Original Article

Innate Immunity

Innate Immunity
2020, Vol. 26(2) 138–145

DOI: 10.1177/1753425919877680
journals.sagepub.com/home/ini
SAGE

Functional characterisation of *Holothuria leucospilota* Fas-associated death domain in the innate immune-related signalling pathways

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Abstract

In this study, the functions of *Holothuria leucospilota* Fas-associated death domain (HLFADD) in the innate immune-related signalling pathways were investigated. The results showed that over-expression of HLFADD in HEK293T cells could activate the transcription factors NF-κB and activator protein-1 (AP-1), and induce the secretion of downstream pro-inflammatory cytokines IL-6, IL-8 and IL-18, suggesting the involvement of the sea cucumber FADD in activating the NF-κB and c-Jun NH_2-terminal kinase-dependent pathways. On the other hand, HLFADD could down-regulate the activations of NF-κB and AP-1 that induced by over-expression of *H. leucospilota* myeloid differentiation factor 88 (HLMyD88), which is supposed to be mediated through its interaction with HLMyD88 to keep the MyD88-dependent TLR signalling at a proper magnitude. The interaction of HLFADD and HLMyD88 were further supported by a co-immunoprecipitation assay. Moreover, HLFADD could activate transcription factor IFN regulatory factor-3 and induced the secretion of downstream IFN-α and IFN-β, indicating that the sea cucumber FADD may also activate the antiviral IFN signalling pathway. In summary, our study may give new insights on the functions of sea cucumber FADD in the innate immune-related signalling pathways.

Keywords

Holothuria leucospilota, Fas-associated death domain, myeloid differentiation factor 88, cytokine, innate immune-related signalling pathway

Date Received: 15 July 2019; accepted: 1 September 2019

Introduction

Fas-associated death domain (FADD) protein is an adaptor protein that is primarily considered as a pivotal signal transducer in the apoptosis pathway mediated by the main death receptors (DRs).[1–3] In vertebrates, especially in mammals, the mechanism for FADD transmitting the extracellular signals mediated by the activated receptor to downstream caspase-8 and ultimately leading to apoptosis has been well studied.[1–3] In invertebrates, the involvement of FADDs in apoptosis has also been illustrated as their mammalian counterparts.[4,5]

In addition to the well-known roles in pro-apoptotic signalling, FADD is also implicated in innate immunity.[6] The FADD-dependent innate immune

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pathways were speculated to be evolutionarily conserved from insects to mammals.[7] To date, a number of reports regarding the activation of the innate immune-related signalling pathways by FADD have been published.[6–13] The activation of NF-κB by FADD has been reported in mammals and other vertebrates.[6–11] In mammalian cells, the activation of transcription factor IFN regulatory factor-3 (IRF-3) by FADD has been found and is supposed to be involved in the antiviral effect of FADD.[7] The fact that early murine embryonic fibroblasts (MEFs) lacking FADD appeared to be obviously sensitive to virus infections again demonstrated the antiviral activity of FADD.[14] In the invertebrate *Drosophila*, FADD has been reported to be involved in the innate immune response to bacterial infection.[15,16] Through activating the NF-κB homologue, the *Drosophila* FADD could induce the expression of antimicrobial genes to defend the invasion of bacteria.[13] In contrast, some other reports published conflicting results to the above findings, that is, that the mammalian FADD could suppress the activation of NF-κB induced by LPS or IL-1β through interacting with myeloid differentiation factor 88 (MyD88),[17,18] a common signalling adaptor protein shared by all TLRs except TLR3 in the TLR/IL-1 receptor (TLR/IL-1R) mediated signalling pathways.[19–21]

In invertebrates, most studies on the role of FADD in innate immunity have focused on *Drosophila*.[4,13,15,16,22] A previous study showed that the *Drosophila* FADD acts downstream of the immune deficiency (IMD) pathway that controls the inducibility of the antibacterial peptide genes, and it is required for the immune defence of *Drosophila* against Gram-negative infection.[16] On the other hand, studies of FADDs in other invertebrate have mainly focused on the response of FADDs to immune challenge.[23,24]

