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1 **Title page**

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3 White spot syndrome virus infection induces Caspase 1-mediated antiviral cell 4 death in crustacean

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6 Geng Yang[1, 2+], Jiajia Wang[1+],Tao Luo[2], Jun Wei[1], Yadong Yang[2], Wenyuan Zhang[2], Xiaobo 7 Zhang[1*]

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10 *1 College of Life Sciences and Laboratory for Marine Biology and Biotechnology of Qingdao 11 National Laboratory for Marine Science and Technology, Zhejiang University, Hangzhou 12 310058, The People's Public of China*

13 *2 Institute of Bioengineering, Zhejiang Academy of Medical Sciences, Hangzhou 310013, The 14 People's Public of China*

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17 * Corresponding author: Prof Xiaobo Zhang

18 Tel: 86-571-88981129

19 Fax: 86-571-88981151

20 Email: zxb0812@zju.edu.cn

21 +: These authors contribute equally to this work.

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24 **Abstract**

25 In vertebrates, pyroptosis is an intensely inflammatory form of programmed cell death which
26 is dependent on Caspase 1 activation and release of cytoplasmic cytokines including IL-1β. This
27 death pathway is critical for controlling pathogenic infection by mobilizing immune cells and
28 stimulating the development of adaptive immune response. In invertebrates, however, due to the
29 lack of adaptive immune response, it is still elusive whether Caspase 1-dependent cell death
30 pathway exists. In this study, our data showed that Caspase 1-mediated cell death was activated
31 by white spot syndrome virus (WSSV) infection to control the virus in shrimp. Caspase 1 had a
32 higher expression level in hemocytes and lymphoid-like organ in shrimp and WSSV infection
33 was significantly promoted upon the inhibition of Caspase 1 enzymatic activity. IL-1β-like
34 protein was identified as the substrate of Caspase 1 and its interaction with Caspase 1 was
35 validated ectopically and endogenously. Moreover, IL-1β like protein was released into
36 extracellular contents under WSSV infection and Prophenoloxidase system was activated,
37 resulting in the reduction of WSSV copies *in vivo*. Our data unraveled a previously unidentified
38 mechanism through which Caspase 1-dependent cell death controlled virus infection in shrimp.
39 Therefore, our study opened the possibility that an invertebrate cytokine network might be
40 operative and regulate host defenses against virus infection as in vertebrates.

41 Key words: shrimp; virus; pyroptosis; Caspase 1; IL-1β-like protein

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47 **Author Summary**

48 The elimination of infected cells by programmed cell death is considered as one of the most
49 important anti-pathogen responses. Pyroptosis is one form of programmed cell death, which is
50 mediated by Caspase 1 activation. Activated Caspase 1 can process the inactive precursors of
51 interleukin 1β (IL-1β) into mature inflammatory cytokines to induce potent immune responses.
52 Meanwhile, pyroptosis also induces morphologic changes, DNA fragmentation and chromatin
53 condensation to destroy infected cells. However, due to the lack of efficient adaptive immune
54 responses, it is still unclear whether Caspase 1-mediated cell death also exits and plays such an
55 important role in defending hosts from pathogen invasion in invertebrates. In our data, Caspase 1
56 gene was characterized and Caspase 1-mediated cell death pathway was validated in shrimp. The
57 Caspase 1-mediated cell death pathway was enhanced by white spot syndrome virus (WSSV)
58 infection to control the virus in shrimp. IL-1β-like protein was cleaved by Caspase 1 and released
59 into extracellular contents under WSSV infection to activate Prophenoloxidase system, leading
60 to the reduction of WSSV copies in shrimp in vivo. On the other hand, silencing of *Caspase 1* or
61 *IL-1β-like* gene facilitated virus infection in shrimp. Our study indicated that Casapase-1-
62 mediated cell death played an important antiviral role in shrimp against WSSV infection and
63 opened the possibility that an invertebrate cytokine network might be operative.

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70 **Introduction**

71 One of the most important anti-pathogen responses consists of the elimination of the infected
72 cells by programmed cell death, a response found in all metazoans (1, 2). Apoptosis is perhaps
73 the most widely recognized programmed cell death, and is defined by the requirement for
74 particular cysteine-dependent aspartate-specific proteases, commonly Caspases 3/7, which
75 produce an orchestrated disassembly of the cell resulting in mounting more severe immune
76 responses (3, 4). It is found that apoptosis plays a very important role in determining the outcome
77 of host-pathogen interactions (5, 6). Other than Caspase 3/7-mediated apoptosis, pyroptosis is a
78 more recently identified pathway of host cell death that is stimulated by a range of pathogen
79 infections and non-infectious stimuli (7, 8).

