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White spot syndrome virus infection activates Caspase 1-mediated cell death in crustacean

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ABSTRACT

In vertebrates, pyroptosis is an intense inflammatory form of programmed cell death. This death pathway is critical for controlling pathogenic infection. In invertebrates, however, due to the lack of adaptive immune response, it is still elusive whether Caspase 1-dependent cell death pathway exists. In this study, our data showed that Caspase 1-mediated cell death was activated by white spot syndrome virus to counteract virus infection. Caspase 1 had a higher expression in hemocytes and lymphoid-like organ in shrimp and WSSV infection was promoted upon the inhibition of Caspase 1 enzymatic activity. IL-1β-like protein was identified as the substrate of Caspase 1 and its interaction with Caspase 1 was validated ectopically and endogenously. Moreover, IL-1β like protein was released into extracellular contents under WSSV infection and Prophenoloxidase system was activated, resulting in the reduction of WSSV. Our data unraveled a previously unidentified mechanism through which Caspase 1-dependent cell death controlled virus infection in shrimp.

1. Introduction

One of the most important anti-pathogen responses consists of the elimination of the infected cells by programmed cell death, a response found in all metazoans (Fink and Cookson, 2005; Jorgensen et al., 2017). Apoptosis is perhaps the most widely recognized programmed cell death, and is defined by the requirement for particular cysteine-dependent aspartate-specific proteases, commonly Caspases 3/7, which produce an orchestrated disassembly of the cell resulting in mounting more severe immune responses (Albert, 2004; Yuan et al., 2016). It is found that apoptosis plays a very important role in determining the outcome of host-pathogen interactions (Gong et al., 2015; Yang et al., 2017). Other than Caspase 3/7-mediated apoptosis, pyroptosis is a more recently identified pathway of host cell death that is stimulated by a range of pathogen infections and non-infectious stimuli (Doitsh et al., 2014; Zhou et al., 2000).

Unlike apoptosis, pyroptosis occurs after Caspase 1 activation. Caspase 1 is usually activated by protein complexes termed inflammasomes in mammals (Yazdi et al., 2010). Two types of inflammasomes formed by Nod-like receptors (NLRs) have been identified. One type is NLRC4 and murine NLRP1b and the other type is NLRP3 (Broz et al., 2010). NLRC4 and murine NLRP1b contain CARD domains that directly interact with the Caspase 1 CARD and then activate Caspase 1 proteolytic activity. In contrast, NLRP3 contains a Pyrin signaling domain instead of a CARD domain (Bao and Shi, 2007). The Pyrin domain binds the Pyrin domain of the adaptor protein ASC which is composed of only a Pyrin and a CARD domain. ASC then recruits Caspase 1 via CARD-CARD interactions and finally activate Caspase 1 proteolytic activity (Srinivasula et al., 2002). Activated Caspase 1 can recognize and process the inactive precursors of interleukin 1β (IL-1β) into mature inflammatory cytokines, which is then secreted into intracellular contents (Srinivasula et al., 2002). IL-1β is an important mediator of the inflammatory response of the host against pathogen infection (Broz et al., 2010). Meanwhile, pyroptosis also induces morphologic changes, DNA fragmentation and chromatin condensation to destroy infected cells (Doitsh et al., 2014; Yazdi et al., 2010).

During pathogen infection, the host benefits from pyroptosis. The compromised cells are eliminated and more severe immune responses are launched, effectively destroying the protective environment where infectious agents can thrive (Case et al., 2009). In Caspase-1-deficient macrophages where pyroptosis is compromised, higher intracellular bacterial loads are detected during *L. pneumophila* infection (Case et al., 2009). Pyroptosis also promotes pathogen clearance by acting as an alarm signal that recruits immune cells to the site of infection (Miao

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et al., 2006). The secretion of IL-1β and IL-18 promotes leukocyte activation and immuno-stimulatory factors are released from lysed cells into the extracellular milieu (Miao et al., 2006). A bunch of released cytosolic products are potent damage- associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1), heat-shock proteins and DNA-chromatin complexes. By the activation of pattern-recognition receptors, these DAMPs accelerate proinflammatory cytokine production (Fernandes-Alnemri et al., 2007). On the other hand, pathogens have evolved effector proteins capable of directly inhibiting Caspase 1 and inflammasome activation, preventing cells from pyroptosis (Sutterwala et al., 2007; von Moltke et al., 2013). Virulent *Pseudomonas* strains express the effector protein ExoU, which blocks Caspase 1 activation and *Y. pseudotuberculosis* T3SS Rho-GTPase activating protein YopE inhibits inflammasome activation (Sutterwala et al., 2007; Master et al., 2008). The host-pathogen interaction mechanisms suggest that the activity of Caspase 1 leading to pyroptosis may represent an important antiviral pathway that prevents replication and spread of viruses to neighboring cells.

Apoptosis serves as a kind of innate immune pathway conserved both in invertebrates and vertebrates, engaging in the host antiviral pathway. But it is not clear that pyroptosis stimulated by a range of pathogen infections and non-infectious stimuli has a role in defending hosts from virus invasion. Pyroptosis is such a potent mechanism to clear intracellular pathogens. However, current studies on the role of pyroptosis in host-virus interactions has not been extensively explored and it is still unclear whether pyroptosis also exits and plays such an important role in defending hosts from pathogen invasion in invertebrates. In this study, we aim to find out whether pyroptosis is also one of the pathways that contributes to eliminating virus in invertebrate. The results revealed that the activity of Caspase 1 in shrimp was enhanced by white spot syndrome virus (WSSV) infection. The silencing of *Caspase 1* or *IL-1β-like* gene facilitated virus infection in shrimp. Our study indicated that Caspase-1-mediated cell death played an important antiviral role in invertebrate.

2. Material and methods

2.1. Shrimp culture and WSSV challenge

Marsupenaeus japonicas shrimp was cultured in groups of 20 individuals in tanks containing aerated seawater at room temperature. The individual shrimp was about 10 g in weight and 10–12 cm in length. To ensure the absence of WSSV in shrimp prior to experimental infection, PCR was performed using WSSV specific primers (5′-TATTG TCTCTCCTGACGTAC-3′ and 5′-CA CATTCTTCACGAGTCTAC-3′). Virus-free shrimp were infected with WSSV (10^5 copies/ml) by injection into the lateral area of the fourth abdominal segment. Three shrimp were randomly collected for each treatment. At different time post-infection, the shrimp hemolymph was collected for later use.

2.2. Rapid amplification of cDNA ends (RACEs)

Total RNAs were extracted from shrimp hemocytes using mirVana miRNA™ Isolation Kit (Ambion, USA). RACE experiments were conducted using 5′-3′ RACE Kit (Roche, USA) according to the manufacturer's protocol. Briefly, cDNA was synthesized and applied for PCR and nest-PCR. PCR was performed using 3′ RACE primers (Caspase 1, 5′-TACAAGATTCCCA ACACGGCCG-3′ and 5′-GCAGGTACGACTCTAGT GCACC-3′; IL-1β like protein gene, 5′- CGGAAATACTCTGGCCTCG TCA-3′ and 5′-CAACCAGAGTGATCGATG-3′) or/and 5′ RACE primers (Caspase 1, 5′-AGGGCACTGGTCAGCCTTGA-3′ and 5′-GAAGAAGAG TTT CGGCTTGC-3′; IL-1β like protein gene, 5′-CAGCTCGTTAAACAGC TCC-3′ and 5′-TTGGT CATTCTGTATCGCTCTC-3′). The PCR procedures were initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by a final elongation at 72 C for 10 min. The PCR products were cloned into pMD-18 vector (TaKaRa, Japan) and subjected to sequencing.

