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Short communication

A novel *TNFAIP8* gene mediates L-arginine metabolism in *Apostichopus japonicus*

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ABSTRACT

Tumor necrosis factor (TNF)-α-induced protein 8 (TNFAIP8) family is a newly identified protein with vital roles in maintaining immune homeostasis. In the current study, we first cloned and characterized a *TNFAIP8* gene from the invertebrate sea cucumber *Apostichopus japonicus*. The gene was designated as *AjTNFAIP8*. The full-length cDNA of *AjTNFAIP8* was 1455 bp long and encoded a matured protein of 201 amino acid residues. Structural analysis indicated that AjTNFAIP8 had a death effector domain (DED)-like domain and composed of six α-helices. Multiple sequence alignment and phylogenetic analysis supported that AjTNFAIP8 is a new member of the TNFAIP8 family. Analysis of basal transcription in five tissues revealed the constitutive expression of *AjTNFAIP8* in the detected tissues with highest expression in the respiratory tree and minimum expression in the tentacle. *Vibrio splendidus* infection and LPS stimulation could significantly downregulate the mRNA expression of *AjTNFAIP8*. More importantly, the transcription of pro-inflammatory molecule *NOS* and its production of NO content were significantly increased after *AjTNFAIP8* silencing, with the suppression of *agmatinase* transcript and arginase activity. These results clearly indicated that *AjTNFAIP8* is an essential negative regulator in innate immunity. Basic information for further exploration of the functional mechanisms of TNFAIP8 family in other marine invertebrate is provided.

1. Introduction

The immune system often encounters numerous alien antigens. Innate and adaptive immunity is needed to eliminate these antigens. However, the strength and the duration of the immune responses should be tightly regulated, because excessive immune reactions potentially lead to lethal inflammatory diseases. Two classes of molecule have been described to maintain immune homeostasis. The first class, such as negative regulator molecules (e.g., SOCS2), inhibitory cytokines (e.g., IL-10), Toll-like receptor (TLR) signal pathways (e.g., CTLA-4), and repressive transcription factors (e.g., Foxp3), limits the activation and expansion of immune cells [1,2]. The second class, such as caspase-8, Bax, and Fas, controls cell death [3,4]. When the expression of these genes is absent or decreased, immune homeostasis fails, resulting in severe inflammatory diseases, which in turn lead to premature death of the organism. Identifying additional genes for maintaining immune homeostasis is crucial, because numerous genes in organism genomes have not yet been identified.

Tumor necrosis factor (TNF)-α-induced protein 8-like 2 (TNFAIP8L2, TIPE2), which belongs to TNFAIP8 family, has been recently identified as an essential negative regulator for maintaining immune homeostasis [2]. TNFAIP8L2 is predominantly expressed in the immune cells of the myeloid and lymphoid tissues and highly expressed in inflammatory tissues [5,6]. This molecule is also lowly expressed in other types of non-immune cells, such as hepatocytes and neurons [7]. Many studies have shown that TNFAIP8L2 expression is associated with the prevention of inflammatory diseases [8–10]. Deletion of the TNFAIP8L2 gene results in the emergence of a higher response to the activation of TLRs and T-cell receptors and release of many inflammatory cytokines [2,11,12]. Numerous studies have reported that the expression levels of IL-2,

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IL-4, IL-6, IL-12, and IFN-γ were significantly increased in TNFAIP8L2-deficient rats [2,12,13]. TNFAIP8L2 is also an inhibitor of MAPK and NF-κB signaling pathways, and this inhibition leads to diminished activity of activator protein-1 (AP-1) and NF-κB [2,5,14]. Activation of the NF-κB pathway in the macrophages would result in serious inflammatory diseases, possibly by affecting the balance of pro-and anti-inflammatory [15]. Lou et al. [16] study indicated that the knockout of TNFAIP8L2 in mice under LPS challenge would lead to the activation of NF-κB and MAPK signaling pathways. This activation which may contribute to increased levels of inducible nitric oxide synthase (iNOS) protein and NO concentration but reduced arginase I and urea production. Transformation of NOS to arginase using the common substrate L-arginine is an essential mechanism to control NO content. This process can avoid excessive NO content and negatively regulate inflammation [17]. Thus, TNFAIP8L2 may play pivotal roles in shifting L-arginine metabolism from production of NO to urea under host inflammatory response. In humans, the abnormal expression of TNFAIP8L2 is associated with infectious diseases and systemic autoimmunity [12]. Therefore, normal expression of TNFAIP8L2 in the immune system is necessary to maintain immune homeostasis.

In addition to TNFAIP8L2, three other members of the TNFAIP8 family are TNFAIP8, TNFAIP8L1, and TNFAIP8L3 [2,5]. TNFAIP8, which is the first characterized member of the TNFAIP8 family, was originally discovered in a primary human head and neck squamous cell carcinoma cell line [18]. Most studies have demonstrated that TNFAIP8 is an apoptosis regulator and plays important roles in inhibiting caspase-mediated apoptosis [19]. Knockdown of TNFAIP8 expression in hematopoietic progenitor cells is sufficient to effectively protect thymocytes against glucocorticoid-induced apoptosis. However, TNFAIP8 overexpression enhanced sensitivity to glucocorticoids, indicating TNFAIP8 is critical to the apoptotic responses of thymocytes to glucocorticoids [20]. In addition, previous studies demonstrated that TNFAIP8 is an oncogene, which plays crucial roles in oncogenesis [21,22]. TNFAIP8 overexpression is correlated with tumor stage and enhanced tumor growth [23]. However, knocking down TNFAIP8 expression in tumor cells would reduce their tumorogenicity [22]. These studies indicated that TNFAIP8 expression or its-mediated signaling pathways may be blocked to selectively suppress tumor formation.