Recently, we cloned an echinoderm *FADD* (*HLFADD*) from the tropical sea cucumber *Holothuria leucospilotai*, and the functions of HLFADD were also preliminarily explored.[5] Our results showed that HLFADD could induce apoptosis in HEK293 cells, and the expression of *HLFADD* transcript was significantly up-regulated by LPS or polyriboinosinic-polyribocytidylic acid (poly (I:C)) challenge, suggesting the roles of echinoderm FADD in innate immunity.[5] Based on the data obtained previously, this study aimed to identify the roles of HLFADD in the innate immune-related signalling pathways to gain new insights into the functions of echinoderm FADD. For this purpose, the functions of HLFADD in activating transcription factors NF-κB, activator protein 1 (AP-1) and IRF-3 was detected by luciferase reporter gene assays. In addition, the inductive effects of HLFADD on the downstream cytokines in the NF-κB, c-Jun NH_2-terminal kinase (JNK) and antiviral IFN signalling pathways were investigated by ELISA assays. Furthermore, the interaction between HLFADD and *H. leucospilota* MyD88 (HLMyD88, cloned by us previously)[25] and the coordinative effects of these two proteins on the NF-κB and JNK pathways were also detected by co-immunoprecipitation (Co-IP) and luciferase reporter gene assays.

Materials and methods

Co-IP

In order to clarify whether HLFADD interacts with HLMyD88, a Co-IP assay was carried out. Logarithmic growth-phase HEK293T cells were inoculated into a T75 cultivating bottle and cultured in DMEM (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2 for 24 h. Then, 15 μg pcDNA3.0/HA/HLFADD and 15 μg pcDNA3.0/Flag/HLMyD88 plasmids which were constructed by us previously[5,26] were co-transfected into the HEK293T cells by using 30 μl Lipofectamine 2000 Invitrogen, Carlsbad, CA according to the manufacturer's instructions. After transfection for 48 h, the HEK293T cells were lysed by using Co-IP lysis buffer (50 mM Tris, pH 7.0, 2 M Imidizole, 3 M KCl, 1 M $MgCl_2$, 5% glycerin, 1% Triton-X-100 and protease inhibitor cocktail; Roche, Mannheim, Germany). A small amount of the lysate was retained for the subsequent Western blot analysis. The rest of the lysate was divided into two equal parts and incubated with rabbit anti-Flag Ab (Sigma–Aldrich, Munich, Germany) as well as IgG (as a control; Sigma–Aldrich) at 4°C overnight, and then incubated with Protein A/G Sepharose (Sigma–Aldrich, St Louis, MO) at 4°C for 4 h. After Co-IP, the Sepharose® beads were centrifuged to the bottom of the tubes and washed three times with lysis buffer. Then, the lysate, the control and the Co-IP proteins were analysed using SDS-PAGE, followed by Western blot detection with Western lightning TM chemiluminescent reagent (Amersham Pharmacia, Helsinki, Finland). In this case, the rabbit anti-HA Ab or the rabbit anti-Flag Ab (Sigma–Aldrich) at 1:400 dilution was used as the primary Ab, respectively. The goat anti-rabbit IgG (Abcam, Cambridge, UK) at 1:1000 dilution was used as the secondary Ab.

Effects of over-expressed HLFADD on innate immune-related signalling pathways

In order to investigate the functional properties of HLFADD in the innate immune-related signalling pathways, luciferase reporter gene assays were

performed as described before.[26,27] First, the HEK293T cells cultured on a 24-well plate were transfected with mixed plasmids (1500 ng/well) which consisted of 450 ng of pcDNA3.0/HA/HLFADD, 450 ng of pcDNA3.0/HA, 580 ng of NF-κB-luc (cat. no.: E8491; Promega, Madison, WI) or AP-1-luc (cat. no.: E411A; Promega) reporter plasmid, and 20 ng of pRL-TK reference plasmid. In this case, 900 ng of pcDNA3.0 blank plasmid was used as the negative control. After transfection for 48 h, the luciferase reporter activity was detected using the Dual-Luciferase® Reporter Assay System (Promega), and the relative luciferase activity was calculated as previously described.[25] The effect of HLFADD on transcription factor IRF3 was tested in the same way as above, except that the dosage of pcDNA3.0/HA/HLFAD was 900 ng and the reporter plasmid was IRF3-luc (cat. no.: P0591; Wuhan Miaoling Bioscience and Technology Co. Ltd, Wuhan, PR China). For the luciferase reporter gene assays, each experiment was performed in triplicate.

