Anti-lipopolysaccharide factor D from kuruma shrimp exhibits antiviral activity

Hai-Shan Jiang¹ · Li-Xia Lv¹ · Jin-Xing Wang¹

Received: 25 December 2020 / Accepted: 17 June 2021 / Published online: 13 July 2021
© The Author(s) 2021

Abstract

Anti-lipopolysaccharide factors (ALFs) exhibit a potent antimicrobial activity against a broad range of bacteria, filamentous fungi, and viruses. In previous reports, seven groups of ALFs (groups A–G) were identified in penaeid shrimp. Among them, group D showed negative net charges and weak antimicrobial activity. Whether this group has antiviral function is not clear. In this study, the ALF sequences of penaeid shrimp were analyzed, and eight groups of ALF family (groups A–H) were identified. The four ALFs including MjALF-C2, MjALF-D1, MjALF-D2, and MjALF-E2 from kuruma shrimp *Marsupenaeus japonicus* were expressed recombinantly in *Escherichia coli*, and the antiviral activity was screened via injection of purified recombinant ALFs into shrimp following white spot syndrome virus (WSSV) infection. Results showed that the expression of Vp28 (WSSV envelope protein) decreased significantly in the MjALF-D2-injected shrimp only. Therefore, MjALF-D2 was chosen for further study. Expression pattern analysis showed that *MjAlf-D2* was upregulated in shrimp challenged by WSSV. The WSSV replication was detected in RNA, genomic DNA, and protein levels using VP28 and *Ie1* (immediate-early gene of WSSV) as indicators in MjALF-D2-injected shrimp following WSSV infection. Results showed that WSSV replication was significantly inhibited compared with that in the rTRX- or PBS-injected control groups. After knockdown of *MjAlf-D2* in shrimp by RNA interference, the WSSV replication increased significantly in the shrimp. All these results suggested that MjALF-D2 has an antiviral function in shrimp immunity, and the recombinant ALF-D2 has a potential application for viral disease control in shrimp aquaculture.

Keywords Anti-lipopolysaccharide factors · Cationic and anionic antimicrobial peptides · White spot syndrome virus · Viral envelope protein

Introduction

White spot syndrome (WSS) in crustaceans is caused by WSS virus (WSSV), which is an enveloped circular double-stranded DNA virus, and it is a severely infectious disease that results in 100% mortality of shrimp within 3–10 days after infection (Peng et al. 2001). WSSV is a virulent pathogen not only to shrimp but also to wider crustacean species. Understanding the molecular mechanisms of the shrimp

immune response against viral infection could provide new strategies for the disease control. Therefore, several studies were carried out, and great progress was achieved with shrimp immunity, especially in the discovery of antiviral effectors (Wang et al. 2014a; Zheng et al. 2019). Among them, antimicrobial peptides (AMPs), as the effector molecules, play important roles in shrimp antiviral immunity.

Several kinds of AMPs have been identified in shrimp, including penaeidins, crustins, anti-lipopolysaccharide factors (ALFs), and stylicins (Tassanakajon et al. 2011, 2018). Most of the peptides with net-positive charges possess antibacterial activity against Gram-positive or -negative bacteria, fungi, and viruses. ALF was first reported in chelicerates (horseshoe crab, *Limulus polyphemus*) as a potent anticoagulant factor (Morita et al. 1985; Ohashi et al. 1984; Tanaka et al. 1982). Since then, ALFs were identified in various crustaceans, such as portunid crabs (Afsal et al. 2012), freshwater prawn (Ren et al. 2012), crayfish (Jiravanichpaisal

Edited by Xin Yu

Hai-Shan Jiang and Li-Xia Lv contributed equally to this work.

✉ Jin-Xing Wang
jxwang@sdu.edu.cn

¹ Shandong Provincial Key Laboratory of Animal Cell and Developmental Biology, School of Life Sciences, Shandong University, Qingdao 266237, China

et al. 2007; Sun et al. 2011) and penaeid shrimp, such as *Litopenaeus vannamei*, *Fenneropenaeus chinensis*, *Penaeus monodon* and *M. japonicus* (Tassanakajon et al. 2018). Besides a conserved signal peptide, the sequences of mature ALF peptides contain a lipopolysaccharide (LPS)-binding domain formed between two conserved cysteine residues. The three-dimensional structure of ALFs consists of three α -helices and four stranded β -sheets (Yang et al. 2009). A central β -hairpin structure formed by 20 amino acid residues between two cysteines is stabilized by a disulfide bond. The central β -hairpin structure is the ALF functional domain which is involved in the recognition and binding of components from microbial cell walls (Schmitt et al. 2016).