80 Unlike apoptosis, pyroptosis occurs after Caspase 1 activation. Caspase 1 is usually activated
81 by protein complexes termed inflammasomes in mammals (9). Two types of inflammasomes
82 formed by Nod-like receptors (NLRs) have been identified. One type is NLRC4 and murine
83 NLRP1b and the other type is NLRP3 (10). NLRC4 and murine NLRP1b contain CARD
84 domains that directly interact with the Caspase 1 CARD and then activate Caspase 1 proteolytic
85 activity. In contrast, NLRP3 contains a Pyrin signaling domain instead of a CARD domain (11).
86 The Pyrin domain binds the Pyrin domain of the adaptor protein ASC which is composed of only
87 a Pyrin and a CARD domain. ASC then recruits Caspase 1 via CARD-CARD interactions and
88 finally activate Caspase 1 proteolytic activity (12). Activated Caspase 1 can recognize and
89 process the inactive precursors of interleukin 1β (IL-1β) into mature inflammatory cytokines,
90 which is then secreted into intracellular contents (12). IL-1β is an important mediator of the
91 inflammatory response of the host against pathogen infection (13). Meanwhile, pyroptosis also

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92 induces morphologic changes, DNA fragmentation and chromatin condensation to destroy
93 infected cells (7, 9).

94 During pathogen infection, the host benefits from pyroptosis. The compromised cells are
95 eliminated and more severe immune responses are launched, effectively destroying the protective
96 environment where infectious agents can thrive (14). In Caspase-1-deficient macrophages where
97 pyroptosis is compromised, higher intracellular bacterial loads are detected during *L.*
98 *pneumophila* infection (14). Pyroptosis also promotes pathogen clearance by acting as an alarm
99 signal that recruits immune cells to the site of infection (15). The secretion of IL-1β and IL-18
100 promotes leukocyte activation and immuno-stimulatory factors are released from lysed cells into
101 the extracellular milieu (15). A bunch of released cytosolic products are potent damage-
102 associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1), heat-
103 shock proteins and DNA-chromatin complexes. By the activation of pattern-recognition
104 receptors, these DAMPs accelerate proinflammatory cytokine production (16). On the other hand,
105 pathogens have evolved effector proteins capable of directly inhibiting Caspase 1 and
106 inflammasome activation, preventing cells from pyroptosis (17, 18). Virulent *Pseudomonas*
107 strains express the effector protein ExoU, which blocks Caspase 1 activation and *Y.*
108 *pseudotuberculosis* T3SS Rho-GTPase activating protein YopE inhibits inflammasome
109 activation (17, 19). The host-pathogen interaction mechanisms suggest that the activity of
110 Caspase 1 leading to pyroptosis may represent an important antiviral pathway that prevents
111 replication and spread of viruses to neighboring cells.

112 Pyroptosis is such a potent mechanism to clear intracellular pathogens. However, current
113 studies on the role of pyroptosis in host-virus interactions has not been extensively explored and
114 it is still unclear whether pyroptosis also exits and plays such an important role in defending

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115 hosts from pathogen invasion in invertebrates. In this study, we aim to find out whether
116 pyroptosis is also one of the pathways that contributes to eliminating virus in invertebrate. The
117 results revealed that the activity of Caspase 1 in shrimp was enhanced by white spot syndrome
118 virus (WSSV) infection. The silencing of *Caspase 1* or *IL-1β-like* gene facilitated virus infection
119 in shrimp. Our study indicated that Casapase-1-mediated cell death played an important antiviral
120 role in invertebrate.

121 **Results**

122 **Involvement of Caspase 1 in the virus infection to shrimp**

123 In order to investigate whether Caspase 1-mediated cell death plays a role in the immune
124 response of invertebrates to virus infection, the profiles of *Caspase 1* gene was characterized in
125 the course of shrimp immunity against WSSV infection. The sequence analysis revealed that the
126 shrimp Caspase 1 contained caspase activity domains (Fig 1A). The expression of the gene in
127 different organs was then examined by real-time PCR. The result showed that Caspase 1
128 expression was most significant in hemocytes followed by lymphoid-like-organ, however, we
129 can barely detect the mRNA of Caspase 1 in muscle and heart (Figure 1B). The expression
130 pattern of the gene was further confirmed by detection of protein levels as shown in Figure 1C.
131 These results indicated the shrimp Caspase 1 might function in immunity system--hemocytes and
132 lymphoid-like organ in shrimp, where fighting off invading pathogens usually took place, so the
133 result indicated that Caspase 1 might play a role in the antiviral cellular pathway..

