-dependent manner (Fig. 5D, 5E).

To further reveal whether the inhibitory activity of bbtTIRC is due to these two TRAF6 binding motifs, we conducted site-directed mutagenesis by replacing the conserved residues 162PVE164 and 257PPE259 into 162AVA164 and 257APA259 (Fig. 5F). In contrast with wild-type bbtTIRC, the bbtTIRC-mutant did not interact with bbtTRAF6 (Fig. 5G), and the capacity of the bbtTIRC-mutant to attenuate the activation of NF-κB mediated by bbtTRAF6 was reduced to 40% (Fig. 5H).

Because K63-linked autopolyubiquitination of TRAF6 is another crucial step in the activation of NF-κB, to investigate whether bbtTIRC could affect the polyubiquitination of bbtTRAF6, we performed ubiquitination assays. Results showed that bbtTIRC could depress the polyubiquitination of bbtTRAF6 and human TRAF6, but not bbt NF-κB essential modulator and human NF-κB essential modulator (Supplemental Fig. 3A-D). Compared with wild-type bbtTIRC, the ability of the bbtTIRC-mutant to depress polyubiquitination of TRAF6 was also attenuated (Fig. 5I). Thus, we suggest that bbtTIRC inhibited the TRAF6-mediated signaling by depressing the polyubiquitination of TRAF6 (Fig. 6).

Discussion

Rudiment of amphioxus TLR signaling

Basal deuterostomes possess many more TIR adaptors than the five seen in vertebrates. For example, the sea urchin possesses 26 potential TIR adaptors, including 4 MyD88-like, 15 SARM1-like, and 7 orphan TIR genes (33). In comparison of the genomes between B. floridai and B. belcheri, we found that the basal chordate amphioxus possesses >40 TIR adaptor proteins, including homologs of MyD88, TICAM, and SARM; adaptors similar to orphan vertebrate TIR genes; and adaptors with novel domain recombination (17). The identification of both TLR and MyD88 in various evolutionary stages, and the functional interaction of amphioxus TLR with MyD88, suggests that the MyD88-dependent pathway is conserved and is crucial for invertebrate TLR signaling (18, 33–35). Because no homolog of vertebrate TICAM1 and TICAM2 was identified in invertebrates, the MyD88-independent pathway was long believed to be a vertebrate
innovation. However, a common ancestor of vertebrate TICAM1 and TICAM2 was identified in amphioxus and shown to specifically activate NF-κB in an MyD88-independent manner (19), suggesting that the primitive MyD88-independent pathway has established in basal chordate. Because vertebrate TICAM1 can induce the production of type I IFN and activate NF-κB, vertebrate MyD88-independent signaling was considered to be originated from the primitive bbtTICAM–NF-κB pathway (19) and coevolved with the emergence of the IFN system and adaptive immunity in vertebrates (36). In this study, we further showed that amphioxus TLRs can selectively bind to bbtTICAM or bbtMyD88 to activate NF-κB (Supplemental Fig. 3E–G). Thus, although both bbtTICAM- and bbtMyD88-dependent pathways converge upon the IKK complex to activate NF-κB in amphioxus, they may associate with distinct TLRs to activate NF-κB, which provides quick and amplified immune responses when amphioxus encounters infections.

In the genome of amphioxus, it is indicated that there are ~39 TLRs, which are far more than that of the vertebrate. Besides the previously characterized bbtSARM, which plays inhibitory roles on both bbtMyD88- and bbtTICAM-dependent pathways by interacting with bbtMyD88 and bbtTICAM in amphioxus. In this study, we further showed that bbtTIRC suppresses bbtMyD88-mediated signaling by interacting with bbtMyD88 and depressing the polyubiquitination of bbtTRAF6, whereas bbtTIRC suppresses the bbtTICAM-mediated activation of NF-κB by interacting with both bbtTICAM and bbtTRAF6. To avoid over-activation, mammals used several spliced variants of TIR adaptors, including MyD88s, a spliced variant of MyD88 that is induced by LPS, and TAG, a spliced variant of the adaptor TRAM. MyD88s did not recruit the downstream adaptor IRAKs (10), whereas TAG displaced the adaptor TRIF, resulting in the negative regulation of vertebrate TLR signaling (37). Our study provides evidence that basal chordate uses expanded TIR adaptors for negative regulation, suggesting the nonredundant roles of the expanded TIR adaptors and the precise regulation of amphioxus TLR signaling. Because both bbtTIRA and bbtTIRC are classical NF-κB target genes, we can speculate that the mechanism depending on bbtTIRA and bbtTIRC may be an effective feedback regulation of amphioxus NF-κB signaling.