The ALFs from penaeid shrimp constitute a diverse and multigenic family of AMPs. They are firstly divided into three groups (groups A–C) on the basis of sequence similarities and phylogenetic analysis (Tassanakajon et al. 2011). Group A consists of anionic and cationic polypeptides, whereas groups B and C are composed of highly cationic polypeptides. Subsequently, group D shrimp ALFs with negative net charges are identified and display impaired LPS-binding activity, and weak antimicrobial activity (Rosa et al. 2013). Then, a new group of ALFs, including MjALF-E1 and -E2 (designated as group E), was identified from *Marsupenaeus japonicus* (Jiang et al. 2015); they exhibit antimicrobial activity against bacteria. In addition, two novel groups of the family (groups F and G) were recently identified in *L. vannamei* (Matos et al. 2018). Group F is composed of cationic peptides with antibacterial and antifungal activities, whereas the antimicrobial activities of group G belonging to anionic peptides have not been identified (Matos et al. 2018). The ALFs with net positively charged LPS-Binding Domain (such as groups B, C, and F), which is highly conserved in sequence of each group, exhibit high antimicrobial activity, and anionic ALFs (such as group D) have weak or no antimicrobial activity. The seven ALFs could be simultaneously identified in a single shrimp, such as *L. vannamei* and *M. japonicus* (Jiang et al. 2015; Matos et al. 2018), possibly suggesting that different ALFs may act synergistically to improve their antimicrobial activity in vivo.

Functional analyses showed that ALF proteins exhibit a potent antimicrobial activity against a wide range of micro-organisms, including Gram-positive and Gram-negative bacteria and filamentous fungi (Rosa et al. 2013). ALFs also play an important role in the defense against viral pathogens in crustaceans. In vivo function analysis in crayfish *Pacifastacus leniusculus* showed that the knock-down expression of the ALF gene by double-stranded RNA (dsRNA) injection enhanced the expression of VP28, the envelope protein of WSSV (Liu et al. 2006). Silencing the LvALF in *L. vannamei* by RNA interference could also increase mortality after WSSV infection (de la Vega

et al. 2008). The synthetic LBD (LPS-binding domain) of FcALF and FcALF2 (group B) from the shrimp *F. chinensis* could inhibit the replication of WSSV in vivo (Li et al. 2014; Liu et al. 2005). The ALFPm3 (group C) identified in *Penaeus monodon* exhibits anti-WSSV function by binding to the target structural protein complex of WSSV and leading to virion damage (Methatham et al. 2017).

Previous studies identified seven groups of ALFs (groups A–G) in kuruma shrimp *M. japonicus*, and the antibacterial functions of some of these ALFs were studied (Jiang et al. 2015). The anionic ALF-Ds showed weak antimicrobial activity (Rosa et al. 2013). Whether the anionic ALFs also has antiviral activity is not clear. In the present study, the phylogenetic tree of ALFs from penaeid shrimp was reconstructed, and a new group of ALF family (group H) was identified. The function of anionic MjALF-D2 in shrimp immunity was analyzed, and the anti-WSSV activity of ALF was authenticated in kuruma shrimp.

Results

Eight groups of the ALF family were identified in penaeid shrimp

Nine ALF members were identified in the transcriptomic sequencing of shrimp *M. japonicus*. Then, 29 sequences of ALFs (GenBank accession numbers listed in Fig. 1A) were collected in four other penaeid shrimp species, namely, *F. chinensis*, *F. penicillatus*, *L. vannamei*, and *P. monodon*, from the GenBank database for reconstruction of phylogenetic trees. The members of the ALF family in penaeid shrimp were divided into eight groups (groups A–H, Fig. 1A), and a new group (group H) was identified. These groups could be classified into five clusters, i.e., cluster I (groups C, B, and F), Cluster II (group H), cluster III (group A), cluster IV (group D), and cluster V (groups E and G). The newly identified group H is composed of cationic ALFs with isoelectric points ranging from 9.14 to 9.80 present in *M. japonicus*, *L. vannamei*, and *Penaeus monodon* (Fig. 1B). The original MjALF-E1 in the previous report (Jiang et al. 2015) was reclassified as MjALF-H in the reconstructed phylogenetic trees (Fig. 1A). The function of MjALF-E1 was studied, and the results revealed that it could strongly bind to bacteria, and has antibacterial activity (Jiang et al. 2015). The original MjALF-D1 (a cationic peptide) was reclassified as group F (MjALF-F) in the phylogenetic trees (Fig. 1A). In summary, eight groups of ALF family were identified in kuruma shrimp *M. japonicus*, and there has not been any functional study conducted for group D member MjALF-D2.

Fig. 1 Phylogenetic tree of ALFs and alignment of group ALF-H. **A** Phylogenetic tree of ALFs from penaeid shrimp constructed via MEGA by using neighbor-joining method. Nine ALF members belong to eight groups of the ALF family identified in *M. japonicus*. **B** Alignment of amino acid sequences of five ALFs in group H. Fc, *Fenneropenaeus chinensis*; Fp, *Fenneropenaeus penicillatus*; Lv, *Litopenaeus vannamei*; Mj, *Marsupenaeus japonicus*; Pm, *Penaeus monodon*. The LPS-binding domain is located at two cysteine residues