134 To explore the role of Caspase 1 in virus infection to shrimp, the expression of Caspase 1
135 was detected at different time points after shrimp were infected with WSSV. The results revealed
136 that the transcriptional level of Caspase 1 was significantly upregulated with the prolonged
137 infection time (Fig 1D). Meanwhile, the protein level of the gene also showed similar expression

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138 patterns during WSSV infection (Figure 1E).Taken together, these results indicate that Caspase-
139 1-mediated cell death is involved in virus infection of shrimp.

140 **Requirement of enzymatic activity of Caspase 1 in virus infection**

141 To determine whether the enzymatic activity of Caspase 1 was associated with virus
142 infection, shrimp were challenged with increasing concentrations of WSSV and hemocytes were
143 collected for the detection of Caspase 1 activity by incubating with the substrate Ac-YVAD-pNA.
144 The results showed that Caspase 1 activity was greatly enhanced while more viruses were used
145 for infection (Fig 2A). Moreover, Caspase 1 activity increased dramatically along with the
146 prolonged infection time (Fig 2B). These results indicated that the enzymatic activity of Caspase
147 1 was actually indispensible in the pathway of virus infection.

148 To explore whether the Caspase 1 activity facilitated or inhibited virus infection, Caspase 1
149 inhibitor Belnacasan was used in combination with WSSV injection or not, and WSSV copy
150 number was then measured by quantitative real-time PCR. The data showed that the WSSV copy
151 number increased significantly upon the inhibition of Caspase 1 enzymatic activity, suggesting
152 that Caspase 1 played an antiviral role possibly conducted by its enzymatic activity.

153 **Interaction between Caspase 1 and IL-1β like protein**

154 In order to reveal the cellular pathway in which Caspase 1 participated to play an antiviral
155 role, we performed Coimmunoprecipitation (Co-IP) to identify the substrate of Caspase 1. The
156 Co-IP data showed that one specific band was discovered after Caspase 1- specific antibody but
157 not IgG was used for CoIP, which was then identified as IL-1β like protein by mass spectrometry
158 (Fig 3A). To further demonstrated the interaction between Caspase 1 and IL-1β like protein,
159 Flag-Caspase 1 and His-IL-1β like protein was respectively cloned into PIZ/V5-His plasmid and
160 co-transfected into insect High Five cells. Anti-Flag IP was performed and then proteins were

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161 analyzed by Western blotting. The result indicated that His-IL like protein was actually detected
162 (Fig 3B), showing the interaction between Caspase 1 and IL-1β like protein. Meanwhile, anti-His
163 IP was also performed and Flag-Caspase 1 could also be found by Western blotting (Fig 3C).
164 These results revealed that the two proteins could interact with each other by their expressions in
165 insect cells.

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

169 Taken together, these findings identify IL-1β like protein is the substrate of Caspase 1 and
170 the two proteins could interact with each other.

171 **Redistribution of IL-1β like protein induced by WSSV infection to activate phenoloxidase**
172 **system**

173 To investigate the functions of Caspase 1 and IL-1β like protein interaction *in vivo*, siRNA
174 respectively targeting *Caspase 1* gene and *IL-1β like protein* gene was designed. The
175 effectiveness of siRNAs was examined by quantitative real-time PCR and Western blotting.
176 From the detected mRNA and protein level, it could be concluded that the siRNAs efficiently
177 knocked down the expressions of *Caspase 1* and *IL-1 like protein* genes (Fig 4A).

178 As reported in mammary cells, IL-1β is firstly interacted with and then cleaved by Caspase 1
179 before it is released into extracellular contents (20), however, in this study, it is still elusive how
180 IL-1β like protein reacts upon virus infection. Therefore, the level of IL-1β like protein in
181 hemocytes and the corresponding plasma before and after WSSV infection was examined.
182 Hemolymph was collected and centrifuged to separate the hymocytes and plasma for the
183 detection of IL-Iβ like protein. Western blotting analysis revealed that the expression level of IL-

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184 1β like protein was increased in the plasma and decreased in hemoctes when shrimp were
185 challenged with WSSV, indicating that IL-1β like protein was released into extracellular contents
186 under virus infection (Fig 4B). In order to determine whether Caspase 1 mediated the release of
187 IL-1 like protein, the expression of Casapse 1 was firstly knocked down by siRNA and then the
188 expression pattern of IL-1β like protein was examined. The results showed that the level of IL-1β
189 like protein in the plasma decreased while the level in the hemocytes increased upon infection
190 with WSSV (Fig 4C). These data showed that the release of IL-1β like protein into plasma was
191 controlled by Caspase 1 in shrimp.