2.3. WSSV Copy number detection by quantitative real-time PCR

To quantify WSSV virions in shrimp, shrimp hemocytes were collected for the extraction of DNA by use of an SQ Tissue DNA kit (Omega Bio-Tek) according to the manufacturer's protocols and subsequently subjected to absolutely quantitative real-time PCR. The primers 5′-CCACCAATTCTACTCATGTACCAAA-3′ and 5′-TCCTTGCAATGGGCA AAATC-3′ were used to amplify a fragment from positions 260,075to 260,138 of the WSSV genome (GenBank accession number AF332093.1). The sequence of the TaqMan probe was 5′-FAM-CTGGGTTA CGAG TCTAA-TAMRA-3′. A linearized plasmid containing DNA fragment from the WSSV genome was quantified and serially diluted 10-fold as an internal standard for real-time PCR. The real-time PCR mixture contained 12.5 μL Ex Taq premix (TaKaRa, Japan), 1 μL of the extracted DNA template or the internal standard plasmid, 0.5 μL of 10 μM (each) primers, and 0.5 μL of 10 μM TaqMan probe. The real-time PCR conditions were set as 95 °C for 1 min followed by 45 cycles of 30 s at 95 °C, 30 s at 52 °C, and 30 s at 72 °C. Due to the sensitivity of quantitative real-time PCR, WSSV copies more than 10^3 could be considered as positive in this study.

2.4. Quantitative real-time PCR for detections of gene expression

Quantitative real-time PCR was performed to quantify gene expression level in shrimp. RNA was extracted from shrimp hemocytes using RNA Isolation kit (Ambion, USA). RNA was reversely transcribed into cDNA by cDNA synthesis kit (Takara, Japan).

The gene expression level was determined using gene-specific primers (Caspase 1, 5′-TTTCAAGGCTGACCAGTGCCCTAC-3′ and 5′-ACC TGCGTCAAGACCCTCACCTC-3′; IL-1β like protein, 5′- GCGTTCCACA TTTCAGATACTCGG-3′ and 5′-TGTAGACC ATTCCAAAGGCTCCC-3′). The 25 μL PCR solution contained 12.5 μL of 2 × Premix Ex Taq (TaKaRa, Japan), 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer and 1 μM DNA template. The stages of PCR program were 95 °C for 5 min, followed by the amplification stage consisting of 40 cycles of 95 °C for 10 s and 60 °C for 30 s.

2.5. Western blot analysis

Proteins were separated by a 12% SDS-polyacrylamide gel, which were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membrane was blocked with 5% nonfat milk in phosphate buffered solution (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM, pH 7.4) for 2 h at room temperature. The blot was incubated with a primary antibody at 4 °C overnight. After washes with PBS, the blot was incubated with the fluorescence-labeled anti-mouse IgG (Cell Signaling Technology, USA) for 2 h at 4 °C. The blot was detected by Odyssey (Licor, USA) for signals. The antibody against Caspase 1 or IL-1β like protein was prepared from mice, which were immunized by injection of the corresponding purified recombinant proteins. The working concentration of the antibody was about 1 μg/ml for Western blot. The antibodies against β-actin or β-tubulin were purchased from Santa Crzu. The dilution ratio was about 5000 for Western blot.

2.6. Annexin V for the detection of hemocyte death

Hemocyte death rate was detected by Annexin V (Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's protocol. Hemocytes were harvested and washed in cold phosphate-buffered saline (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM, pH 7.4). The hemocytes were then resuspended in 1 × annexin-binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4) at ~ 1×10^6 cells/ml, followed by the addition of 5 μL of Alexa Fluor 488

annexin V and 1 μL of 100 μg/ml PI (propidium iodide). The sample was incubated at room temperature for 15 min in the dark. Subsequently 400 μL of 1× annexin-binding buffer was added to the sample and measured by flow cytometry at 530 nm and 575 nm.

2.7. TdT-mediated dUTP nick end labeling (TUNEL) assay

TUNEL system (Promega, USA) was conducted according to the manufacturer's instruction with some modifications. Briefly, shrimp hemocytes were mixed with equal volume of heparin sodium (40 mg/ml) and mounted onto a poly-L-lysine coated glass slide (Sigma, USA), followed by incubation for 10 min at 4 °C. The hemocytes were fixed with 4% methanol-free paraformaldehyde for 10 min at 4 °C. Subsequently the hemocytes were rinsed twice with PBS and permeabilized with 0.2% Triton X-100 for 5 min. After rinsing slides with PBS twice at room temperature, the hemocytes were covered with 100 μL of equilibration buffer at room temperature for 10 min. The hemocytes were incubated with rTdT mix containing green fluorescein-12-dUTP for 60 min at 37 °C and then counterstained with PI. The reactions were terminated by immersing the slide in 2 × SSC for 15 min. The slide was air-dried and mounted with anti-fade solution (Invitrogen, USA) to assess fluorescence by microscopy.

2.8. Caspase 1 activity detection

Caspase 1 Activity Assay Kit was used to detect Caspase 1 activity according to the manufacturer's instructions (Beyotime, China). Briefly, shrimp hemocytes were collected and washed with PBS. Then, the hemocytes were lysed in 100 μL ice-cold lysis buffer for 15 min and centrifuged at 15,000 × *g* (4 °C) for 10 min. The supernatant was incubated with the substrate Ac-YVAD-pNA (2 mM) for 2 h at 37 °C. The reaction was detected by a spectrophotometer at OD_{405}. Caspase 1 inhibitor VX-765 (APExBIO, USA) was used to inhibit Caspase 1 activity in shrimp. The inhibitor was injected into shrimp at 100 μg/g shrimp in combination with WSSV injection or not.

2.9. Plasmid construction

The open reading frame (ORF) of Caspase 1 was amplified by PCR using sequence-specific primers (Caspase 1, 5′-TCGggatccATGGATTAC AAGGACGACGATGACAAGCCCCAAGGC TTTGCAAGGATGT-3′ and 5′-CTCGAGcgATACTTCTGCGTGAAGTACACCTTC-3′; IL-1β like protein gene, 5′-CCTggatccGCCACCATGCAGGCGGTAACAGATGGCA-3′ and 5′-AAG gcggccgcCTGTCCTGAAGACTTCCAT-3′). The amplified fragment was then cloned into pIZ-V5-His plasmid (Invitrogen, USA).

2.10. Cell culture and plasmids transfection

Insect High Five cells (Invitrogen, USA) were cultured and maintained at 27 °C in Express Five serum-free medium (Invitrogen) containing L-glutamine (Invitrogen). Cells were co-transfected with plasmids expressing Flag-Caspase 1 and His-IL-1β like protein according to the manufacture's instruction. Briefly, when the cells reached about 70% confluence in a 6-well plate, 2 μg for each indicated plasmid and 6 μL Cellfectin Reagent were diluted in 100 μL Grace's medium, respectively. The diluted plasmids and Cellfectin Reagent were then mixed gently and incubated for 30 min at room temperature. The mixture was added to the cells and incubated in a 27 °C incubator for 5 h before the medium was replaced by fresh Express Five serum-free medium. Cells were finally subjected to further research 48 h after transfection.

2.11. Co-immunoprecipitation

Insect High Five cells (Invitrogen, USA) were collected and lyzed in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2% NP40, 1 mM PMSF, 1 × protease inhibitor, pH 8.0). The lysate was then centrifuged three times for 5 min each to remove all the undissolved contents. The supernatant was incubated with anti-Flag or anti-His antibodies (Santa Cruz, USA) overnight at 4 °C. Protein A + G agarose beads (Invitrogen, USA) were incubated with the lysate for 2 h at 4 °C. After washed three times with ice-cold wash buffer (50 mM Tris-HCl, 200 mM NaCl, 0.1% NP40, 1 mM PMSF, 1 × protease inhibitor, pH 8.0), the immuno-complexes were eluted by 100 mM glycine and subjected to Western blotting for the detection of indicated proteins.