Antiviral activity screening using recombinant MjALFs

Four recombinant ALF(rALF) polypeptides, including MjALF-C2 (GenBank accession no. KU213608), MjALF-D1 (KU160499), MjALF-D2 (MT977630), and MjALF-E2 (KY627761), were successfully expressed in *Escherichia coli*. After the recombinant ALFs were purified via Ni-NTA chromatography (Fig. 2A), the antiviral effect of the rALFs was screened initially by injecting the rALF and WSSV mixture into shrimp; viral replication was detected at 36 h post injection using the expression of Vp28 (the envelope protein of WSSV) as the indicator. The results showed that WSSV replication in gills of the rMjALF-D2-injected shrimp was inhibited significantly compared with that in other rALFs and the controls (Fig. 2B). For further confirmation of the results, PBS and thioredoxin (TRX)-tag protein purified from the parent vector pET-32a(+) were used as controls in the antiviral activity analysis. The results showed that compared with PBS and TRX, rMjALF injection significantly

Fig. 2 Recombinant expression and purification of four MjALFs and antiviral activity analysis in shrimp. **A** Purification of MjALFs expressed in *E. coli*. and Lane 1, total proteins of *E. coli* with pET32a/Alf plasmids without induction; lane 2, total proteins of *E. coli* with pET32a/Alf after IPTG induction; lane 3, purified MjALFs; and lane M, standard protein markers. **B** Expression of Vp28 in gills of rMjALF-injected shrimp challenged by WSSV, as analyzed by qPCR using β -Actin as internal control. **C** qPCR was used to analyze the expression of Vp28 (encoding the envelope protein of WSSV) in hemocytes of rMjALF-D2-injected shrimp challenged by WSSV. PBS and rTRX injection were used as controls. Asterisks indicate statistical significance (* $P < 0.05$; ** $P < 0.01$) analyzed by Student's *t* test

inhibited WSSV replication in shrimp hemocytes (Fig. 2C). All the results suggested that MjALF-D2 may possess anti-WSSV function in shrimp.

MjAlf-D2 was detected in all tissues tested, and upregulated in shrimp challenged by WSSV

The tissue distribution of *MjAlf-D2* in the hemocytes, heart, hepatopancreas, gills, stomach, and intestine of untreated shrimp was detected by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The results showed that *MjAlf-D2* was detected in all tissues examined, and it was highly expressed in gills, stomach, and intestine (Fig. 3A). The expression patterns of *MjAlf-D2* in different tissues of shrimp challenged by WSSV were analyzed by quantitative real-time PCR (qPCR), and the results showed

Fig. 3 Tissue distribution and expression patterns of *MjAlf-D2* in shrimp. **A** Tissue distribution of *MjAlf-D2* in normal shrimp analyzed by RT-PCR. **B** Expression pattern of *MjAlf-D2* in hemocytes of shrimp challenged by WSSV was analyzed by qPCR. **C** Expression pattern of *MjAlf-D2* in intestine of WSSV-challenged shrimp analyzed by qPCR. **D** Expression pattern of *MjAlf-D2* in gills of shrimp challenged by WSSV. Student's *t* test was used to analyze statistical significances (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

that after WSSV challenge, *MjAlf-D2* was significantly upregulated in the hemocytes (Fig. 3B), intestine (Fig. 3C), and gills (Fig. 3D) of shrimp. The expression of *MjAlf-D2* was upregulated from 6 h, reached the highest level at 12 h, and started to recover from 36 h post WSSV infection. These results suggested that *MjAlf-D2* was responsible for WSSV infection in shrimp.

Recombinant *MjAlf-D2* inhibited the replication of WSSV in vivo

WSSV proliferation in RNA, genomic DNA, and protein levels was detected in r*MjAlf-D2*-injected shrimp following WSSV infection, with *Vp28* and *Ie1* (immediate-early gene of WSSV) as indicators, using qPCR to confirm the anti-WSSV activity of r*MjAlf-D2*. The results showed that compared with PBS and TRX injection control, the r*MjAlf-D2*-injected group showed significant reduced expression of *Vp28* and *Ie1* in gills at 36 h (Fig. 4A) and 48 h (Fig. 4B) post WSSV injection. The expression of *VP28* in gills of the shrimp was also detected at the protein level by Western blotting, and similar results were obtained (Fig. 4C). These results suggested that r*MjAlf-D2* inhibits WSSV replication in shrimp.

VP28 and *Ie1* expression increased significantly in *MjAlf-D2*-silenced shrimp

MjAlf-D2 RNAi was performed in vivo to further confirm the role of *MjAlf-D2* in shrimp antiviral immunity. *MjAlf-D2* could be knocked down by approximately 65% by the injection of *MjAlf-D2* dsRNA (7 µg/g shrimp) compared

with the *dsGfp* injected in the controls (Fig. 5A). The *MjAlf-D2*-silenced shrimp were challenged with WSSV, and the expression of *Vp28* and *Ie1* was detected at mRNA, genomic DNA, and protein levels at 36 and 48 h post WSSV injection. The results showed that the expression of *VP28* increased in gills of shrimp at the protein level compared with that of *dsGfp* (Fig. 5B). The expression of *Vp28* and *Ie1* at RNA and genomic DAN levels was also analyzed by qPCR. The results showed that this expression increased significantly in the *MjAlf-D2*-knockdown shrimp challenged by WSSV at 36 h (Fig. 5C) and 48 h (Fig. 5D) post viral injection. All the results suggested that *MjAlf-D2* exerts important roles in shrimp antiviral immunity.