192 To further determine whether IL-1β like protein was required to be cleaved by Caspase 1
193 before releasing into extracellular contents, the enzymatic activity of Caspase 1 was inhibited by
194 Belnacasan and then the level of IL-1β like protein was examined. The result showed that the
195 level of IL-1β like protein didn't increase in the plasma, suggesting that the enzymatic activity of
196 Caspase 1 was required for the release of IL-1β like protein and that IL-1β like protein needed
197 cleavage by Caspase 1 before releasing into extracellular contents (Fig 4C).

198 Prophenoloxidase (PO) activating system is an important identification and defense system
199 for eliminating viruses in crustaceans (21). To find out whether Caspase 1 mediated cell death
200 could influence PO system, Caspase 1 and IL-1β like protein were respectively knocked down
201 and then the PO activity was measured with or without WSSV challenge. As expected, WSSV
202 challenge greatly induced the PO activity (Fig 4D). However, either Caspase 1 knockdown or IL-
203 1β like protein knockdown partially inhibited the PO activation (Fig 4D), suggesting that
204 Caspase 1 mediated cell death could enhance PO activity upon WSSV infection.

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205 Taken the above data together, these findings demonstrate that the releasing of IL-1β like
206 protein is dependent on the enzymatic activity of Caspase 1, resulting in the activation of PO
207 system and cell damage which prevent shrimp from virus infection.

208 **Negative correlation of Caspase 1 and IL-1β like protein expressions with virus infection**

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

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

217 **Discussion**

218 The outbreak of viral diseases is a major concern preventing the development of shrimp
219 aquaculture industry. WSSV is one of the most virulent shrimp viruses and the processes of
220 pathogenesis are extremely complex. Due to the lack of effective therapeutics to control virus, it
221 is of great importance to understand viral pathogenesis and host responses at the molecular level
222 in order to prevent virus invasion. So far, some studies have demonstrated that many genes and
223 pathways are involved in host defense against WSSV, including Ran protein, the Ras-activated
224 endocytosis process, the RNA interference pathway and apoptosis (22-25). Apoptosis is maybe
225 the best described form of cell death that is mediated by the activation of the apoptotic caspase
226 enzymes Caspase 3/7 and the antiviral role of apoptosis has been discovered both in invertebrates
227 and vertebrates. Currently, pyroptosis, which is mediated by Caspase 1 activation, is thought to

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228 have the capacity of eliminating viruses only in vertebrates (26). In vertebrates, pyroptosis
229 activation can result in not only the production of activated inflammatory cytokines, but also
230 rapid cell death characterized by plasma-membrane rupture and release of proinflammatory
231 intracellular contents(26). On the other hand, viruses including HIV have evolved mechanisms to
232 inhibit pyroptosis, enhancing their ability to persist and cause disease (27). Ultimately, it is the
233 competition between host and pathogen to regulate pyroptosis, and the outcome dictates life or
234 death to the host or to viruses. Based on the critical role of Caspase 1 in the defense of a wide
235 range of virus infections in vertebrates, in this study, Caspase 1 gene was first cloned from
236 shrimp and the activity of Caspase 1 was then characterized. Our data showed that Caspase 1
237 activity was upregulated upon WSSV infection and the knockdown of Caspase 1 facilitated virus
238 infection. Compared with the function of pyroptosis in vertebrates, our study revealed that
239 Caspase 1-mediated cell death also contributed to eliminating virus in crustacean. Our findings
240 indicated that the antiviral function of Caspase 1 was conservative although the sophisticated
241 immune responses of shrimp have not been developed yet.

242 In vertebrates, the activated Caspase 1 recognizes and cleaves IL-1β into the mature form.
243 The release of mature IL-1β further enhances the inflammatory response by stimulating immune
244 cell activation and more cytokine secretion (28), leading to critical physiological consequences
245 such as fever and hypotension. However, the cleavage and release of IL-1β is not indispensible
246 for caspase 1-mediated cell death as demonstrated by IL-1β-/- knockout animals (29). In our
247 study, the substrate of Caspase 1, which was named IL-1β like protein, was firstly identified. The
248 results showed that WSSV infection induced the redistribution of IL-1β like protein into plasma
249 of shrimp, indicating that IL-1β like protein was released into extracellular contents upon virus
250 infection. The released IL-1β like protein, instead of triggering inflammation responses, activated

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251 phenoloxidase system of shrimp, an important invertebrate defense mechanism against virus
252 infection. These data demonstrated that the function IL-1β like protein participating in Caspase
253 1- mediated cell death in shrimp was similar with that in vertebrates, and that IL-1β like protein
254 functioned as cytokine-like molecules in invertebrates. Although invertebrates including shrimp
255 lack adaptive immune system, several putative functional analogues of inflammatory cytokines
256 have been identified so far. For example, tumor necrosis factor (TNF)-like molecules and
257 interleukin-1 (IL-1)-like molecules are detected in a variety of invertebrates, such as insects,
258 echinoderms and protochordates (30, 31). Moreover, chemokine IL-8 and transforming growth
259 factor β1 are vertebrate cytokines involved in inflammatory processes and molecules cross-
260 reacting with the two proteins using antibodies have been identified in mollusks (32). In addition,
261 IL-2-like activity is detected in protochordates and echinoderms which have hematopoietic
262 organs and T-like cells (33). In this study, our data suggested that an invertebrate cytokine
263 network might be operative and regulate host defense mechanisms against virus infection as in
264 vertebrates.