The structure, evolution, and function of TNFAIP8 family members are currently the hot topic in various vertebrates because of their diverse regulator roles in innate and adaptive immunity. Among the invertebrate TNFAIP8 family, only one molecule, denoted as Sigmar, has been characterized from the fruit fly *Drosophila melanogaster* [24]. Sigmar functions in the modulation of JNK signaling, cytoskeletal rearrangement, and autophagy during salivary gland development [24,25]. However, the roles of this family on immune responses under pathogen infection or disease outbreaks are poorly understood. In the present study, we used a marine echinoderm species, sea cucumber (*Apostichopus japonicus*, Echinodermata, Holothuroidea), as a model to first investigate the molecular mechanism of *TNFAIP8* gene under immune responses. *A. japonicus* has become one of the most important marine aquaculture species in China [26]. Recently, the wild *A. japonicus* population in China rapidly declined due to outbreaks of infectious diseases [27], such as skin ulceration syndrome (SUS), which is the most contagious and lethal disease in sea cucumber culture industry [28,29]. Meantime, sea cucumber is a transitional organism from invertebrate to vertebrate and closer to cephochordata. Thus, this species is considered to be an ideal model for investigating the evolution of innate immunity. The current study was performed to explore the role of *TNFAIP8* in *A. japonicus* to demonstrate the possible involvement of the *TNFAIP8* family in the innate immunity of invertebrates.

2. Materials and methods

2.1. Animals and challenge experiments

Healthy adult *A. japonicus*, with average wet weight of 125 ± 15 g, were collected from Dalian Pacific Aquaculture Company (Dalian, China) and quarantined in aerated natural seawater with salinity of 28‰ at 16 °C for at least three days. The *V. splendidus* strain used in this assay was previously isolated from *A japonicus* individuals with diagnosed SUS. All *A. japonicus* individuals were randomly assigned to five treatment groups. Four groups, with 10 individuals in each group, were infected by immersion with live *V. splendidus* at a final concentration of 10^7 CFU/mL. Coelomic fluids from five individuals were randomly sampled at 6, 24, and 48 h post infection. The last group with 10 individuals was used as control group, and samples were collected at 0 h. Coelomic fluids were collected through a 100 μm sterile nylon mesh and then centrifuged at 800 × *g* for 5 min to harvest the coelomocytes. In addition, other tissues, including muscle, tentacle, respiratory tree, and intestine, were collected from healthy individuals for spatial expression analysis. Each experiment was performed with five replicates, and the samples were stored at −80 °C for further analysis.

2.2. Cloning and characterization of the full-length TNFAIP8 from A. japonicus (AjTNFAIP8)

Information for the partial sequence of *TNFAIP8* gene could be found in our previously established *A. japonicus* transcriptome database [30]. Homolog searching with BLASTx in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that this partial sequence contained the complete 5′-end, but lacked the 3′-end with poly(A) tail as shown in homologs from other species. A gene-specific 3′-end cloning primer for *AjTNFAIP8* (Table 1) was designed to clone the 3′-end of the gene using 3′-Full RACE Kit (TaKaRa, Japan) following the manufacturer's instructions. *AjTNFAIP8* 3′-specific primer, combined with cDNA library primer in RACE kit, was used to generate the PCR products for the 3′-end of *AjTNFAIP8*. Purified PCR products were sequenced at Sangon, Inc. (Shanghai, China). Full sequences of *AjTNFAIP8* were assembled with DNAMAN7 (Lynnon Biosoft, USA) and confirmed with BLASTx in NCBI. The deduced amino acid sequence based on the full length *AjTNFAIP8* sequence was further analyzed with bioinformatics tools provided by the Expert Protein Analysis System (http://www.expasy.org/), including the prediction of the signal peptide and protein domains with Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) and estimation of the molecular mass (MM) and theoretical protein isoelectric point (*pI*) using ProtParam (http://www.expasy.ch/tools/protparam.html). Similarities of AjTNFAIP8 protein with homologs were determined by multiple alignments using the ClustalW2 multiple alignment program (http://www.ebi.ac.uk/clustalw/) and Multiple Align Show program (http://www.bio-soft.net/sms/index.html). The three-dimensional (3D) protein structure was predicted by Protein Homology/analogY Recognition Engine V 2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/login.html). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6.0 program.

2.3. Response of A. japonicus coelomocytes to stimulation of lipopolysaccharides (LPS) in vitro

Coelomocytes were isolated and cultured from *A. japonicus* as described by Xing et al. [31] and Gu et al. [32]. The coelomic fluids were filtered through a 100 μm sterile nylon mesh to remove non-coelomocyte tissue debris and then mixed with an equal volume of anticoagulant solution (0.02 M EGTA, 0.48 M NaCl, 0.019 M KCl, and