2.12. Mass spectrometry analysis

Protein bands were excised from SDS-PAGE gel and subjected to in-gel digestion by trypsin. MALDI (matrix-assisted laser desorption/ionization)-TOF (time of flight) spectra of the peptides were obtained via time-of-flight delayed extraction MALDI MS (Bruker Autoflex). A nitrogen laser (337 nm) was used to irradiate the sample. Spectra were acquired in reflectron mode in the mass range of 600–3500 Da. A near point calibration was applied and a mass tolerance of 100 ppm used. Data mining was performed using the Mascot search engine against shrimp protein database established from the combined published shrimp transcriptome.

2.13. Synthesis of siRNAs and RNAi assay

Based on the sequences of *Caspase 1* and *IL-1β like protein* genes, siRNAs were designed to specifically target the genes and then were synthesized in vitro using a commercial kit according to the manufacturer's instructions (TaKaRa, Japan). The siRNAs used were Caspase 1-siRNA-1 (5′-AAACCACUCGGAAUGCGAUGCG-3′), Caspase 1-siRNA-2 (5′-CCAGGCCUACAA GAUUCCCAAC-3′) and IL-1β-like-protein-siRNA (5′-GGGAAGGCTTTGAGGTCCTTGT-3′). The scrambled siRNA (5′-UUC UCCGAACGUGUCACGUTT-3′) not targeting any genes was used as the control. The formation of double-stranded RNAs was monitored by determining the size in agarose gel electrophoresis. The synthesized siRNAs were dissolved in siRNA buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) and quantified by Nanodrop 2000. The RNA interference (RNAi) assay was conducted in shrimp by the injection of a siRNA into the lateral area of the fourth abdominal segment at 30 μg/shrimp using a syringe with a 29-gauge needle. The siRNA (15 μg) and WSSV (10^5 copies/ml) were co-injected into virus-free shrimp at a volume of 100 μL per shrimp. At 12 h after the co-injection, the siRNA (15 μg) (100 μL /shrimp) was injected into the same shrimp. For each treatment, 20 shrimp were used. At different times after the last injection, the shrimp hemocytes were collected. Three shrimp specimens from each treatment, randomly selected, were collected for analysis. The assays were biologically repeated for three times.

2.14. Phenoloxidase activity detection

Hemolymph was centrifuged at 500 × *g* and the plasma was used for the detection of phenoloxidase activity. The reaction contained 300 μL 0.1 M K_3PO_4 (pH6.0), 10 μL 0.01 M L-dopa, (pH 6.0) and 10 μL plasma. After incubation at 28 °C for 2 min, the activity of phenoloxidase was measured by a spectrophotometer at OD492. 10 μL water was used instead of plasma to set up a baseline and shrimp plasma without any treatments was set as a control.

2.15. Shrimp mortality analysis

Shrimp with different treatments were cultured at 20 shrimp/treatment. Shrimp mortality was examined every day. The experiment was biologically repeated three times.

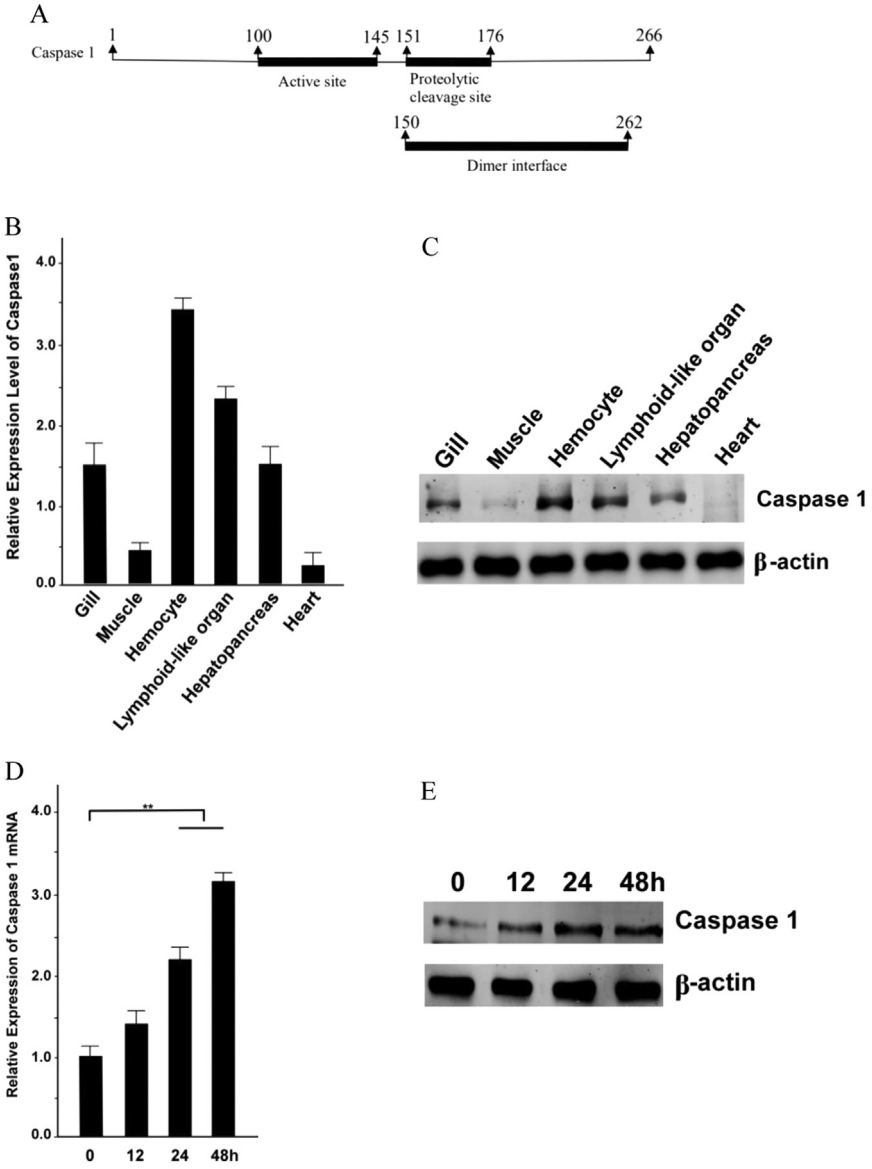

Fig. 1. Involvement of Caspase 1 in the virus infection to shrimp. (A) Sequence analysis of shrimp Caspase 1. *Casapse 1* gene was cloned from shrimp hemocytes. Shrimp Caspase 1 contained three domains including active site, proteolytic cleavage site and dimer interface. (B) Detection of Caspase 1 mRNA in different tissues of shrimp. The Caspase 1 mRNA was examined by quantitative real-time PCR. Shrimp β-actin was used as an internal control. (C) Examination of Caspase 1 protein in different shrimp tissues. The Caspase 1 protein was detected by Western blot. Shrimp β-actin was used as an internal control. (D) Expression level of Caspase 1 mRNA in shrimp challenged with virus. Shrimp were challenged with WSSV. At different time post-infection, the Caspase 1 mRNA of shrimp hemocytes were analyzed by quantitative real-time PCR (**, $p < 0.01$). (E) Determination of Caspase 1 protein level in WSSV-infected shrimp. Proteins were extracted from virus-infected shrimp hemocytes and analyzed by Western blot. The numbers indicated the time post-infection. Shrimp β-actin was used as an internal control. (F) Detection of Caspase 1 knockdown efficiency by quantitative real-time PCR. Shrimp were respectively injected with two siRNAs and Caspase 1 mRNA was analyzed by quantitative real-time PCR. (G) Examination of Caspase 1 knockdown efficiency by Western blot. (H) Impact of Caspase 1 silencing on virus infection. WSSV copy number was measured by quantitative real-time PCR at 48 h after shrimp were treated with Caspase 1 siRNA with or without WSSV infection. Because of the sensitivity of quantitative real-time PCR, more than 100 viral copies could be detected.

2.16. Statistical analysis

The data from three independent experiments were analyzed by one-way analysis of variance (ANOVA) to calculate the mean and standard deviation (SD) of the triplicate assays.

