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Toll-like receptor 2 of tongue sole *Cynoglossus semilaevis*: Signaling pathway and involvement in bacterial infection

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ABSTRACT

Toll-like receptor (TLR) 2 is a member of the TLR family that plays a pivotal role in innate immunity. In mammals, TLR2 is known to recognize specific microbial structures and trigger MyD88-dependent signaling to induce various cytokine responses. In this study, we examined the expression and function of the tongue sole *Cynoglossus semilaevis* TLR2, CsTLR2. CsTLR2 is composed of 898 amino acid residues and shares 25.6%–27.3% overall sequence identities with known teleost TLR2. CsTLR2 is a transmembrane protein with a toll/interleukin-1 receptor domain and eight leucine-rich repeats. Expression of CsTLR2 occurred in multiple tissues and was upregulated during bacterial infection. Stimulation of the CsTLR2 pathway led to enhanced expression of MyD88-dependent signaling molecules. Recombinant CsTLR2 (rCsTLR2) corresponding to the extracellular region was able to bind to a wide range of bacteria. Under both *in vitro* and *in vivo* conditions, rCsTLR2 significantly reduced bacterial infection. These observations add new insights into the signaling and function of teleost TLR2.

1. Introduction

Pattern-recognition receptors (PRRs) are a group of conserved molecules that mediate recognition of microbial components by the innate immune system of both vertebrates and invertebrates [1]. Toll-like receptors (TLRs), a family of evolutionarily conserved type I transmembrane proteins, are one of best characterized PRRs [2,3]. They contain an extracellular domain (ECD) with a tandem array of leucine-rich repeat (LRR) motifs and an intracellular toll/interleukin-1 receptor (TIR) domain connected by a linker [1,4,5]. In the ECD, the LRR motif is involved in recognition of pathogen-associated molecular patterns (PAMPs) present in microbes and thus defines the specificity of the TLR, while the cytoplasmic TIR domain harbors conserved amino acids involved in signal transduction and localization of the TLR [6,7]. Upon recognizing the ligand by LRR, the signaling pathway is activated through TIR domain by homotypic protein interaction with adaptor molecules also containing a TIR domain in the cytoplasm [8]. So far, all reported mammalian TLRs recruit the adaptor protein Myeloid differentiation factor 88 (MyD88) except for TLR3, which recruits TIR domain-containing adaptor inducing IFN-β (TRIF) [9,10]. Activation of TLR induces the recruitment of MyD88 to the receptor complex, which in turn activates several downstream adaptor molecules including IL-1 receptor associated protein kinase (IRAK), tumor necrosis factor receptor-associated factor 6 (TRAF6), and transforming growth factor-β-activated kinase 1 (TAK1), resulting in the activation of NF-κB and transcription of inflammatory genes [11]. To date, 19 distinct TLR genes have been identified from various animal species, and in mammals most of the TLRs have been shown to recognize distinct PAMPs [12–15].

In mammalian vertebrates, TLR2 is best known as a receptor recognizing lipopeptides, lipoteichoic acid (LTA), and peptidoglycans (PGN) [16–18]. In addition, TLR2 also interacts with a wide range of additional ligand types including zymosan derived from yeast, glycosylphosphatidylinositols (GPIs) from protozoan parasites, and lipopolysaccharide (LPS) of Gram-negative bacteria [19–22]. Upon ligand recognition, TLR2 stimulates MyD88-dependent signaling pathway and activates NF-κB and mitogen activated protein kinase (MAPK), which lead to induction of

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antimicrobial defense [23–26]. Recent studies have shown that TLR2 may function together with TLR4 [27]. Additionally, TLR2 can function alone as a homodimer or as a heterodimer with TLR1 or TLR6 to recognize a variety of PAMPs [2,28–30].

In fish, a number of works on TLR2 has been carried out in zebrafish [31,32], Japanese flounder [33], pufferfish [34], channel catfish [35], European common carp [36], mrigal [37], rohu [38], and orange-spotted grouper [39,40]. Fish TLRs generally are homologous to mammalian TLRs in structure and functional property, however, they possess some specific features and differ from each other in detailed expression patterns [31,32,35,41–46]. In Japanese flounder, TLR2 was modulated in expression by peptidoglycan and poly I:C treatment [33]; in zebrafish, catfish, orange-spotted grouper, and rohu, TLR2 expression was regulated by bacterial pathogens such as *Mycobacterium marinum*, *Vibrio alginolyticus*, and *Edwardsiella ictulari* [31,35,38,40].

Half-smooth tongue sole (*Cynoglossus semilaevis*) is a species of flatfish mainly distributed in the Yellow Sea and the East China Sea. The history of tongue sole farming in China is short. However, due to its high economic value, tongue sole has become an important commercial fish species in China. With the rapid development of the industry, diseases caused by bacterial pathogens such as Vibrio and Edwardsiella have been reported [47]. To date, the information about half smooth tongue sole (*Cynoglossus semilaevis*) TLRs is scarce. In this study, we analyzed the tongue sole TLR2 (named CsTLR2) in the aspects of expression, signaling pathway, and antibacterial potential.