**Novel TIR adaptors negatively regulated amphioxus TLR signaling at distinct aspects**

In addition to the regulation at the adaptor level, posttranslation modification of the key molecules in TLR signaling is a key mechanism of feedback regulation, including ubiquitination and phosphorylation. K63 autopolyubiquitination of TRAF6 in conjunction with the E2 enzyme Uev1a/Ubc13 is essential to activate NF-κB signaling, whereas the deubiquitinases A20 and CYLD (38, 39), which remove the K63-linked polyubiquitin chains from TRAF6, can suppress such activation (40). In addition, A20 binding and inhibitor of NF-κB (ABIN-1) and ABIN-3 contain a ubiquitin binding domain and may contribute to the termination of the NF-κB response downstream of TRAF6 by recruiting A20 to ubiquitylated IKKγ (41). Our previous study showed that bbtABIN2 can compete with bbtTRAF6 for the K63-linked ubiquitin chains to negatively regulate NF-κB activation. In this study, we observed that bbtTIRC binds human TRAF6 and bbtTRAF6 to depress their polyubiquitination. In vertebrates, phosphorylation of a series of kinases is another crucial step in the release and subsequent translocation of NF-κB into the nucleus. In this study, when the 635K within the STYKc domain of bbtTIRA was replaced with 635R, the suppression of bbtTICAM-mediated activation of NF-κB was eliminated, indicating that the STYKc domain of bbtTIRA is important for the regulation of NF-κB. Studies in vertebrates have shown that Ab1, an Ab2-like tyrosine kinase, can mediate the phosphorylation of inhibitor of NF-κB α at Tyr505 and increase its stability (42), resulting in the inhibition of NF-κB. Because the STYKc domain in bbtTIRA is homologous with the tyrosine kinase of Ab2, further characterization of the relationships of bbtTIRA with inhibitor of NF-κB α or other substrates should help to define the distinct functions of bbtTIRA in anti-inflammation.

In conclusion, to our knowledge, this study provides the first evidence that the expanded TIR adaptors in basal chordate play nonredundant roles in the activation of NF-κB, adding a further layer of complexity to amphioxus innate immunity, not only with respect to the diversity of receptor recognition, but also in the cytoplasmic regulation (Fig. 6).

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Inventory of Supplemental Information

1. **Table S1.** TIR adaptors in distinct evolutionary species.

2. **Figure S1.** The sequence analyses and expression patterns of bbtTIRA, bbtTIRB, bbtTIRC and bbtTIRD.

3. **Figure S2.** The cellular distributions and the signal transduction regulations of bbtTIRA, bbtTIRB, bbtTIRC and bbtTIRD.

4. **Figure S3.** BbtTIRC inhibited the polyubiquitination of TRAF6, and bbtTLRs selectively bound to bbtTICAM or bbtMyD88 to activate NF-κB.
Table S1. TIR adaptors in distinct evolutionary species.

<table>
<thead>
<tr>
<th>Catalog</th>
<th>D.m</th>
<th>Amphioxus</th>
<th>Human being</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S.p</td>
<td>Bf(models)</td>
</tr>
<tr>
<td>TIR-like genes or adaptors</td>
<td>2</td>
<td>26</td>
<td>62</td>
</tr>
<tr>
<td>MyD88-like (DEATH-TIR)</td>
<td>1</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>SARM1-like</td>
<td>1</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td>TIRAP-like</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>TICAM2-like</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CARD-TIR</td>
<td>4</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>CARD-TIR-ResIII-Helicase_C-RIG-I_C-RD</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CARD-TIR-LRR</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Death-TIR-Death</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DEATH-TIR-TIR</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>DED-CARD-TIR</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DED-TIR-Glycos_transf_1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Helicase_C-DEAD</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>LRR_1-2xMBT-Ras</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LRR-CCP-TM-TIR</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>LRR-Death-TIR</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>TIR</td>
<td>7</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>TIR-TIR</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>TIR-2xOAS1_C</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TIR-Death-5xLRR_1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TIR-Death-7xAnk</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TIR-Glycos_transf_1</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TIR-HEAT</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TIR-NB-ARC-10xWD40</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>TIR-Pkinase_Tyr</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TIR-2xTSP_1-EGF</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TPR-TIR</td>
<td>7</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Other TIR genes</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