Coordinative effect of HLFADD and HLMyD88 on NF-κB and JNK signalling pathways

The coordinative effects of HLFADD and HLMyD88 on NF-κB and AP-1 signalling pathways were also investigated by using a luciferase reporter gene assay. The HEK293T cells cultured on a 24-well plate were transfected with mixed plasmids (1500 ng/well) which consisted of 450 ng of pcDNA3.0/HA/HLFADD, 450 ng of pcDNA3.0/Flag/HLMyD88, 580 ng of NF-κB-luc or AP-1-luc reporter plasmid and 20 ng of pRL-TK reference plasmid (Promega). In this case, pcDNA3.0 blank plasmid was used as the negative control, and each experiment was performed in triplicate.

ELISA assay

To explore whether the secretion of cytokines could be induced by HLFADD, 1 μg of pcDNA3.0/HA/HLFADD plasmid (experimental group) or pcDNA3.0/HA blank plasmid (negative control group) was transfected into the HEK293T cells, and the supernatants from the transfected cells were collected at 24 and 48 h post transfection. Then, the concentrations of IL-6, IL-8, IL-18, IFN-α and IFN-β in the collected samples were determined by using human IL-6, IL-8, IL-18, IFN-α and IFN-β ELISA Kits (Elabscience Biotechnolog Co. Ltd, Wuhan, PR China) according to the manufacturer's procedures. In this case, the un-transfected HEK293T cells were used as the blank control. The experiment was performed in triplicate.

Data transformation and statistical analysis

For the luciferase reporter assay and ELISA assay, data are expressed as the mean ± standard error (SE) and were analysed using Student's *t*-test followed by Fisher's least significant difference (LSD) test with IBM SPSS (v11.0) Statistics for Windows vXX (IBM Corp., Armonk, NY).

Results

Interaction between HLFADD and HLMyD88

The Co-IP proteins by Flag Ab were detected by Western blot. When the rabbit anti-HA Ab and the rabbit anti-Flag Ab were used as the primary Abs, the recombinant HLFADD protein with HA tag (~31 kD; Figure 1a) and the recombinant HLMyD88 protein with Flag tag (~37 kD; Figure 1b) were detected, respectively, indicating the interaction between HLFADD and HLMyD88 (Figure 1).

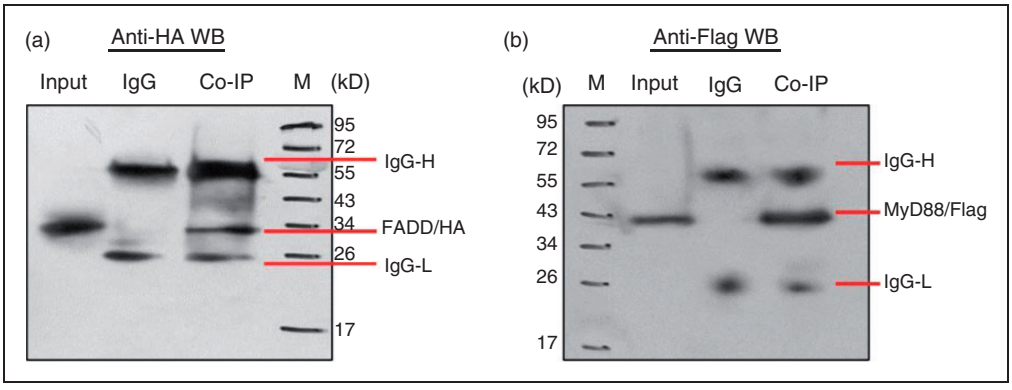

Figure 1. Co-IP of *H. leucospilota* HLFADD and HLMyD88 proteins. (a) Western blot detection by using anti-HA Ab as the primary Ab. (b) Western blot detection by using anti-Flag Ab as the primary Ab.