3. Results

3.1. Involvement of Caspase 1 in the virus infection to shrimp

In order to investigate whether Caspase 1-mediated pathway plays a role in the immune response of invertebrates to virus infection, the profiles of *Caspase 1* gene was characterized in the course of shrimp immunity against WSSV infection. The sequence, which was analyzed by BLAST, revealed that the shrimp Caspase 1 (GenBank accession no MK227438) contained Caspase activity domains, including active site, proteolytic cleavage site and dimer interface (Fig. 1A). The expression of the gene in different organs was then examined by real-time PCR. The result showed that Caspase 1 expression was most significant in hemocytes followed by lymphoid-like-organ, however, we can barely detect the mRNA of Caspase 1 in muscle and heart (Fig. 1B). The expression pattern of the gene was further confirmed by detection of protein levels as shown in Fig. 1C. These results indicated the shrimp Caspase 1 might function in immunity system–hemocytes and

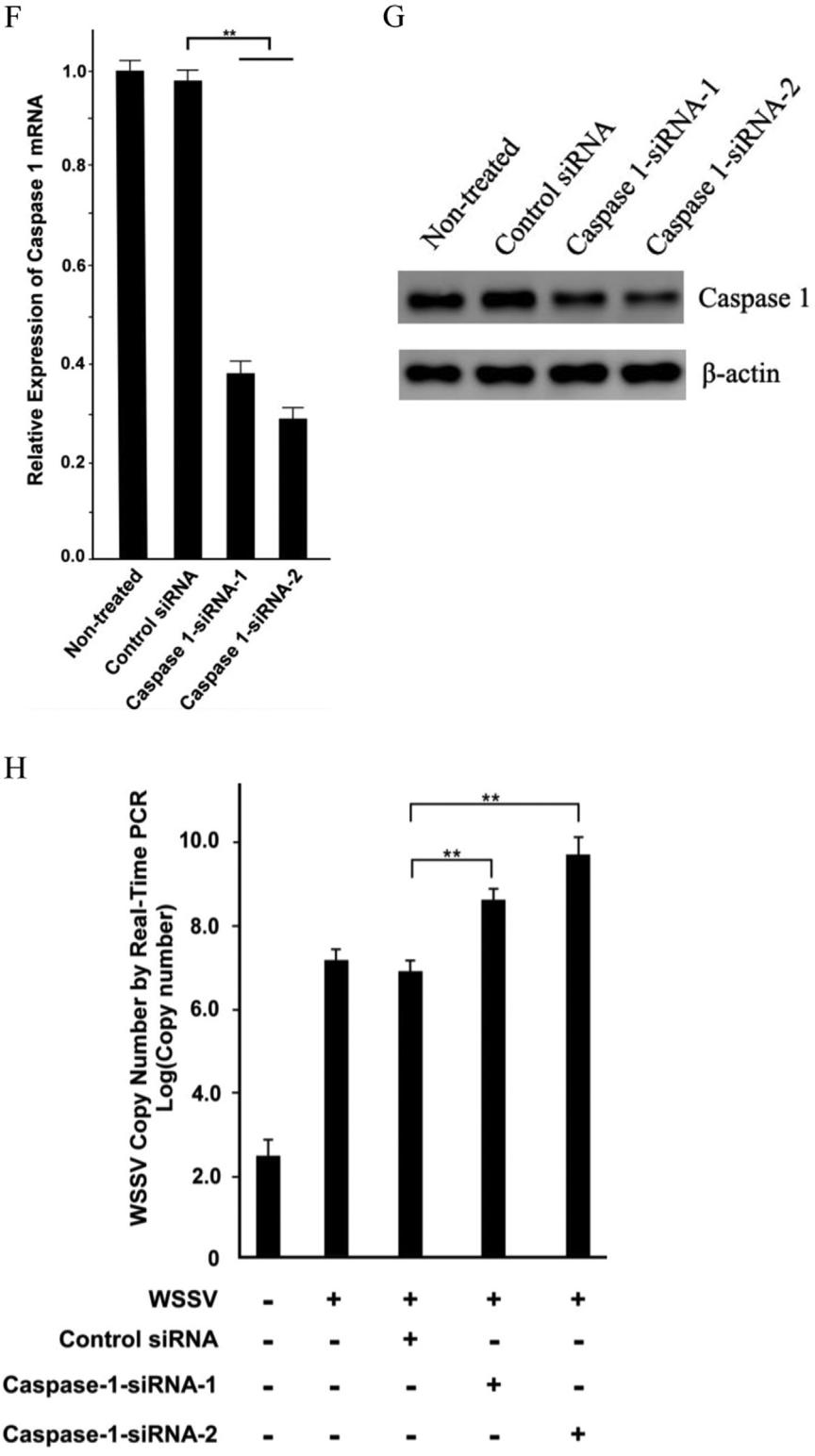

Fig. 1. (*continued*)

lymphoid-like organ in shrimp, where fighting off invading pathogens usually took place, so the result indicated that Caspase 1 might play a role in the antiviral cellular pathway.

To explore the role of Caspase 1 in virus infection to shrimp, the expression of Caspase 1 was detected at different time points after shrimp were infected with WSSV. The results revealed that the transcriptional level of Caspase 1 was significantly upregulated with the prolonged infection time (Fig. 1D). Meanwhile, the protein level of the gene also showed similar expression patterns during WSSV infection (Fig. 1E). These data indicated that Caspase 1 expression (both mRNA and protein) was induced upon WSSV infection. To further explore the function of Caspase 1, two siRNAs specifically targeting different sites of Caspase 1 were designed and injected into shrimp to knock down Caspase 1. Real-time PCR and Western blotting showed both siRNAs could silence the expression of Caspase 1 while Caspase 1-siRNA-2 had a better knockdown efficiency (Figs. 1F and 1G). Under this condition,