Discussion

In this study, the phylogenetic tree of the ALF family from penaeid shrimp was reconstructed, and a new group (group H) was identified. Eight groups of ALF family (A–H) were found simultaneously in kuruma shrimp *M. japonicus*, and the antiviral activity of ALFs in the shrimp was screened using recombinant ALF polypeptides. The anionic ALF-D2 possessed anti-WSSV activity, and this inhibitory activity was further confirmed by RNAi and “overexpression” of *MjAlf-D2* in shrimp. The results suggested that *MjAlf-D2* has a potential application for viral disease control in shrimp aquaculture.

Shrimp have an efficient and sophisticated innate immune system that provides protection against invading pathogens. In immune responses, shrimp recognize pathogens via germline-encoded proteins called pattern recognition receptors by sensing pathogen-associated molecular patterns from

Fig. 4 Recombinant MjALF-D2 inhibits WSSV replication in shrimp. **A, B** Mixture of rMjALF-D2 and WSSV was injected into shrimp. Expression of *Vp28* (upper panel) and *Ie1* (lower panel) in mRNA and DNA levels was analyzed by qPCR at 36 h (A) and 48 h (B) in gills post WSSV injection. PBS and TRX-tag (rTRX) injection were used as controls. **C** Western blotting was used to analyze the expression of VP28 at protein level in gills of shrimp at 36 and 48 h after

WSSV and recombinant protein infection β -Actin (ACTB) was used as loading control. The bands on the membranes of western blot were digitalized using ImageJ software based on three independent repeats (lower panel). The qPCR data were analyzed by Student's *t* test and asterisks indicated significant difference (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$)

different micro-organisms (Wang and Wang 2013). Cellular and humoral immunities are initiated in the animals against pathogens by phagocytosis, nodulation, encapsulation, melanization, extracellular traps (Ng et al. 2013) (belonging to cellular responses), or by secretion of AMPs, inhibitors of proteinases, and reactive oxygen species or reactive nitrogen species (belonging to humoral responses) (Bi et al. 2015; Ng et al. 2013; Tassanakajon et al. 2018; Yang et al. 2016a). The AMPs are considered as major humoral immune effectors against invading pathogens by killing or controlling the invading micro-organisms. Among different classes of AMPs, the ALFs are the diverse family of AMP in penaeid shrimp. ALFs exhibit broad-spectrum and powerful antimicrobial activity against major shrimp pathogens. Previous studies identified seven groups of ALFs in *M. japonicus* (Jiang et al. 2015; Matos et al. 2018). Groups A and C were found in a broad range of tissues, including hemocytes, heart, hepatopancreas, gills, stomach, and intestine. ALF-Bs had a high expression level in hemocytes, heart, and

intestine. MjALF-D had a low expression in hemocytes, and upregulated by WSSV challenge. MjALF-E had low expression level in hepatopancreas. Group F (originally designated as MjALF-D1) was discovered in heart, gill, stomach, and intestine, and had a high expression in stomach. Group G (originally designated as MjALF-A2, GenBank accession no. MT977629) was mainly distributed in the stomach (Jiang et al. 2015). In the present study, group H was identified as a new group of the ALF family in *M. japonicus*, *L. vannamei*, and *P. monodon*. Eight groups of ALFs were present in a single shrimp, indicating that the different ALFs may act synergistically to improve their antimicrobial activities in vivo. Simultaneous exposure of an invading pathogen to multiple different AMPs may be a critical mechanism for the host effective immune response. A recent study in fruit fly using CRISPR gene editing to knock out all known inducible AMPs showed that AMPs have synergistic, additive, and highly specific microbicidal effects in *Drosophila* (Hanson

Fig. 5 Expression of *Vp28* and *Ie1* increased significantly in *MjAlf-D2*-silenced shrimp. **A** Efficiency analysis of *MjAlf-D2* RNAi in shrimp analyzed by qPCR. **B** Western blotting was performed to analyze the expression of VP28 in gills of *MjAlf-D2*-knockdown shrimp at 36 and 48 h post WSSV challenge. The western blot results were digitalized using ImageJ software based on three independent repeats, and shown in the lower panel of b. **C** Expression of *Vp28* (upper

panel) and *Ie1* (lower panel) in mRNA and genomic DNA levels in gills of shrimp at 36 h post WSSV infection analyzed by qPCR. **D** Expression of *Vp28* (upper panel) and *Ie1* (lower panel) in mRNA and genomic DNA levels in gills of shrimp at 48 h post WSSV infection was analyzed by qPCR. Significant differences were indicated by asterisks (* $P < 0.05$; ** $P < 0.01$) analyzed by Student's *t* test

et al. 2019). A similar situation may exist in penaeid shrimp, such as *M. japonicus*.

The antibacterial mechanisms of AMPs are well studied. The bacterial membrane is the main target for most cationic AMPs. Four main models based on interactions between AMPs and bacterial membranes have been proposed: carpet, toroidal pore, aggregate, and barrel-stave models (Jenssen et al. 2006). Some AMPs have activity against enveloped and non-enveloped viruses, and the mechanisms are different based on different AMPs and viruses. A common mechanism of action against enveloped viruses is similar with antibacterial mechanisms, that is, AMPs can destabilize the viral envelope by interaction with the envelope, and damage the virions, thus inhibiting infectivity (Mookherjee et al. 2020). ALFs have direct interaction with the envelope protein of WSSV. For example, the recombinant WSSV189 protein interacts with ALFPm3 (Suraprasit et al. 2014). The

antiviral activity of ALFs may be mediated by direct interaction with WSSV proteins. The antiviral mechanism of *MjALF-D2* may be similar with that of other AMPs, i.e., it damages the virions by interaction with the viral envelope.