Effect of HLFADD on the NF-κB signalling pathway

The results of the luciferase reporter gene assay showed that HLFADD could significantly enhance the activity of NF-κB luciferase reporter in HEK293T cells ($P < 0.05$). When the NF-κB luciferase reporter was co-transfected with HLFADD, the luciferase activity was 1.87-fold up-regulated compared to the control group (Figure 2a). Compared to the empty vector pcDNA3.0, the over-expression of HLMyD88 could significantly increase the activities of NF-κB luciferase reporter ($P < 0.001$), with a 3.58-fold increase in luciferase activity (Figure 2a). However, when HLFADD and HLMyD88 were co-transfected with NF-κB luciferase reporter, the luciferase activity was 2.82-fold up-regulated, indicating that HLFADD could down-regulate the NF-κB activation induced by HLMyD88.

Effect of HLFADD on the JNK signalling pathway

As shown in Figure 2b, when AP-1 luciferase reporter was co-transfected with either HLFADD or HLMyD88, the AP-1 luciferase activities were 1.27- or 1.65-fold up-regulated, respectively, compared to the control group ($P < 0.05$). However, when HLFADD and HLMyD88 were co-transfected with AP-1 luciferase reporter, the luciferase activity was 1.39-fold up-regulated, indicating that HLFADD could down-regulate the AP-1 activation induced by HLMyD88.

Effect of HLFADD on the IFN signalling pathway

As shown in Figure 2c, when the IRF3-luc luciferase reporter was co-transfected with HLFADD, the luciferase activity was 1.32-fold up-regulated compared to the control group, indicating that HLFADD could significantly enhance the activity of IRF3 luciferase reporter in HEK293T cells ($P < 0.05$)

Induction of cytokines by HLFADD

To investigate the effects of HLFADD on secretion of cytokines, pcDNA3.0/HA/HLFADD and pcDNA3.0/HA were transfected into HEK293T cells. As shown in Figure 3, HLFADD could induce the secretion of IL-6 (Figure 3a), IL-8 (Figure 3b), IL-18 (Figure 3c), IFN-α (Figure 3d) and IFN-β (Figure 3e). The results showed that there was no significant difference ($P > 0.05$) in the concentrations of all the cytokines between the blank and negative control groups. On the contrary, the concentrations of IL-6, IL-8, IL-18, IFN-α and IFN-β in the HLFADD transfected groups were 2.86-/2.69-fold, 2.53-/2.47-fold, 1.62-/1.52-fold, 3.36-/3.25-fold and 2.53-/2.48-fold higher than those in the blank/negative control groups, respectively, at 24 h after transfections. The increases in cytokine concentrations in the HLFADD-transfected groups at 48 h after transfections were similar to those at 24 h after transfections. In this case, the fold increases for IL-6, IL-8, IL-18, IFN-α and IFN-β concentrations to the blank/negative control groups were 2.93/2.80, 2.09/2.05, 1.31/1.26, 3.53/3.44 and 2.94/2.88, respectively.

Discussion

In mammals, FADD is a multifunctional protein. Besides the widely known role in apoptosis, FADD participates in a variety of non-apoptotic processes, such as innate immune signalling, embryogenesis, haematopoiesis, proliferation and cell-cycle progression.[6,28–30] Recently, we cloned an echinoderm FADD named *HLFADD* from the sea cucumber