265 **Material and methods**

266 **Shrimp culture and WSSV challenge**

267 *Marsupenaeus japonicas* shrimp was cultured in groups of 20 individuals in tanks containing
268 aerated seawater at room temperature. The individual shrimp was about 10g in weight and 10 to
269 12 cm in length. To ensure the absence of WSSV in shrimp prior to experimental infection, PCR
270 was performed using WSSV specific primers (5'-TATTGTCTCTCCTGACGTAC-3' and 5'-CA
271 CATTCTTCACGAGTCTAC-3'). Virus-free shrimp were infected with WSSV (10^5 copies/ml)
272 by injection into the lateral area of the fourth abdominal segment. Three shrimp were randomly

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273 collected for each treatment. At different time post-infection, the shrimp hemolymph was
274 collected for later use.

275 **Rapid amplification of cDNA ends (RACEs)**

276 Total RNAs were extracted from shrimp hemocytes using mirVana miRNA™ Isolation Kit
277 (Ambion, USA). RACE experiments were conducted using 5'-3' RACE Kit (Roche, USA)
278 according to the manufacturer's protocol. Briefly, cDNA was synthesized and applied for PCR
279 and nest-PCR. PCR was performed using 3' RACE primer, 5' RACE primer or/and gene-
280 specific primers. The amplified DNAs were sequenced.

281 **Quantitative real-time PCR for detections of virus copies and gene expression**

282 Quantitative real-time PCR was performed to quantify WSSV copies and gene expression
283 level in shrimp. DNA or RNA was extracted from shrimp hemocytes using SQ tissue DNA kit
284 (Omega-BioTek, USA) or RNA Isolation kit (Ambion, USA). RNA was reversely transcribed
285 into cDNA by cDNA synthesis kit (Takara, Japan).

286 The WSSV copies were evaluated with WSSV-specific primers (5'-TTGGTTTCAG
287 CCCGAGATT-3' and 5'-CCTTGGTCAGCCCCTTGA-3'). The gene expression level was
288 determined using gene-specific primers (Caspase 1, 5'-TTTCAAGGCTGACCAGT GCCCTAC-
289 3' and 5'-ACCTGCGTCAAGACCCTCACCTC-3'; IL-1β, 5'- GCGTTCC
290 ACATTTCAGATACTCGG-3' and 5'-TGTAGACCATTCCAAAGGCTCCC-3'). The 25μL
291 PCR solution contained 12.5μL of 2×Premix Ex Taq (TaKaRa, Japan), 0.5μL of 10μM forward
292 primer, 0.5μL of 10μM reverse primer and 1μM DNA template. The stages of PCR program
293 were 95°C for 5 min, followed by the amplification stage consisting of 40 cycles of 95°C for 10 s
294 and 60°C for 30 s.

295 **Western blot analysis**

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296 Proteins were separated by a 12% SDS-polyacrylamide gel, which were then transferred onto
297 a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membrane was blocked
298 with 5% nonfat milk in phosphate buffered solution (PBS) for 2 h at room temperature. The blot
299 was incubated with a primary antibody at 4°C overnight. After washes with PBS, the blot was
300 incubated with the fluorescence-labeled anti-mouse IgG (Cell Signaling Technology, USA) for 2
301 h at 4°C. The blot was detected by Odyssey (Licor, USA) for signals.

302 **Caspase 1 activity detection**

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

310 **Cell culture and plasmids transfection**

311 Insect High Five cells (Invitrogen, USA) were cultured and maintained at 27°C in Express
312 Five serum-free medium (Invitrogen) containing L-glutamine (Invitrogen). Cells were co-
313 transfected with plasmids expressing Flag-Caspase 1 and His-IL-1β like protein according to the
314 manufacture's instruction. Briefly, when the cells reached about 70% confluence in a 6-well
315 plate, 2μg for each indicated plasmid and 6μl Cellfectin Reagent were diluted in 100 μl Grace's
316 medium, respectively. The diluted plasmids and Cellfectin Reagent were then mixed gently and
317 incubated for 30 minutes at room temperature. The mixture was added to the cells and incubated

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318 in a 27°C incubator for 5 h before the medium was replaced by fresh Express Five serum-free
319 medium. Cells were finally subjected to further research 48 h after transfection.