Shrimp aquaculture has grown rapidly and become a major global industry that contributes significantly to socioeconomic development in many countries in Asia and the Americas. However, a succession of bacterial and viral diseases affects the sustainable development of the industry. Shrimp AMPs are small peptides, which are important effectors against a wide spectrum of pathogens in the innate immune response of shrimp. Antibiotics have been used widely in aquaculture, resulting in the development and spread of resistance. Antibiotic resistance has become a global problem due to the increased multidrug-resistant pathogens along with a gradually decline in the discovery of new antibiotics for use in human and animal health

(Mookherjee et al. 2020). AMPs are potential alternative of antibiotics as they affect a wide spectrum of pathogens, including viruses. WSSV is the most devastating virus in shrimp aquaculture. It infects all cultured penaeid shrimp species, and causes substantially economic losses globally. The potential use of ALFs in shrimp disease control has been reported previously (Supungul et al. 2017). Administering rALFPm3 could increase shrimp survival after WSSV infection. In the present study, the injection of rMjALF-D2 could inhibit WSSV replication in shrimp, and thus be beneficial to shrimp aquaculture. Therefore, shrimp ALFs have potential therapeutic uses to overcome severe viral disease outbreaks in shrimp aquaculture.

Materials and methods

Immune challenge, tissue collection and total RNA extraction

M. japonicus shrimp (8–10 g/individual) were bought from the market in Jinan, Shandong Province, China, and cultured as described previously (Niu et al. 2019). The shrimp were acclimated for 48 h in tanks with aerated artificial seawater (salinity between 24 (w/v) and 26) at ~24 °C, and randomly taken for use in the following experiments. For pathogen challenge, the shrimp were divided into two groups (30 individuals per group): one group was injected with WSSV (3.2×10^5 copies per shrimp) and the other (control group) was injected with same volume of PBS (Jiang et al. 2013). Hemolymph was collected from five shrimp using sterile syringes with anticoagulant buffer (450 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L EDTA, 100 mmol/L HEPES, pH 7.45), and then the hemocytes were collected after centrifugation at 800 g for 6 min at 4 °C. The other tissues were dissected with scissors and forceps from at least three shrimp, and placed on ice for RNA or protein extraction. The total RNAs from hemocytes and other organs (heart, hepatopancreas, gills, stomach and intestine) of the challenged and control shrimp were extracted using Unizol reagent (Bio-star, Shanghai, China). cDNA was synthesized with a pair of primers (SMART F and Oligo anchor R) using a Revert Aid First Strand cDNA synthesis kit (Fermentas, Burlington, Canada).

Before challenge experiments, we randomly took three shrimp to detect if the shrimp are WSSV-free samples using RNA from hemocytes and intestines to analyze the expression of Vp28 mRNA with specific primers Vp28F and Vp28R by qPCR. The PCR was performed with the following procedures: 95 °C for 10 min; 40 cycles at 95 °C for 10 s and 60 °C for 50 s; and then a melting period from 65 to 95 °C. The data obtained were analyzed using the cycle

threshold ($2^{-\Delta\Delta C_T}$) method. β -Actin was used as the internal control gene.

Phylogenetic analysis

The full-length coding sequences of MjALFs were obtained from hemocyte-, intestine-, and hepatopancreas-transcriptomic sequencing of *M. japonicus* in the laboratory. The ALF full-length sequences of other shrimp were collected from GenBank databases. Neighbor-joining analysis was conducted via MEGA 7, and bootstrap sampling was reiterated 500 times (Kumar et al. 2016).

Semi-quantitative RT-PCR and quantitative real-time (qPCR) analysis

The tissue distribution of MjALF-D2 in different tissues of non-injected shrimp were analyzed via RT-PCR with specific primers MjAlf RTF and RTR. β -actin was amplified as an internal control, with a pair of primers β -actin F and β -actin R.

qPCR was performed to analyze the transcriptional profiles of MjAlf-D2 at different timepoints after WSSV challenge. The profile of the PCR was 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 60 s, and read at 75 °C for 2 s; melting curve analysis was performed from 68 to 95 °C. The assay was repeated three times, and data were calculated using the $2^{-\Delta\Delta C_T}$ method. Significant differences were analyzed using Student's *t* test. The figures were created on Graphpad Prism 5 software.

Expression and purification of four recombinant ALFs

The mature peptide sequences of four MjALFs, including MjALF-C2, MjALF-D1, MjALF-D2, and MjALF-E1, were amplified with different primers (Table 1) via RT-PCR; cloned into pET-32a (+); and transformed into *E. coli* Rosetta cells. The recombinant proteins were purified by affinity chromatography using High-affinity Ni-IDA Resin (Genscript, Nanjing, China). The parent vector pET-32a (+) was used to express the TRX-tag protein in the Rosetta cells. The control protein (TRX) was also purified using the same methods described above with High-affinity Ni-IDA Resin (Genscript, Nanjing, China).