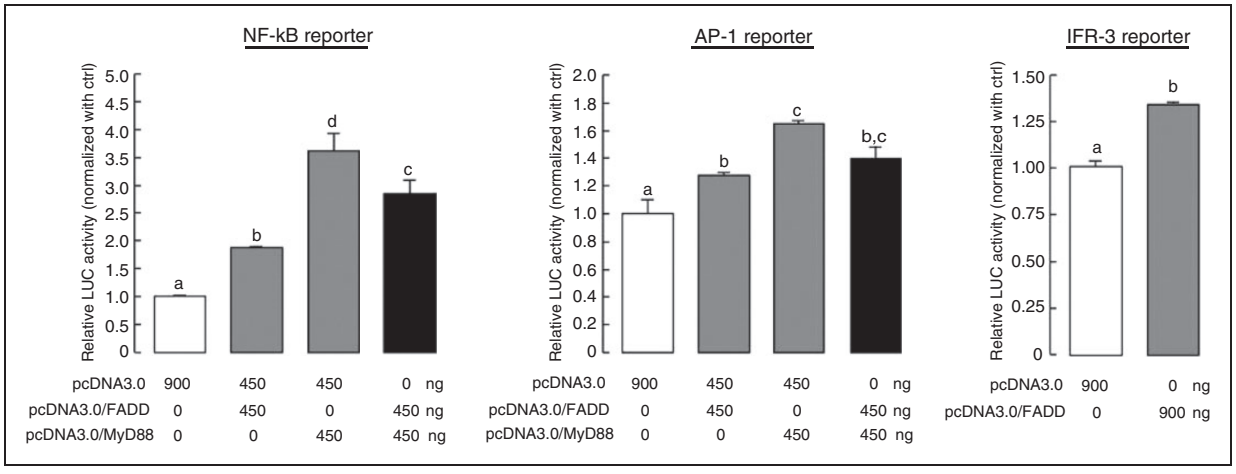

Figure 2. Effects of over-expressed HLFADD on the activation of NF-κB, activator protein-1 and IRF3. The same letter represents a similar expression level ($P > 0.05$), and a different letter represents significant difference of expression levels between two groups ($P < 0.05$). The data are expressed as the mean±standard error ($n = 3$).