2. Materials and methods

2.1. Fish

Clinically healthy tongue sole (average 9.3 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Before experiment, fish were acclimatized in the laboratory for two weeks and confirmed to be without pathogen infection in liver, kidney, and spleen by plate count as reported previously [48]. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, USA) at the dose of 0.1 g/l.

2.2. Sequence analysis

The cDNA sequence (GenBank accession no. XP_008322030) and amino acid sequence of CsTLR2 were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI). Domain search was performed with the conserved domain search program of NCBI. The theoretical molecular mass and theoretical isoelectric point were predicted by using EditSeq in the DNASTAR (Madison, WI) software package. Multiple sequence alignment was created with DNAMAN.

2.3. Quantitative real time reverse transcription-PCR (qRT-PCR)

qRT-PCR was performed as reported previously [49]. Briefly, tissues (intestine, blood, spleen, kidney, gill, liver, brain, heart, and muscle) were taken aseptically from five tongue sole and used for total RNA extraction with EZNA Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China). The expression level of *CsTLR2* was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with beta-actin (ACTB) as an internal reference [50]. To examine *CsTLR2* expression in response to bacterial infection, *Edwardsiella tarda* was cultured in Luria-Bertani broth (LB) medium at 28 °C to an OD_{600} of 0.8; the cells were washed with PBS and resuspended in PBS to 1×10^6 CFU/ml. Tongue sole were divided randomly into two groups (20/group) and injected intraperitoneally (i.p.) with 50 μl *E. tarda* or PBS. At 6 h, 12 h, 24 h, and 48 h post-infection, five fish were taken for tissue collection, and *CsTLR2* expression was determined by qRT-PCR as above.

To examine the effect of Pam3CSK4 on the expression of the genes of TLR2 pathway, tongue sole peripheral blood lymphocytes (PBL) were prepared and maintained in 96-well culture plates (1×10^6 cells/well) containing L-15 medium (Thermo Scientific HyClone, Beijing, China) as reported previously [51]. The cells were added with Pam3CSK4 (InvivoGen, San Diego, USA) (100 ng/ml) or PBS (control) and incubated at 22 °C for 18 h. After incubation, the mRNA levels of the immune genes, *CsTLR2*, *MyD88*, *IRAK4*, *TRAF6*, *TAK1*, *NF-κB/p105*, *tumor necrosis factor-α* (*TNF-α*), *interleukin-1β* (*IL-1β*), and *IL-8* was determined by qRT-PCR as above. All experiments were performed three times.

2.4. Plasmid construction and protein purification

To construct pETCsTLR2, which expresses the extracellular region of CsTLR2 (residues 40 to 630), the coding sequence of this region was amplified by PCR with primers F1 (5′—GATATCATG CCAACACCCCCATCCACCTC— 3′, underlined sequence, EcoRV site) and R1 (5′—GATATCGTCACAGGACACTTCGCTGGGC— 3′, underlined sequence, EcoRV site); the PCR products were ligated with the T—A cloning vector T-Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the *CsTLR2*-containing fragment, which was inserted into pET259 [52] at the SwaI site. Protein purification and analysis were performed as described previously [49]. Briefly, *Escherichia coli* BL21(DE3) (TransGen Biotech Beijing, China) was transformed separately with pETCsTLR2 and pET32a (Novagen, San Diego, USA), which expressing Trx; the transformants were cultured in LB medium at 37 °C to mid-logarithmic phase, and isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM. After growing at 16 °C for an additional 10 h, the cells were harvested by centrifugation (4200 *g*), and His-tagged proteins were purified using Ni-NTA Agarose (QIAGEN, Valencia, USA) as recommended by the manufacturer.

2.5. Interaction between rCsTLR2 and bacteria

The bacteria used in this study have been reported previously [49]. Bacteria were cultured in LB medium to OD_{600} 0.8 and resuspended in PBS to 10^8 CFU/ml. Bacteria-protein interaction was determined by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence microscopy as reported previously [49].

2.6. Effect of rCsTLR2 on in vitro and in vivo bacterial infection

Both *in vitro* and *in vivo* infection were performed as reported previously [49]. In brief, for *in vitro* infection, *E. tarda* was resuspended in 100 μg/ml fluorescein isothiocyanate (FITC) (Tiangen, Beijing, China) and incubated at 37 °C for 2 h, the cells were then collected by centrifugation and washed with PBS. rCsTLR2 and rTrx were added to the cells to the concentration of 25 μg/ml. After incubation at 22 °C for 1 h, the cells were washed and resuspended in L-15 medium (Thermo Scientific HyClone, Beijing, China) to 1×10^8 cells/ml. One milliliter of PBL (~10^7 cells) was mixed with 100 μl *E. tarda*, rCsTLR2-treated *E. tarda*, rTrx-treated *E. tarda*, or L-15 medium (control). The mixture was incubated in the dark for 2 h, and the cells were collected by centrifugation and washed with PBS. Extracellular fluorescence was quenched by adding 1 ml 0.125% trypan blue in PBS, followed by incubation at 22 °C for 5 min. The