Antivirus activity analysis of recombinant ALFs

WSSV inoculation was prepared following the previous method with a few modifications (Yang et al. 2016b). The gills (1 g) of WSSV-infected crayfish *Procambarus clarkii* were homogenized in 10 ml PBS (140 mmol/L NaCl,

Table 1 Primers used in the study

Primer	Sequence (5'-3')	Direction
β -actinF	AGTAGCCGCCCTGGTTGTAGAC	Forward
β -actinR	TTCTCCATGTCGTCCAGT	Reverse
MjALF-D2RNAi-F	GCGTAATACGACTCACTATAGGAGCACCTACAGTCATCAC	Forward
MjALF-D2RNAi-R	GCGTAATACGACTCACTATAGGAATCTCAAAGTTATTCTA	Reverse
Gfp RNAi-F	TAATACGACTCACTATAGGGGGTGGTCCCAATTCTCGTGGAAC	Forward
Gfp RNAi-R	TAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGC	Reverse
MjAlf-D2RT-F	CGCAGGCTTATGGAGGAC	Forward
MjAlf-D2RT-R	AGGTGACAGTGCCGAGGA	Reverse
MjAlf-C2EXF	TACTCAGAATTCCAGGGGTGGGAGGCACTCGTGCCA	Forward
MjAlf-C2EXR	TACTCACTCGAGTTACTGATTAAACCAAGCCT	Reverse
MjAlf-D1EXF	TACTCAGAATTCCAAATATGGGAGACGCTGAT	Forward
MjAlf-D1EXR	TACTCACTCGAGTTACTTGTGAGCCACGCCT	Reverse
MjAlf-D2EXF	TACTCAGAATTCCAGGGACTAAAGGACTTTTTAT	Forward
MjAlf-D2EXR	TACTCACTCGAGCTACACGATATATGGTTTTG	Reverse
MjAlf-E2EXF	TACTCAGAATTCTATGATGACGTCACCCAATCC	Forward
MjAlf-E2EXR	TACTCACTCGAGTTACAGCCACTCTGCCGCTT	Reverse
Vp28F	AGCTCCAACACCTCCTCCTTCA	Forward
Vp28R	TTACTCGGTCTCAGTGCCAGA	Reverse
Ie1F	GACTCTACAAATCTCTTTGCCA	Forward
Ie1R	CTACCTTTGCACCAATTGCTAG	Reverse

2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4 , and 1.8 mmol/L KH_2PO_4 ; pH 7.4), and then centrifuged at 5000 g for 10 min at 4 °C. The supernatant was filtered through a 0.45 μm filter (Jinlong, Tianjin, China). The viral titer was determined via qPCR on the basis of a previous report (Wang et al. 2014b). *M. japonicus* were divided into three groups, and the shrimp in each group was injected with PBS, TRX, or *MjALFs* (30 μg). One hour later, the shrimp was challenged with WSSV (2.5×10^5 copies). Genomic DNA, mRNA, and proteins were extracted from the gills of WSSV-infected and control shrimp at 36 and 48 h post WSSV injection. The genomic DNA was extracted using the MagExtractor Genomic DNA purification kit (Toyobo, Shanghai, China), and WSSV replication was analyzed using qPCR, with Vp28, a WSSV envelope protein, as an indicator.

RNA interference assay

The dsRNAs of *MjALF-D2* or green fluorescent protein (GFP) were synthesized using the DNA template amplified with specific primers *MjAlf-D2RNAiF*, *RNAiR*, *GfpF*, and *GfpR* linked to the T7 promoter (Table 1) by T7 RNA polymerase (Fermentas, Thermo Fisher Scientific, USA). The shrimp were randomly divided into two groups. Each shrimp in the two groups was injected with 30 μg of *dsMjAlf-D2* or *dsGfp* two times every 24 h. Total RNA was extracted from the gills of the shrimp at 48 h post second injection using TRIzol (TransGen, Beijing, China) to detect the RNAi efficiency. Then, WSSV (2.5×10^5 copies) was injected into the

MjAlf-D2-silenced shrimp at 48 h post second *dsRNA* injection. Genomic DNA, mRNA, and proteins were extracted from the gills of the shrimp at 36 and 48 h post WSSV injection using the method described above for the detection of WSSV replication.

Western blotting

Western blotting was used to detect WSSV replication by using VP28 antibody. The gills of *MjAlf-D2*-RNAi shrimp were homogenized in the buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride; pH 7.5), and the homogenate was centrifuged at 10,000 g for 10 min at 4 °C. The proteins of the sample were separated by 12.5% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked with 3% nonfat milk in TBS (10 mmol/L Tris-HCl and 150 mmol/L NaCl; pH 7.5) for 1 h, and incubated for 2 h with 1/100 diluted antiserum against VP28 in TBS with 3% nonfat milk. Then, after washing to remove the free, nonspecifically binding antiserum, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution in TBS) was added and incubated for 2 h. The membrane was put in the reaction system (10 ml of TBS with 45 μl of NBT and 35 μl of BCIP) in the dark for 5 min to visualize the signal. The antibody against the VP28 and β -actin used in Western blotting was prepared in the laboratory. The bands on the membrane of western blot

of three independent repeats were digitalized by scanning using ImageJ software.