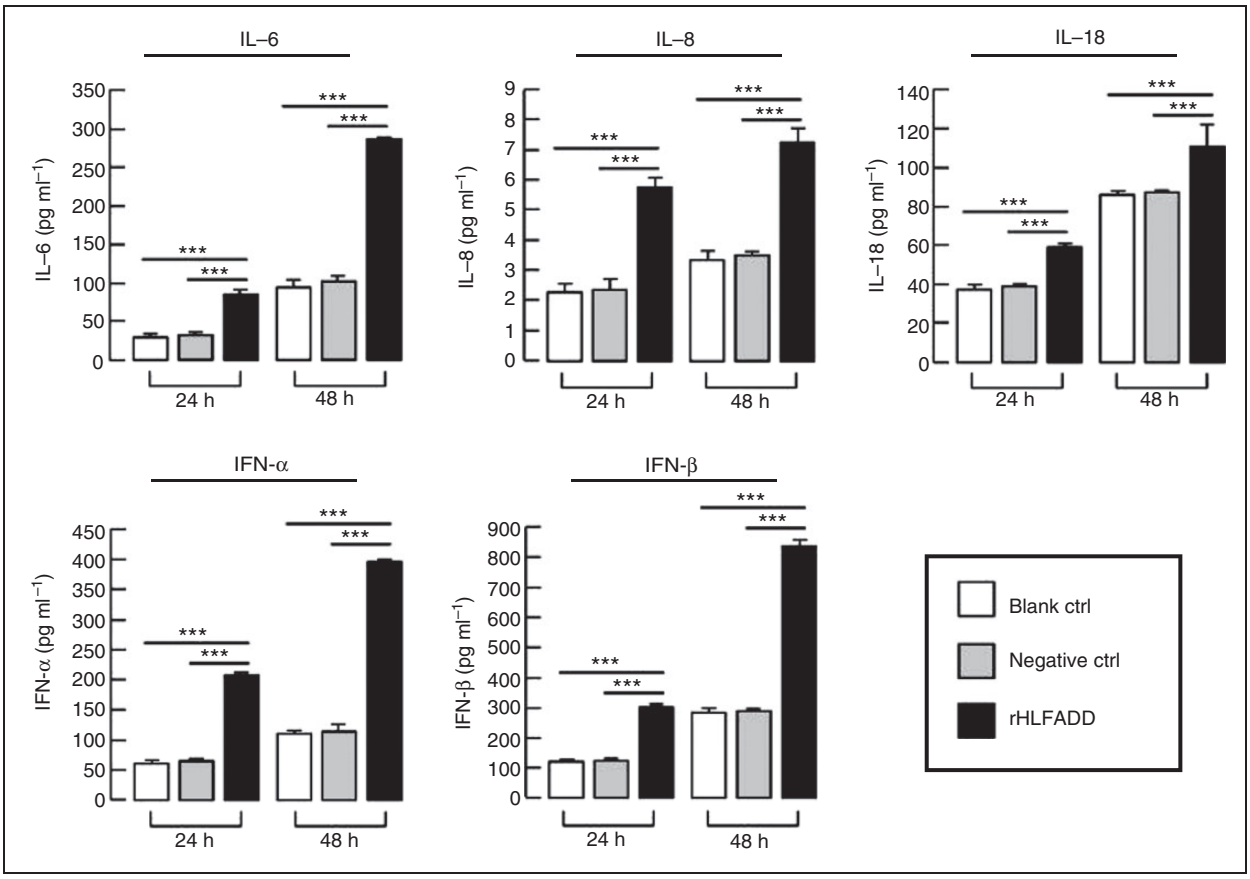

Figure 3. Induction of IL-6, IL-8, IL-18, IFN-α and IFN-β by over-expressed HLFADD. The data are expressed as the mean ± standard error ($n = 3$). ***$P < 0.001$.

H. leucospilota and found that over-expression of HLFADD protein could induce apoptosis.[5] When the *H. leucospilota* coelomocytes were challenged with LPS or poly (I:C), the expression of *HLFADD* mRNA was significantly up-regulated, suggesting the potential roles of HLFADD in the innate immune defence of sea cucumber.[5] In order to explore the functions of HLFADD further, the roles of HLFADD in the innate immune-related signalling pathways were explored in this study.

Our previous study showed that *H. leucospilota* MyD88 (HLMyD88) could activate transcription factors (NF-κB and AP-1), and the over-expression of HLMyD88 could induce the secretion of pro-inflammatory cytokines (such as IL-1β and TNF-α).[25] The current study showed that the over-expression of HLFADD could also induce the secretion of cytokines, including IL-6, IL-8, IL-18, IFN-α and IFN-β.

Several studies in vertebrates and the invertebrate *Drosophila* have reported that the over-expressed FADD can result in the activation of NF-κB.[6–11,13] In rat carotid arteries *in vivo*, the transcript expression of *IL-8*, a target gene of the NF-κB signalling, is up-regulated following FADD expression, and the release of IL-8 protein was also induced by FADD.[9] Our current study showed that the over-expressed HLFADD in HEK293T cells could activate the transcription factor NF-κB and induce the secretion of the pro-inflammatory cytokines IL-8 and IL-6. In mammals, the expression of IL-6 and IL-8 is mainly controlled by the NF-κB signalling pathway.[31,32] Therefore, we speculated that the over-expression of HLFADD could activate the NF-κB signalling pathway. In addition, the over-expressed HLFADD could also activate transcription factor AP-1 and induce the secretion of the pro-inflammatory cytokine IL-18. AP-1 is the transcription factor for JNK signalling pathway, which has been found to be involved in the regulation of IL-18 expression and the mediating of IL-18 secretion.[33,34] Thus, the current study may provide evidence for the activation of the JNK signalling pathway by over-expressed HLFADD.

Although the activation of NF-κB induced by FADD has been shown by multiple experiments, the results have been conflicting. It has been reported that FADD down-regulated the activation of NF-κB which