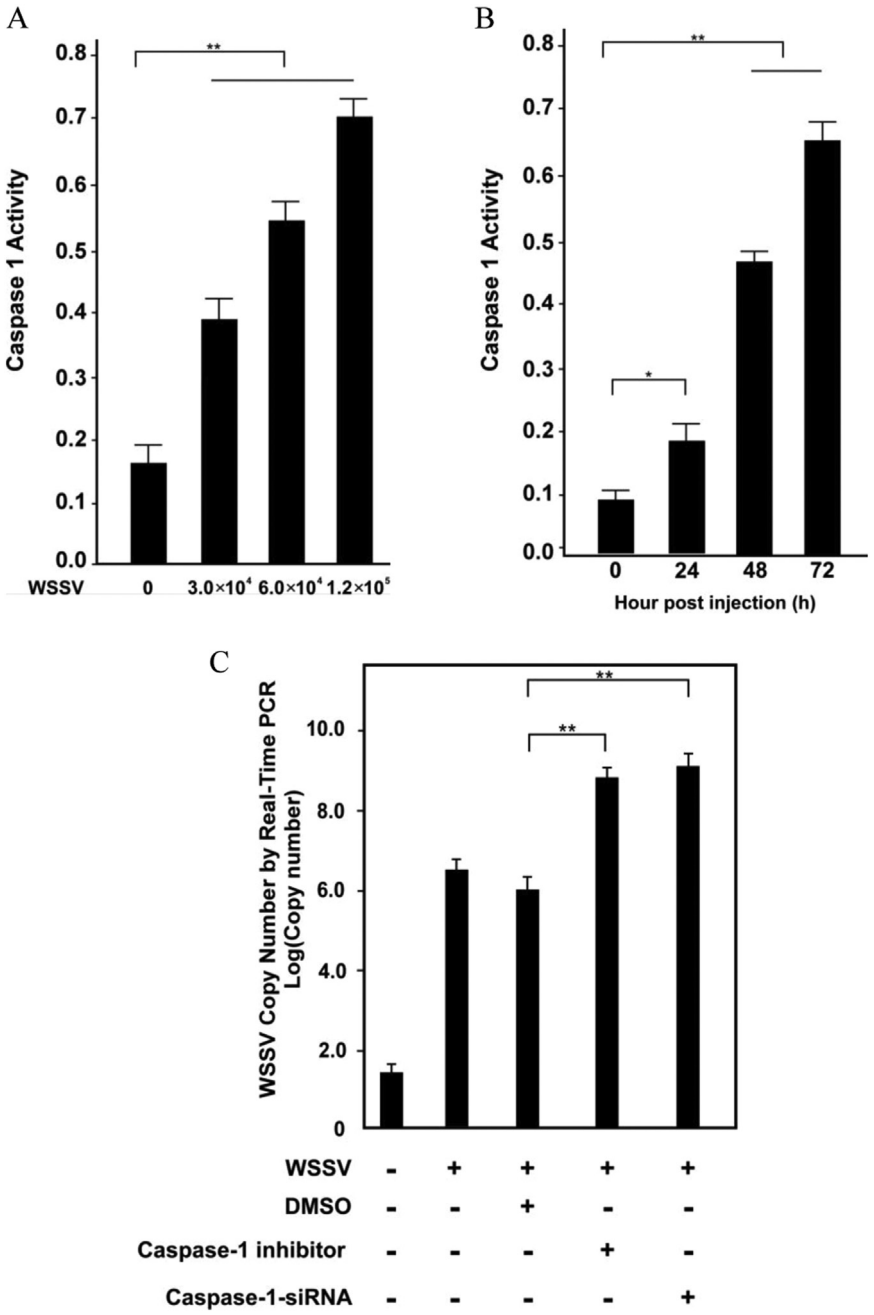

Fig. 2. Caspase-1 mediated cell death participating in the antiviral responses. (A) Influence of WSSV concentration on Caspase 1 activity. Caspase 1 activity was measured at OD_{450} after shrimp were challenged with increasing WSSV concentration. Caspase 1 activity was detected at 48 h post-infection. (B) Effects of virus infection on Caspase 1 activity. Caspase 1 activity was measured at different time points after shrimp were challenged with WSSV. (C) Impact of inhibition of Caspase 1 activity on virus infection. WSSV copy number was measured by quantitative real-time PCR at 48 h after shrimp were treated with Caspase 1 inhibitor with or without WSSV infection. (D) Inhibition or reduction of Caspase 1 on the hemocyte death examined by Annexin V. Hemocytes with the indicated treatments were collected and stained with Annexin V and PI. The hemocytes were analyzed by flow cytometry. (E) Inhibition or reduction of Caspase 1 on the hemocyte death examined by TUNEL. Hemocytes with the indicated treatments were collected for the TUNEL assay. The samples were examined by fluorescent microscopy.

we examined the impact of Caspase 1 reduction on WSSV replication. The results showed that WSSV copy number increased upon Caspase 1 knockdown especially upon those treated with Caspase 1-siRNA-2 (Fig. 1H), suggesting that Caspase-1 was involved in the antiviral immune pathway of shrimp.

3.2. Caspase-1 mediated cell death participating in the antiviral responses

To further determine how Caspase 1 is involved in the antiviral pathway, we first examined whether the enzymatic activity of Caspase 1 was associated with virus infection. Shrimp were challenged with increasing concentrations of WSSV and hemocytes were collected for the detection of Caspase 1 activity by incubating with the substrate Ac-YVAD-pNA. The results showed that Caspase 1 activity was greatly enhanced while more viruses were used for infection (Fig. 2A). Moreover, Caspase 1 activity increased dramatically along with the prolonged infection time (Fig. 2B). To explore whether the Caspase 1 activity facilitated or inhibited virus infection, Belnacasan (VX-765), which has been demonstrated as a potent Caspase 1 inhibitor (McKenzie et al., 2018; Do Carmo et al., 2018), was used to inhibit

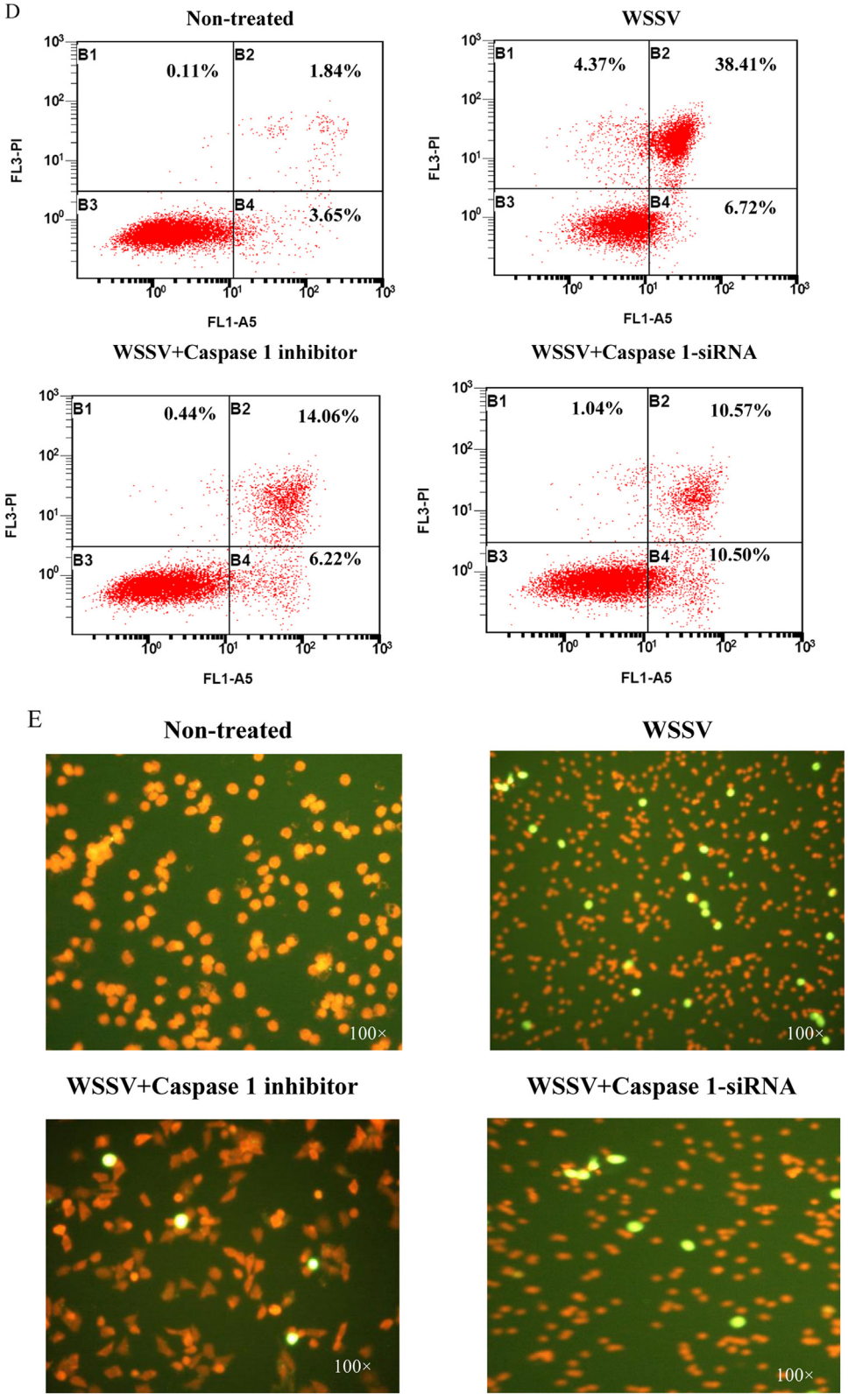

Fig. 2. (*continued*)

Caspase 1 activity in combination with WSSV injection or not. DMSO and Caspase 1 siRNA were respectively set as a negative and a positive control. The result showed that DMSO treatment group didn't show the increase of WSSV copy number compared with the group infected with WSSV only, however, WSSV copy number significantly elevated in the VX-765 treated group, the increase level of which is similar with that in the Caspase 1 knockdown group. The data suggested that the enzymatic activity of Caspase 1 was critical in the antiviral pathway (Fig. 2C).