Statistical analysis

Data are presented as mean $\pm$ SD ($n = 3$). Statistical evaluation of significant differences between experimental groups was conducted using Student's t test. The P values are defined as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Acknowledgements This research is supported by grants from the National Natural Science Foundation of China (grant nos. 31630084 and 31930112), and the National Key Research and Development Program of China (grant no. 2018YFD0900502).

Author contributions HSJ and JXW conceived and designed the study. HSJ and LXL performed the experiments, HSJ, LXL and JXW contributed to data analysis and interpretation. HSJ and JXW wrote the manuscript. All authors edited and approved the final manuscript.

Declarations

Conflict of interest All the authors declare that there are no conflicts of interest.

Animal and human rights statement The shrimp used in our study are consumed frequently by the wider community, so the approval according to the regulations on the use of shrimp is unnecessary.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Afsal VV, Antony SP, Chaithanya ER, Singh IS, Philip R (2012) Two isoforms of anti-lipopolysaccharide factors identified and characterized from the hemocytes of portunid crabs, *Portunus pelagicus* and *Scylla tranquebarica*. *Mol Immunol* 52:258–263
- Bi WJ, Li DX, Xu YH, Xu S, Li J, Zhao XF, Wang JX (2015) Scavenger receptor B protects shrimp from bacteria by enhancing phagocytosis and regulating expression of antimicrobial peptides. *Dev Comp Immunol* 51:10–21
- de la Vega E, O'Leary NA, Shockey JE, Robalino J, Payne C, Browdy CL, Warr GW, Gross PS (2008) Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. *Mol Immunol* 45:1916–1925
- Hanson MA, Dostalova A, Ceroni C, Poidevin M, Kondo S, Lemaitre B (2019) Synergy and remarkable specificity of antimicrobial peptides *in vivo* using a systematic knockout approach. *Elife* 8:e44341
- Jenssen H, Hamill P, Hancock RE (2006) Peptide antimicrobial agents. *Clin Microbiol Rev* 19:491–511
- Jiang HS, Sun C, Wang T, Zhao XF, Wang JX (2013) A single whey acidic protein domain containing protein (SWD) inhibits bacteria invasion and dissemination in shrimp *Marsupenaeus japonicus*. *Fish Shellfish Immunol* 35:310–318
- Jiang HS, Zhang Q, Zhao YR, Jia WM, Zhao XF, Wang JX (2015) A new group of anti-lipopolysaccharide factors from *Marsupenaeus japonicus* functions in antibacterial response. *Dev Comp Immunol* 48:33–42
- Jiravanichpaisal P, Lee SY, Kim YA, Andren T, Soderhall I (2007) Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: characterization and expression pattern. *Dev Comp Immunol* 31:441–455
- Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33:1870–1874
- Li S, Guo S, Li F, Xiang J (2014) Characterization and function analysis of an anti-lipopolysaccharide factor (ALF) from the Chinese shrimp *Fenneropenaeus chinensis*. *Dev Comp Immunol* 46:349–355
- Liu F, Liu Y, Li F, Dong B, Xiang J (2005) Molecular cloning and expression profile of putative antilipopolysaccharide factor in Chinese shrimp (*Fenneropenaeus chinensis*). *Mar Biotechnol* (NY) 7:600–608
- Liu H, Jiravanichpaisal P, Soderhall I, Cerenius L, Soderhall K (2006) Antilipopolysaccharide factor interferes with white spot syndrome virus replication in vitro and in vivo in the crayfish *Pacifastacus leniusculus*. *J Virol* 80:10365–10371
- Matos GM, Schmitt P, Barreto C, Farias ND, Toledo-Silva G, Guzman F, Destoumieux-Garzon D, Perazzolo LM, Rosa RD (2018) Massive gene expansion and sequence diversification is associated with diverse tissue distribution, regulation and antimicrobial properties of anti-lipopolysaccharide factors in shrimp. *Mar Drugs* 16:381
- Methatham T, Boonchuen P, Jaree P, Tassanakajon A, Somboonwivat K (2017) Antiviral action of the antimicrobial peptide ALFPm3 from *Penaeus monodon* against white spot syndrome virus. *Dev Comp Immunol* 69:23–32
- Mookherjee N, Anderson MA, Haagsman HP, Davidson DJ (2020) Antimicrobial host defence peptides: functions and clinical potential. *Nat Rev Drug Discov* 19:311–332
- Morita T, Ohtsubo S, Nakamura T, Tanaka S, Iwanaga S, Ohashi K, Niwa M (1985) Isolation and biological-activities of *Limulus* anti-coagulant (Anti-Lps Factor) which interacts with lipopolysaccharide (LPS). *J Biochem* 97:1611–1620
- Ng TH, Chang SH, Wu MH, Wang HC (2013) Shrimp hemocytes release extracellular traps that kill bacteria. *Dev Comp Immunol* 41:644–651
- Niu GJ, Wang S, Xu JD, Yang MC, Sun JJ, He ZH, Zhao XF, Wang JX (2019) The polymeric immunoglobulin receptor-like protein from *Marsupenaeus japonicus* is a receptor for white spot syndrome virus infection. *PLoS Pathog* 15:e1007558
- Ohashi K, Niwa M, Nakamura T, Morita T, Iwanaga S (1984) Anti-LPS factor in the horseshoe crab, *Tachypleus tridentatus*: Its hemolytic activity on the red blood cell sensitized with lipopolysaccharide. *FEBS Lett* 176:207–210
- Peng SE, Lo CF, Lin SC, Chen LL, Chang YS, Liu KF, Su MS, Ko GH (2001) Performance of WSSV-infected and WSSV-negative *Penaeus monodon* postlarvae in culture ponds. *Dis Aquat Organ* 46:165–172
- Ren Q, Zhang Z, Li XC, Jie D, Hui KM, Zhang CY, Wang W (2012) Three different anti-lipopolysaccharide factors identified from