320 **Co-immunoprecipitation**

321 Cells were collected and lyzed in ice-cold lysis buffer (50mM Tris-HCl, 150mM NaCl, 0.2%
322 NP40, 1mM PMSF, 1×protease inhibitor, pH 8.0). The lysate was then centrifuged three times
323 for 5 min each to remove all the undissolved contents. The supernatant was incubated with
324 indicated antibody overnight at 4°C. Protein A+G agarose beads (Invitrogen, USA) were
325 incubated with the lysate for 2 h at 4°C. After washed three times with ice-cold wash buffer
326 (50mM Tris-HCl, 200mM NaCl, 0.1% NP40, 1mM PMSF, 1×protease inhibitor, pH 8.0), the
327 immuno-complexes were eluted by 100 mM glycine and subjected to Western blotting for the
328 detection of indicated proteins.

329 **Synthesis of siRNAs and RNAi assay**

330 Based on the sequences of *Caspase 1* and *IL-1β like protein* genes, siRNAs were designed to
331 specifically target the genes and then were synthesized in vitro using a commercial kit according
332 to the manufacturer's instructions (TaKaRa, Japan). The siRNAs used were Caspase 1-siRNA
333 (5'-AAACCACUCGGAAUGCGAUGCG-3') and IL-1β-like-protein-siRNA (5'-
334 GGGAAGGCTTTGAGGTCCTTGT-3'). The scrambled siRNA not targeting any genes was
335 used as the control. The formation of double-stranded RNAs was monitored by determining the
336 size in agarose gel electrophoresis. The synthesized siRNAs were dissolved in siRNA buffer (50
337 mM Tris-HCl, 100 mM NaCl, pH 7.5) and quantified by Nanodrop 2000. The RNA interference
338 (RNAi) assay was conducted in shrimp by the injection of a siRNA into the lateral area of the
339 fourth abdominal segment at 30 μg/shrimp using a syringe with a 29-gauge needle. The siRNA
340 (15μg) and WSSV (10^5 copies/ml) were co-injected into virus-free shrimp at a volume of 100μL

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341 per shrimp. At 12 h after the co-injection, the siRNA (15μg) (100μL /shrimp) was injected into
342 the same shrimp. For each treatment, 20 shrimp were used. At different times after the last
343 injection, the shrimp hemocytes were collected. Three shrimp specimens from each treatment,
344 randomly selected, were collected for analysis. The assays were biologically repeated for three
345 times.

346 **Phenoloxidase activity detection**

347 Hemolymph was centrifuged and the plasma was used for the detection of phenoloxidase
348 activity. The reaction buffer (0.1M K3PO4, 0.01M L-dopa, pH 6.0) and plasma was mixed and
349 then incubated at 28°C for 2 min. The activity of phenoloxidase was measured by a
350 spectrophotometer at OD492.

351 **Shrimp mortality analysis**

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

354 **Statistical analysis**

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

357 **Acknowledgements**

358

359 **References**

360 1. Fink SL & Cookson BT (2005) Apoptosis, pyroptosis, and necrosis: mechanistic
361 description of dead and dying eukaryotic cells. *Infection and immunity* 73(4):1907-1916.
362 2. Jorgensen I, Rayamajhi M, & Miao EA (2017) Programmed cell death as a defence
363 against infection. *Nature reviews. Immunology* 17(3):151-164.

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364 3. Albert ML (2004) Death-defying immunity: do apoptotic cells influence antigen
365 processing and presentation? *Nature reviews. Immunology* 4(3):223-231.
366 4. Yuan J, Najafov A, & Py BF (2016) Roles of Caspases in Necrotic Cell Death. *Cell*
367 167(7):1693-1704.
368 5. Gong Y*, et al.* (2015) The role of miR-100 in regulating apoptosis of breast cancer cells.
369 *Scientific reports* 5:11650.
370 6. Yang G, Gong Y, Wang Q, Wang L, & Zhang X (2017) miR-100 antagonism triggers
371 apoptosis by inhibiting ubiquitination-mediated p53 degradation. *Oncogene* 36(8):1023-
372 1037.
373 7. Doitsh G*, et al.* (2014) Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1
374 infection. *Nature* 505(7484):509-514.
375 8. Zhou X*, et al.* (2000) Nitric oxide induces thymocyte apoptosis via a caspase-1-
376 dependent mechanism. *Journal of immunology* 165(3):1252-1258.
377 9. Yazdi AS*, et al.* (2010) Nanoparticles activate the NLR pyrin domain containing 3
378 (Nlrp3) inflammasome and cause pulmonary inflammation through release of IL-1alpha
379 and IL-1beta. *Proceedings of the National Academy of Sciences of the United States of*
380 *America* 107(45):19449-19454.
381 10. Broz P*, et al.* (2010) Redundant roles for inflammasome receptors NLRP3 and NLRC4 in
382 host defense against Salmonella. *The Journal of experimental medicine* 207(8):1745-
383 1755.
384 11. Bao Q & Shi Y (2007) Apoptosome: a platform for the activation of initiator caspases.
385 *Cell death and differentiation* 14(1):56-65.