To determine whether Caspase 1 initiated cell death to inhibit virus replication, we then examined the cell death of hemocytes by Annexin V and TUNEL upon inhibition or knockdown of Caspase 1. The results showed that WSSV infection induces dramatic cell death as shown in

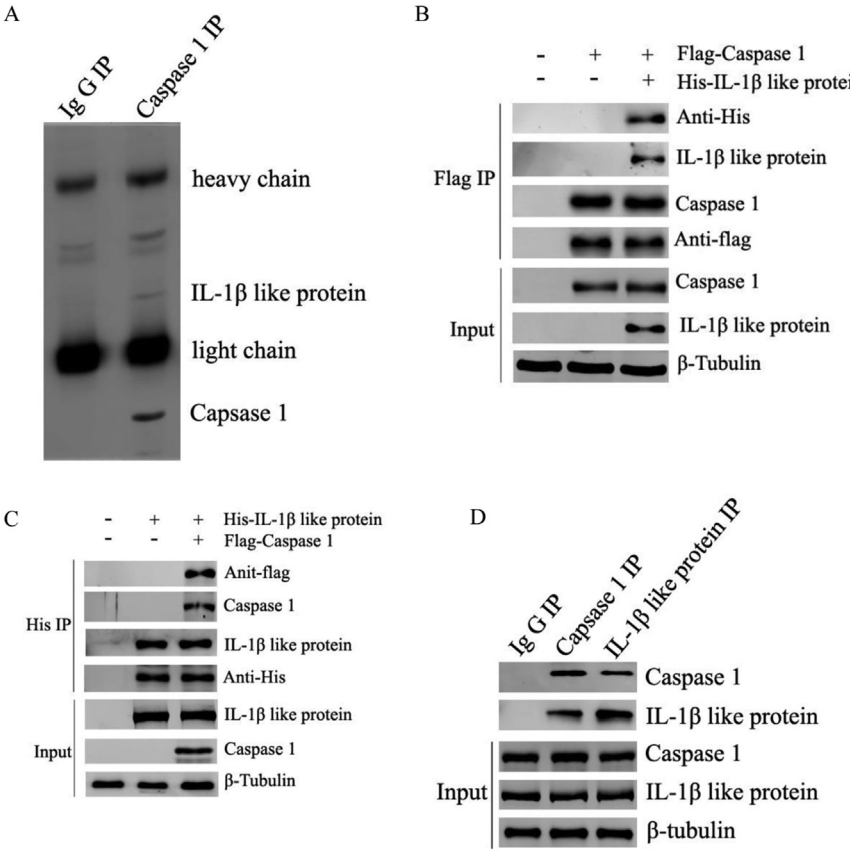

Fig. 3. Interaction between Caspase 1 and IL-1β like protein. (A) SDS-PAGE analysis of the elute from Caspase 1 IP by silver staining. Caspase 1 IP was performed and the immunoprecipitate was analyzed by SDS-PAGE by silver staining. The proteins were identified by mass spectrometry. (B) Validation of Flag-Caspase 1 and His-IL-1β like protein by Anti-Flag IP in insect High Five cells. The Flag-Caspase 1 and His-IL-1β like protein constructs were co-transfected into insect cells and Anti-Flag IP was performed. The elute was analyzed by Western blot. β-tubulin was used as an internal control. (C) Validation of Flag-Caspase 1 and His-IL-1β like protein by Anti-His IP in High Five cells. Flag-Caspase 1 and His-IL-1β like protein were cotransfected into High Five cells and Anti-His IP was performed and the elutes was analyzed by western blot. β-tubulin was used as a control. (D) Endogenous Caspase 1 and His-IL-1β like protein interaction by IP. Caspase 1 or IL-1β like protein IP was performed respectively and the immunoprecipitates were analyzed by Western blot. β-tubulin was used as an internal control.

Fig. 2D. However, both Caspase 1 inhibitor and Caspase 1 siRNA partially inhibited cell death stimulated by virus infection. Similar pattern was also detected as the hemocytes were examined by TUNEL assay (Fig. 2E). In combination, these data demonstrated that Caspase 1-mediated cell death played an important role in antiviral immune responses.

3.3. Interaction between Caspase 1 and IL-1β like protein

In order to reveal the cellular pathway in which Caspase 1 participated to play an antiviral role, we performed co-immunoprecipitation (Co-IP) to identify the substrate of Caspase 1. The Co-IP data showed that one specific band was discovered after Caspase 1- specific antibody but not IgG was used for CoIP, which was then identified as IL-1β like protein by mass spectrometry (Fig. 3A). To further demonstrated the interaction between Caspase 1 and IL-1β like protein, Flag-Caspase 1 and His-IL-1β like protein (shrimp IL-1β GenBank accession no MK227439) was respectively cloned into PIZ/V5-His plasmid and co-transfected into insect High Five cells. Anti-Flag IP was performed and then proteins were analyzed by Western blotting. The result indicated that His-IL-1β like protein was actually detected (Fig. 3B), showing the interaction between Caspase 1 and IL-1β like protein. Meanwhile, anti-His IP was also performed and Flag-Caspase 1 could also be found by Western blotting (Fig. 3C). These results revealed that the two proteins could interact with each other by their expressions in insect cells.

To further confirm the interaction Caspase 1 and IL-1β like proteins in vivo, endogenous IP was performed using Caspase 1 or IL-1β like protein antibody upon the viral infection. The results showed that IL-1β like protein was detected by Caspase 1 endogenous IP and vice versa (Fig. 3D).

Taken together, these findings demonstrate IL-1β like protein and Caspase 1 could interact with each other in vitro and in vivo.

3.4. Redistribution of IL-1β like protein induced by WSSV infection to activate phenoloxidase system

To further investigate the functions of Caspase 1 and IL-1β like protein interaction in vivo, siRNA targeting *IL-1β like protein* gene was used. The effectiveness of siRNA was also examined by quantitative real-time PCR and Western blotting. From the detected mRNA and protein level, it could be concluded that the siRNA efficiently knocked down the expression of *IL-1 like protein* genes (Fig. 4A).

As reported in mammary cells, IL-1β is firstly interacted with and then cleaved by Caspase 1 before it is released into extracellular contents (Strowig et al., 2012), however, in this study, it is still elusive how IL-1β like protein reacts upon virus infection. Therefore, the level of IL-1β like protein in hemocytes and the corresponding plasma before and after WSSV infection was examined. Hemolymph was collected and centrifuged to separate the hemocytes and plasma for the detection of IL-Iβ like protein. Western blotting analysis revealed that the expression level of IL-1β like protein was increased in the plasma and decreased in hemocytes when shrimp were challenged with WSSV, indicating that IL-1β like protein was released into extracellular contents under virus infection (Fig. 4B). In order to determine whether Caspase 1 mediated the release of IL-1β like protein, the expression of Casapse 1 was firstly knocked down by siRNA and then the expression pattern of IL-1β like protein was examined. The results showed that the level of IL-1β like protein in the plasma decreased while the level in the hemocytes increased upon infection with WSSV (Fig. 4C). These data showed that the release of IL-1β like protein into plasma was controlled by Caspase 1 and the secreted IL-1beta might be cleaved by Caspase 1 in shrimp.