had been induced by either LPS or IL-1β,[18] based on the results that TNF-α-, TRAIL- and Fas ligand-induced NF-κB activity was dramatically reduced in a FADD-deficient Jurkat cell line.[35] The researchers considered that the ability of FADD to promote or inhibit NF-κB activation is stimulus and/or signalling pathway specific.[18] There are other reports that FADD negatively regulates LPS signalling by suppressing the activation of the JNK pathway.[17] It was speculated that the negative regulation of the NF-κB and JNK pathways by FADD might be caused by the interaction between FADD and MyD88.[17,18] MyD88 is a pivotal adapter molecule which can activate the signalling cascades of NF-κB and MAPKs, such as JNK.[19,36,37] Once PAMPs were recognised by TLRs, MyD88 was recruited.[38] Then, MyD88 in turn recruits IL-1 receptor-associated kinase (IRAK) 4, IRAK2 and IRAK1 and phosphorylates IRAK1.[39] The activated IRAKs then bind to TNF receptor-associated factor 6 (TRAF6) and initiate the cascaded responses belonging to two distinct signalling pathways, leading to the activation of transcription factors NF-κB and AP-1.[40–42] The over-expression of FADD significantly reduces the detectable MyD88/IRAK1 interaction, whereas a complete loss of FADD enhances the MyD88/IRAK1 interaction.[17] The interaction between FADD and MyD88 appears to reduce the stability of the MyD88/IRAK1 interaction, thereby attenuating the activation of NF-κB and JNK pathways that are induced by LPS challenge.[17] There is a possibility that FADD occupies the binding site where IRAKs bind to MyD88 and competes with IRAKs for binding to MyD88, thereby precluding the interaction between IRAKs and MyD88.[17] In the current study, the interaction between HLFADD and HLMyD88 and the coordinating effects of these two proteins on the NF-κB and JNK pathways were further detected by Co-IP and luciferase reporter gene assays. Our results showed that HLFADD could interact with HLMyD88 and down-regulate the NF-κB and AP-1 activation induced by HLMyD88. We speculated that the interaction between HLFADD and HLMyD88 may weaken the activations of NF-κB and AP-1 that are induced by HLMyD88.

On one hand, the over-expressed HLFADD activates the transcription factors NF-κB and AP-1; on the other hand, through the interaction with HLMyD88, HLFADD down-regulates NF-κB and AP-1 activation induced by HLMyD88, suggesting HLFADD may provide a safety check of the innate immune response by conferring a proper magnitude to the MyD88-dependent TLR signalling.

In mammalian cells, FADD is involved in innate immune mechanisms which are independent of the TLR signalling pathways.[7] A complex named 'innateosome' that comprises FADD, receptor-interacting protein 1 and TANK-binding kinase1 can activate the transcription factor IRF-3, resulting in the production of IFN-α and IFN-β and mediation of antiviral responses.[7] In this study, our results showed that HLFADD could activate transcription factor IRF-3 and subsequently induce the secretion of IFN-α and IFN-β, indicating that the sea cucumber FADD may also activate the antiviral IFN signalling pathway. Murine embryonic fibroblasts lacking FADD were very sensitive to viral infection.[14] The roles of HLFADD in the antiviral IFN signalling pathway would be helpful for sea cucumber against viral infections.

In conclusion, the over-expressed HLFADD could active transcription factors NF-κB and AP-1 and induced the secretion of cytokines IL-6, IL-8 and IL-18 in HEK293T cells, suggesting that the sea cucumber FADD might active NF-κB and JNK pathways. On the other hand, HLFADD could also down-regulate the activation of transcription factors NF-κB and AP-1 that induced by HLMyD88 through the interaction with HLMyD88 to keep MyD88-dependent TLR signalling at a proper magnitude. In addition, HLFADD could activate transcription factor IRF-3 and induce the secretion of IFN-α and IFN-β, indicating that the sea cucumber FADD may also activate the antiviral IFN signalling pathway. In summary, our study provides new insights on the functions of HLFADD in innate immune-related signalling pathways.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship and/or publication of this article: This study received funding from the National Key R&D Program of China (2018YFD0901605), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA13020205), the Science and Technology Promoting Projects for Oceanic and Fishery in Guangdong Province (SDYY-2018-01), the Science and Technology Research Programs of Guangdong Province (2016A020210136, 2017B030314052, 2018A030313857), the Scientific Research Projects of City-affiliated Universities in Guangzhou (1201421216) and the Institution of South China Sea Ecology and Environmental Engineering, Chinese Academy of Sciences (ISEE2018PY03).

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