D.m: *Drosophila melanogaster*; S.p: *Strongylocentrotus purpuratus*; Bf: *Branchiostoma floridae*; Bbe: *Branchiostoma belcheri*. ANK, Ankyrin repeat, PF00023; Arm, PF00514; CARD, PF00619; Death, PF00531; DED, PF01335; Glycos_transf_1, PF00534; HEAT, PF02985; Helicase_C, PF00271; LRR_1, PF00560; MBT, malignant brain tumor, PF02820; NB-ARC, PF00931; OAS1_C, PF10421; Pkinase_Tyr, PF07714; Pro_isomerase, PF00160; Ras, PF00071; ResIII, PF04851; RIG-I_C-RD, PF11648; SAM_1, SAM, sterile alpha motif.
PF00536; SEFIR, PF08357; TIR, Toll/IL-1R homology, PF01582; TPR_1, PF00515; TSP_1, PF00090; WD40, PF00400.
FIGURE S1. The sequence analyses and expression patterns of bbtTIRA, bbtTIRB, bbtTIRC
and bbtTIRD. (A) Sequence alignments of STYKc domain from representative organisms ABL2 proteins and bbtTIRA. (B) The conserved three boxes and BB loop in the TIR domains. Sequence alignments based on TIR domains of MyD88 from mammal, fish, amphioxus, fruit fly, the TIR domains of thale cress genes, and the TIR domain of bbtTIRA, bbtTIRB, bbtTIRC and bbtTIRD. Representative organisms are: Hs: Homo sapiens; Mm: Mus musculus; Dr: Danio rerio; Ci: Ciona intestinalis; Dm: Drosophila melanogaster; Cs: C. elegans; AT: Arabidopsis thaliana. (C) RT-PCR showed that transcripts of bbtTIRA, bbtTIRB, bbtTIRC and bbtTIRD are abundant in the digestive tissues of normal amphioxus. The results are presented as expression relative to that in muscles. (D) Quantitative real-time PCR (RT-PCR) analysed the expression of bbtTIRA, bbtTIRB, bbtTIRC and bbtTIRD after challenged with LPS and LTA. (E) RT-PCR analyses of expression patterns of bbtTIRA, bbtTIRB, bbtTIRC and bbtTIRD after LPS and LTA mixture challenging in the presence or absence of the NF-κB inhibitor helenalin. Results were presented as “fold induction” of mRNA expression in triplicate from two parallel experiments, using 2-ΔΔC_{i} method. Endogenous control for quantification was cytoplasmic β-actin.
**FIGURE S2.** The cellular distributions and the signal transduction regulations of bbtTIRA, bbtTIRB, bbtTIRC and bbtTIRD. (A, B) The results showed that bbtTIRB and bbtTIRD did not co-localize with the mitochondrial marker. Moreover, bbtTIRB and bbtTIRD did not co-localize with the endoplasmic reticulum marker. Immunofluorescence microscopy images were taken from HeLa cells cotransfected with GFP-fused mitochondrial marker PHB1 or RFP-fused endoplasmic reticulum marker ERM, together with HA-tagged pCMV-bbtTIRB, bbtTIRD, and stained with anti-HA and Alexa Fluor 532 Ab or Alexa Fluor 488 Ab. (C-E) Reporter assays showed that bbtTIRA, bbtTIRB and bbtTIRC did not have an effect on the induction of type I interferon or on the activation of AP1 and NF-κB. HsTICAM1 (Human
TICAM1) was used as positive control. All reporter assays performed in HEK293T cells, and data are shown as the means ± SD of three samples per treatment. The results were confirmed by at least three separate experiments. Values were considered to be significant at P < 0.05. Student’s t-test (two tailed distribution, two-sample unequal variance) was used for the calculation of all P-values. *P < 0.05 and **P < 0.01. (F, G) BbtTIRA did not colocalize with bbtMyD88, while bbtTIRB and bbtTIRC did not colocalize with bbtTICAM. Immunofluorescence microscopy images were taken from HeLa cells cotransfected with GFP-fused bbtMyD88 or bbtTICAM, together with HA-tagged pCMV-bbtTIRA, bbtTIRB, and bbtTIRC, and stained with anti-HA and Alexa Fluor 532 Ab. All confocal assays were confirmed by at least three separate experiments.
FIGURE S3. BbtTIRC inhibited the polyubiquitination of TRAF6, and bbtTLRs selectively bound to bbtTICAM or bbtMyD88 to activate NF-κB. (A, B) bbtTIRC strongly inhibited TRAF6 polyubiquitination, and it had the same effect on HsTRAF6 (human TRAF6). (C, D) bbtTIRC had no effect on the ubiquitination of NEMO. HEK293T cells were co-transfected with HA-ubiquitin, bbtTIRC-Myc and Flag-TRAF6 or Flag-NEMO expression vectors as indicated. (E). The TIR domain of bbtTLRL (bbtTLRL-TIR) cloned from amphioxus. bbtTLRL (corresponding to B. floridae gene ID:236291). (F). bbtTLRL-TIR alone could not activate NF-κB. However, when it co-expressed with bbtMyD88, it can mount the bbtMyD88
mediated activation of NF-κB. (G). bbTLRL-TIR combined with bbMyD88 selectively, but not bbTICAM. All reporter assays performed in HEK293T cells are shown as the means ± SD of three samples per treatment, and values were considered significant when *P < 0.05. The results were confirmed by at least three separate experiments.