To further determine whether IL-1β like protein was required to be cleaved by Caspase 1 before releasing into extracellular contents, the enzymatic activity of Caspase 1 was inhibited by VX-765 and then the level of IL-1β like protein was examined. The result showed that the level of IL-1β like protein didn't increase in the plasma, suggesting that

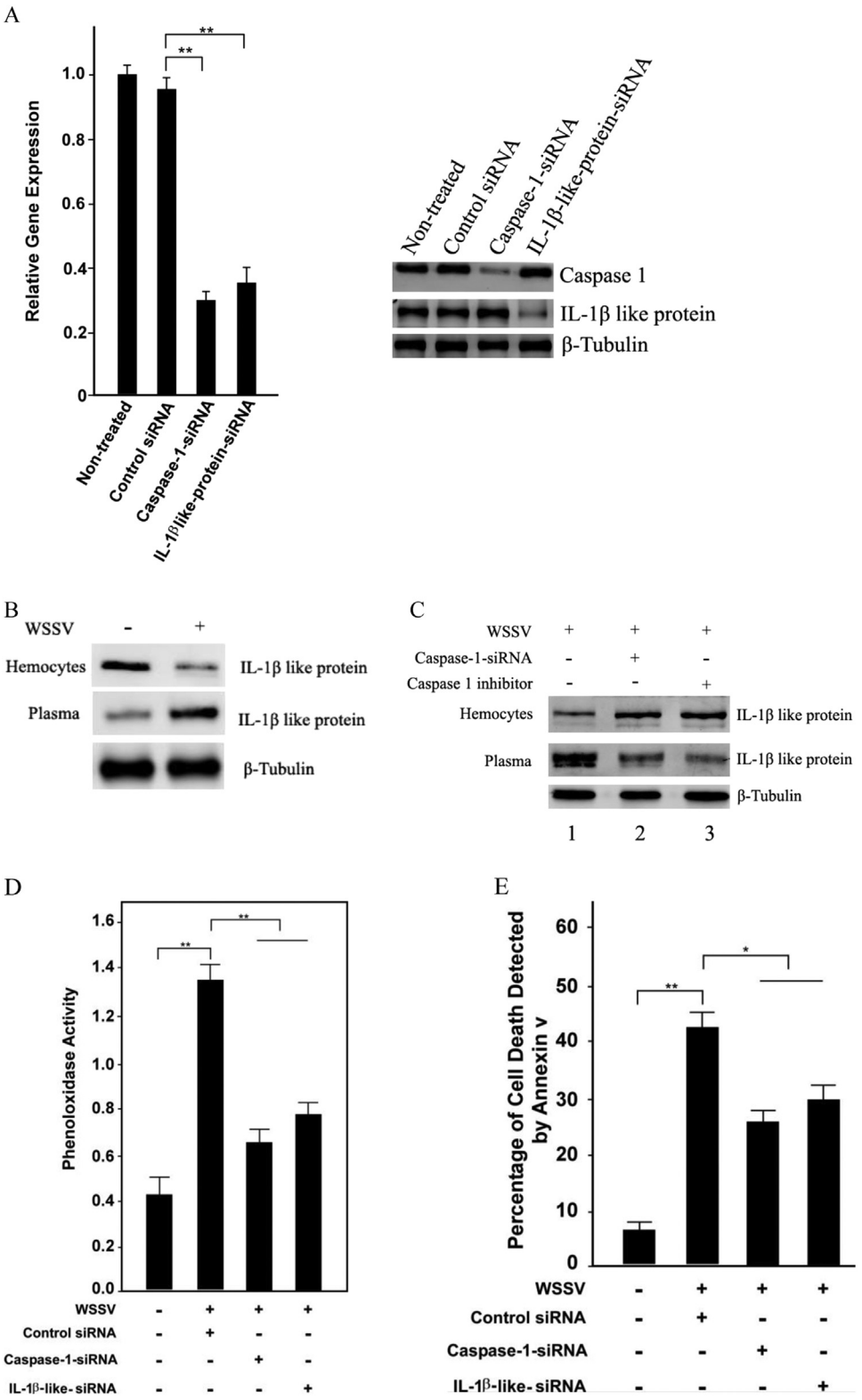

Fig. 4. Redistribution of IL-1β like protein induced by WSSV infection to activate phenoloxidase system. (A) Detection of Caspase 1 and IL-1β like protein knockdown efficiency. The mRNA and protein levels of Caspase 1 and IL-1β like protein were respectively detected by quantitative real-time PCR (left) and Western blot (right) at 48 h after shrimp were injected with indicated siRNAs. (B) Detection of IL-1β like protein expression in hemocytes or plasma after WSSV infection. The expression level of IL-1β like protein in hemocytes or plasma was examined by Western blot with or without WSSV infection. Samples were collected at 48 h after WSSV infection. (C) Evaluation of IL-1β like protein expression in hemocytes or plasma after WSSV infection with or without Caspase 1 inhibition. The expression level of IL-1β like protein in hemocytes or plasma was detected by Western blot after WSSV-infected shrimp were treated with Caspase 1 siRNA or inhibitor. Caspase 1 inhibitor or siRNA was immediately injected into shrimp after WSSV infection. Samples were collected 48 h after injection. (D) Examination of phenoloxidase activity upon knockdown of Caspase 1 or IL-1β like protein in shrimp. Phenoloxidase activity was measured at OD_{492} after shrimp were treated with either Caspase-1-siRNA or IL-1β-like-protein-siRNA at 48 h after WSSV injection. (E) Impact of knockdown of Caspase 1 or IL-1β like protein on the cell death of hemocytes. Cell death rate was measured by Annexin V after shrimp were treated with either Caspase-1-siRNA or IL-1β-like-protein-siRNA at 48 h after WSSV injection.

the enzymatic activity of Caspase 1 was required for the release of IL-1β like protein (Fig. 4C).

Prophenoloxidase (PO) activating system is an important identification and defense system for eliminating viruses in crustaceans (Sutthangkul et al., 2015). To find out whether Caspase 1 mediated cell death could influence PO system, Caspase 1 and IL-1β like protein were respectively knocked down and then the PO activity was measured with or without WSSV challenge. As expected, WSSV challenge greatly induced the PO activity (Fig. 4D). However, either Caspase 1 knockdown or IL-1β like protein knockdown partially inhibited the PO activation (Fig. 4D). We then detected cell death of hemocytes by Annexin V under the same treatments as shown in Fig. 4D. The results indicated that the cell death rate was partially inhibited when either Caspase 1 or IL-1β like protein was knocked down (Fig. 4E).

Taken the above data together, these findings demonstrate that the releasing of IL-1β like protein is dependent on Caspase 1 resulting in the