- giant freshwater prawn, *Macrobrachium rosenbergii*. Fish Shellfish Immunol 33:766–774
- Rosa RD, Vergnes A, de Lorget J, Goncalves P, Perazzolo LM, Sauné L, Romestand B, Fievet J, Gueguen Y, Bachère E, Destoumieux-Garzón D (2013) Functional divergence in shrimp anti-lipopolysaccharide factors (ALFs): from recognition of cell wall components to antimicrobial activity. PLoS ONE 8:e67937
- Schmitt P, Rosa RD, Destoumieux-Garzón D (2016) An intimate link between antimicrobial peptide sequence diversity and binding to essential components of bacterial membranes. Biochim Biophys Acta 1858:958–970
- Sun C, Xu WT, Zhang HW, Dong LP, Zhang T, Zhao XF, Wang JX (2011) An anti-lipopolysaccharide factor from red swamp crayfish, *Procambarus clarkii*, exhibited antimicrobial activities *in vitro* and *in vivo*. Fish Shellfish Immunol 30:295–303
- Supungul P, Jaree P, Somboonwiwat K, Junprung W, Proespraiwong P, Mavichak R, Tassanakajon A (2017) A potential application of shrimp antilipopolysaccharide factor in disease control in aquaculture. Aquaculture Res 48:809–821
- Suraprasit S, Methatham T, Jaree P, Phiwsaiya K, Senapin S, Hirono I, Lo CF, Tassanakajon A, Somboonwiwat K (2014) Anti-lipopolysaccharide factor isoform 3 from *Penaeus monodon* (ALFPm3) exhibits antiviral activity by interacting with WSSV structural proteins. Antiviral Res 110:142–150
- Tanaka S, Nakamura T, Morita T, Iwanaga S (1982) Limulus anti-LPS factor - an anticoagulant which inhibits the endotoxin-mediated activation of *Limulus* coagulation system. Biochem Biophys Res Commun 105:717–723
- Tassanakajon A, Amparyup P, Somboonwiwat K, Supungul P (2011) Cationic antimicrobial peptides in penaeid shrimp. Mar Biotechnol (NY) 13:639–657
- Tassanakajon A, Rimphanitchayakit V, Visetnan S, Amparyup P, Somboonwiwat K, Charoensapsri W, Tang S (2018) Shrimp humoral responses against pathogens: antimicrobial peptides and melanization. Dev Comp Immunol 80:81–93
- Wang XW, Wang JX (2013) Pattern recognition receptors acting in innate immune system of shrimp against pathogen infections. Fish Shellfish Immunol 34:981–989
- Wang PH, Huang T, Zhang X, He JG (2014a) Antiviral defense in shrimp: from innate immunity to viral infection. Antiviral Res 108:129–141
- Wang XW, Xu YH, Xu JD, Zhao XF, Wang JX (2014b) Collaboration between a soluble C-type lectin and calreticulin facilitates white spot syndrome virus infection in shrimp. J Immunol 193:2106–2117
- Yang Y, Boze H, Chemardin P, Padilla A, Moulin G, Tassanakajon A, Pugniere M, Roquet F, Destoumieux-Garzón D, Gueguen Y, Bachère E, Aumelas A (2009) NMR structure of rALF-Pm3, an anti-lipopolysaccharide factor from shrimp: model of the possible lipid A-binding site. Biopolymers 91:207–220
- Yang HT, Yang MC, Sun JJ, Shi XZ, Zhao XF, Wang JX (2016a) Dual oxidases participate in the regulation of intestinal microbiotic homeostasis in the kuruma shrimp *Marsupenaeus japonicus*. Dev Comp Immunol 59:153–163
- Yang MC, Shi XZ, Yang HT, Sun JJ, Xu L, Wang XW, Zhao XF, Wang JX (2016) Scavenger receptor C mediates phagocytosis of white spot syndrome virus and restricts virus proliferation in shrimp. PLoS Pathog 12:e1006127
- Zheng SC, Xu JY, Liu HP (2019) Cellular entry of white spot syndrome virus and antiviral immunity mediated by cellular receptors in crustaceans. Fish Shellfish Immunol 93:580–588