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386 12. Srinivasula SM, *et al.* (2002) The PYRIN-CARD protein ASC is an activating adaptor
387 for caspase-1. *The Journal of biological chemistry* 277(24):21119-21122.
388 13. Broz P, von Moltke J, Jones JW, Vance RE, & Monack DM (2010) Differential
389 requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine
390 processing. *Cell host & microbe* 8(6):471-483.
391 14. Case CL, Shin S, & Roy CR (2009) Asc and Ipaf Inflammasomes direct distinct
392 pathways for caspase-1 activation in response to Legionella pneumophila. *Infection and*
393 *immunity* 77(5):1981-1991.
394 15. Miao EA, *et al.* (2006) Cytoplasmic flagellin activates caspase-1 and secretion of
395 interleukin 1beta via Ipaf. *Nature immunology* 7(6):569-575.
396 16. Fernandes-Alnemri T, *et al.* (2007) The pyroptosome: a supramolecular assembly of ASC
397 dimers mediating inflammatory cell death via caspase-1 activation. *Cell death and*
398 *differentiation* 14(9):1590-1604.
399 17. Sutterwala FS, *et al.* (2007) Immune recognition of Pseudomonas aeruginosa mediated
400 by the IPAF/NLRC4 inflammasome. *The Journal of experimental medicine*
401 204(13):3235-3245.
402 18. von Moltke J, Ayres JS, Kofoed EM, Chavarria-Smith J, & Vance RE (2013)
403 Recognition of bacteria by inflammasomes. *Annual review of immunology* 31:73-106.
404 19. Master SS, *et al.* (2008) Mycobacterium tuberculosis prevents inflammasome activation.
405 *Cell host & microbe* 3(4):224-232.
406 20. Strowig T, Henao-Mejia J, Elinav E, & Flavell R (2012) Inflammasomes in health and
407 disease. *Nature* 481(7381):278-286.

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408 21. Sutthangkul J*, et al.* (2015) Suppression of shrimp melanization during white spot
409 syndrome virus infection. *The Journal of biological chemistry* 290(10):6470-6481.
410 22. Liu W, Han F, & Zhang X (2009) Ran GTPase regulates hemocytic phagocytosis of
411 shrimp by interaction with myosin. *Journal of proteome research* 8(3):1198-1206.
412 23. Han F & Zhang X (2007) Characterization of a ras-related nuclear protein (Ran protein)
413 up-regulated in shrimp antiviral immunity. *Fish & shellfish immunology* 23(5):937-944.
414 24. Xu J, Han F, & Zhang X (2007) Silencing shrimp white spot syndrome virus (WSSV)
415 genes by siRNA. *Antiviral research* 73(2):126-131.
416 25. Wang L, Zhi B, Wu W, & Zhang X (2008) Requirement for shrimp caspase in apoptosis
417 against virus infection. *Developmental and comparative immunology* 32(6):706-715.
418 26. Schroder K & Tschopp J (2010) The inflammasomes. *Cell* 140(6):821-832.
419 27. Deeks SG (2011) HIV infection, inflammation, immunosenescence, and aging. *Annual*
420 *review of medicine* 62:141-155.
421 28. Hilbi H, Chen Y, Thirumalai K, & Zychlinsky A (1997) The interleukin 1beta-converting
422 enzyme, caspase 1, is activated during Shigella flexneri-induced apoptosis in human
423 monocyte-derived macrophages. *Infection and immunity* 65(12):5165-5170.
424 29. Fantuzzi G*, et al.* (1996) Effect of endotoxin in IL-1 beta-deficient mice. *Journal of*
425 *immunology* 157(1):291-296.
426 30. Caselgrandi E, Kletsas D, & Ottaviani E (2000) Neutral endopeptidase-24.11 (NEP)
427 deactivates PDGF- and TGF-beta-induced cell shape changes in invertebrate
428 immunocytes. *Cell biology international* 24(2):85-90.

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429 31. Wittwer D, Franchini A, Ottaviani E, & Wiesner A (1999) Presence of IL-1- and TNF-
430 like molecules in Galleria mellonella (Lepidoptera) haemocytes and in an insect cell line
431 Fromestigmene acraea (Lepidoptera). *Cytokine* 11(9):637-642.