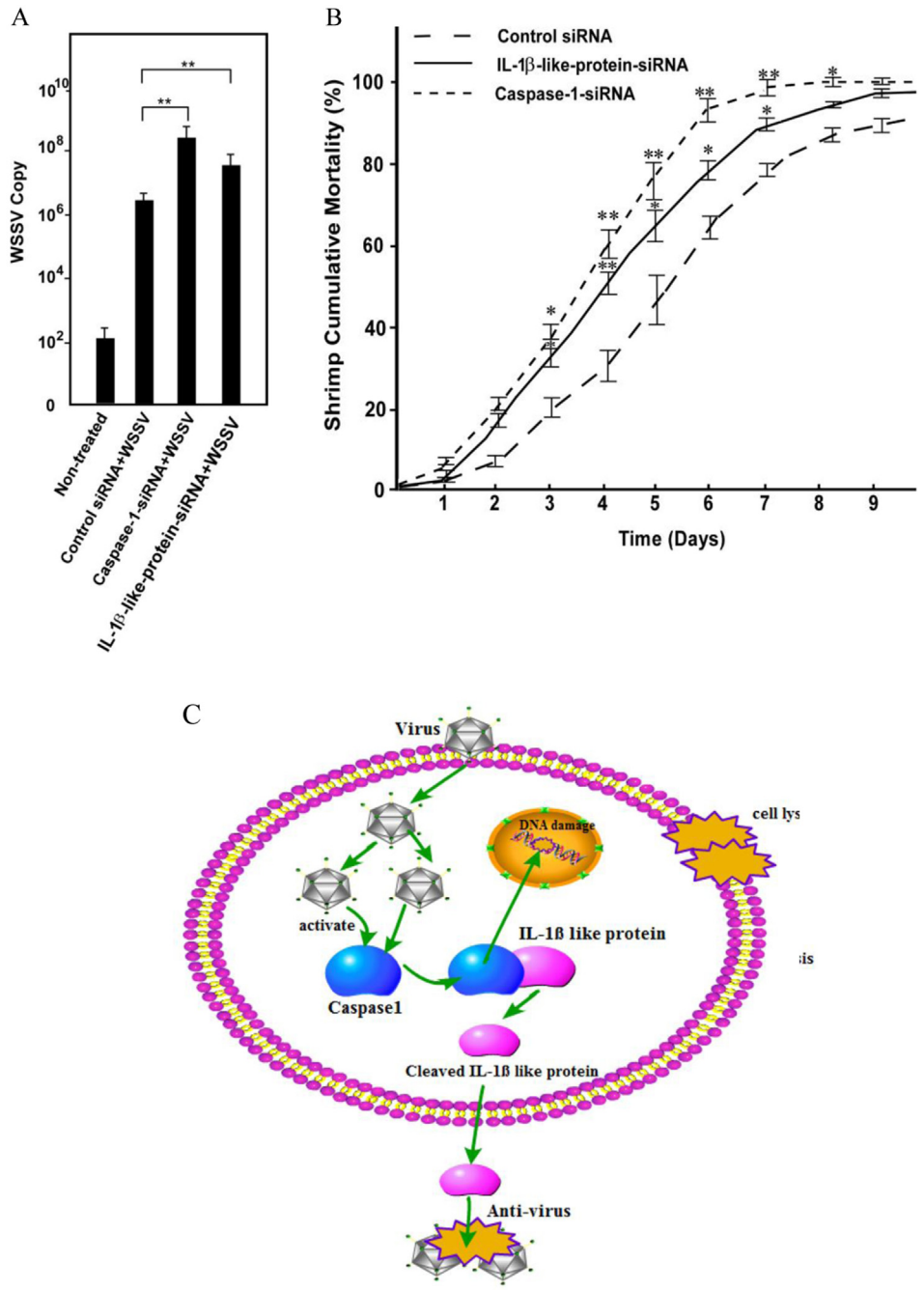

Fig. 5. Negative correlation of Caspase 1 and IL-1β like protein expressions with virus infection. (A) Detection of WSSV copies after knockdown of Caspase 1 or IL-1β like protein genes. WSSV copy number in Caspase-1-siRNA or IL-1β-like-protein-siRNA treated shrimp was measured by quantitative real-time PCR. (B) Monitoring of shrimp cumulative mortality upon knockdown of Caspase 1 or IL-1β like protein genes. (C) A model for the Caspase 1-mediated antiviral pathway in shrimp. Upon WSSV infection, Caspase 1 was activated and then interacted with IL-1β like protein, facilitating its releasing into plasma. Phenoloxidase was then activated for controlling virus infection. In all panels, the statistical significance of difference between treatments were indicated with asterisks (*, $p < 0.05$; **, $p < 0.01$).

activation of PO system and cell damage, which prevent shrimp from virus infection.

3.5. Negative correlation of Caspase 1 and IL-1β like protein expressions with virus infection

To investigate the influence of Caspase 1-mediated cell death on virus infection, WSSV copy number was detected upon knockdown of either *Caspase 1* or *IL-1β like protein* gene in shrimp. The results showed that the silencing of Caspase 1 or IL-1β like protein significantly increased the copy number of WSSV compared to the control, indicating that Caspase 1-mediated cell death played an important antiviral role in shrimp immunity (Fig. 5A), which was further demonstrated by the shrimp cumulative mortality analysis (Fig. 5B).

Collectively, these findings reveal a novel pathway in which Caspase 1-mediated cell death induces PO activation and plays a critical antiviral role in shrimp immunity (Fig. 5C).

4. Discussion

The outbreak of viral diseases is a major concern preventing the development of shrimp aquaculture industry. WSSV is one of the most virulent shrimp viruses and the processes of pathogenesis are extremely complex. Due to the lack of effective therapeutics to control virus, it is of great importance to understand viral pathogenesis and host responses at the molecular level in order to prevent virus invasion. So far, some studies have demonstrated that many genes and pathways are involved in host defense against WSSV, including Ran protein, the Ras-activated endocytosis process, the RNA interference pathway and apoptosis (Liu et al., 2009; Han and Zhang, 2007; Xu et al., 2007; Wang et al., 2008). Apoptosis is maybe the best described form of cell death that is mediated by the activation of the apoptotic caspase enzymes

Caspase 3/7 and the antiviral role of apoptosis has been discovered both in invertebrates and vertebrates. Currently, pyroptosis, which is mediated by Caspase 1 activation, is thought to have the capacity of eliminating viruses only in vertebrates (Schroder and Tschopp, 2010). In vertebrates, pyroptosis activation can result in not only the production of activated inflammatory cytokines, but also rapid cell death characterized by plasma-membrane rupture and release of proinflammatory intracellular contents (Schroder and Tschopp, 2010). On the other hand, viruses including HIV have evolved mechanisms to inhibit pyroptosis, enhancing their ability to persist and cause disease (Deeks, 2011). Ultimately, it is the competition between host and pathogen to regulate pyroptosis, and the outcome dictates life or death to the host or to viruses. Based on the critical role of Caspase 1 in the defense of a wide range of virus infections in vertebrates, in this study, Caspase 1 gene was first cloned from shrimp and the activity of Caspase 1 was then characterized. Our data showed that Caspase 1 activity was upregulated upon WSSV infection and the knockdown of Caspase 1 facilitated virus infection. Compared with the function of pyroptosis in vertebrates, our study revealed that Caspase 1-mediated cell death also contributed to eliminating virus in crustacean. The results showed that the inhibition of caspase 1 activity could not fully stop cell death, suggesting the existence of other pathways, such as apoptosis, which could mediate cell death. In this context, our findings indicated that the antiviral function of Caspase 1 was conservative although the sophisticated immune responses of shrimp have not been developed yet.

In vertebrates, the activated Caspase 1 recognizes and cleaves IL-1β into the mature form. The release of mature IL-1β further enhances the inflammatory response by stimulating immune cell activation and more cytokine secretion (Hilbi et al., 1997), leading to critical physiological consequences such as fever and hypotension. However, the cleavage and release of IL-1β is not indispensible for caspase 1-mediated cell death as demonstrated by IL-1β-/- knockout animals (Fantuzzi et al., 1996). In our study, the substrate of Caspase 1, which was named IL-1β like protein, was firstly identified. The results showed that WSSV infection induced the redistribution of IL-1β like protein into plasma of shrimp, indicating that IL-1β like protein was released into extracellular contents upon virus infection. The released IL-1β like protein, instead of triggering inflammation responses, activated phenoloxidase system of shrimp, an important invertebrate defense mechanism against virus infection. These data demonstrated that the function IL-1β like protein participating in Caspase 1- mediated cell death in shrimp was similar with that in vertebrates, and that IL-1β like protein functioned as cytokine-like molecules in invertebrates. In vertebrates, the Caspase-1 activation can lead to induction of pyroptosis by cleavage of Gasdermin D. However, Gasdermin D gene is not conservative among species and shrimp might employ other proteins instead of Gasdermin D. Although invertebrates including shrimp lack adaptive immune system, several putative functional analogues of inflammatory cytokines have been identified so far. For example, tumor necrosis factor (TNF)-like molecules and interleukin-1 (IL-1)-like molecules are detected in a variety of invertebrates, such as insects, echinoderms and protochordates (Caselgrandi et al., 2000; Wittwer et al., 1999). Moreover, chemokine IL-8 and transforming growth factor β1 are vertebrate cytokines involved in inflammatory processes and molecules cross-reacting with the two proteins using antibodies have been identified in mollusks (Ottaviani et al., 2000). In addition, IL-2-like activity is detected in protochordates and echinoderms which have hematopoietic organs and T-like cells (Aliprantis et al., 2000). In this study, our data suggested that an invertebrate cytokine network might be operative and regulate host defense mechanisms against virus infection as in vertebrates.

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