432 32. Ottaviani E, Franchini A, Malagoli D, & Genedani S (2000) Immunomodulation by
433 recombinant human interleukin-8 and its signal transduction pathways in invertebrate
434 hemocytes. *Cellular and molecular life sciences : CMLS* 57(3):506-513.

435 33. Aliprantis AO, Yang RB, Weiss DS, Godowski P, & Zychlinsky A (2000) The apoptotic
436 signaling pathway activated by Toll-like receptor-2. *The EMBO journal* 19(13):3325-
437 3336.

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452 # Figure legends

453 **Fig 1. Involvement of Caspase 1 in the virus infection to shrimp.** (A) Sequence analysis of
454 shrimp Caspase 1. *Casapse 1* gene was cloned from shrimp hemocytes. Shrimp Caspase 1
455 contained three domains including active site, proteolytic cleavage site and dimer interface. (B)
456 Detection of Caspase 1 mRNA in different tissues of shrimp. The Caspase 1 mRNA was
457 examined by quantitative real-time PCR. Shrimp β-actin was used as an internal control. (C)
458 Examination of Caspase 1 protein in different shrimp tissues. The Caspase 1 protein was
459 detected by Western blot. Shrimp β-actin was used as an internal control. (D) Expression level of
460 Caspase 1 mRNA in shrimp challenged with virus. Shrimp were challenged with WSSV. At
461 different time post-infection, the Caspase 1 mRNA of shrimp hemocytes were analyzed by
462 quantitative real-time PCR (*, p<0.01). (E) Determination of Caspase 1 protein level in WSSV-
463 infected shrimp. Proteins were extracted from virus-infected shrimp hemocytes and analyzed by
464 Western blot. The numbers indicated the time post-infection. Shrimp β-actin was used as an
465 internal control.

466 **Fig 2. Requirement of enzymatic activity of Caspase 1 in virus infection.** (A) Influence of
467 WSSV concentration on Caspase 1 activity. Caspase 1 activity was measured at OD_{450} after
468 shrimp were challenged with increasing WSSV concentration. Caspase 1 activity was detected
469 at 48 h post-infection. (B) Effects of virus infection on Caspase 1 activity. Caspase 1 activity was
470 measured at different time points after shrimp were challenged with WSSV. (C) Impact of
471 inhibition of Caspase 1 activity on virus infection. WSSV copy number was measured by
472 quantitative real-time PCR at 48 h after shrimp were treated with Caspase 1 inhibitor with or
473 without WSSV infection.

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

486 **Fig 4. Redistribution of IL-1β like protein induced by WSSV infection to activate**
487 **phenoloxidase system.** (A) Detection of Caspase 1 and IL-1β like protein knockdown efficiency.
488 The mRNA and protein levels of Caspase 1 and IL-1β like protein were respectively detected by
489 quantitative real-time PCR (left) and Western blot (right) at 48 h after shrimp were injected with
490 indicated siRNAs. (B) Detection of IL-1β like protein expression in hemocytes or plasma after
491 WSSV infection. The expression level of IL-1β like protein in hemocytes or plasma was
492 examined by Western blot with or without WSSV infection. Samples were collected at 48 h after
493 WSSV infection. (C) Evaluation of IL-1β like protein expression in hemocytes or plasma after
494 WSSV infection with or without Caspase 1 inhibition. The expression level of IL-1β like protein
495 in hemocytes or plasma was detected by Western blot after WSSV-infected shrimp were treated
496 with Caspase 1 siRNA or inhibitor. Caspase 1 inhibitor or siRNA was immediately injected into

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497 shrimp after WSSV infection. Samples were collected 48 h after injection. (D) Examination of
498 phenoloxidase activity upon knockdown of Caspase 1 or IL-1β like protein in
499 shrimp. Phenoloxidase activity was measured at OD_{492} after shrimp were treated with either
500 Caspase-1-siRNA or IL-1β-like-protein-siRNA at 48 h after WSSV injection.

501 **Fig 5. Negative correlation of Caspase 1 and IL-1β like protein expressions with virus**
502 **infection.** (A) Detection of WSSV copies after knockdown of Caspase 1 or IL-1β like protein
503 genes. WSSV copy number in Caspase-1-siRNA or IL-1β-like-protein-siRNA treated shrimp
504 was measured by quantitative real-time PCR. (B) Monitoring of shrimp cumulative mortality
505 upon knockdown of Caspase 1 or IL-1β like protein genes. (C) A model for the Caspase 1-
506 mediated antiviral pathway in shrimp.

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520 **Fig 1**

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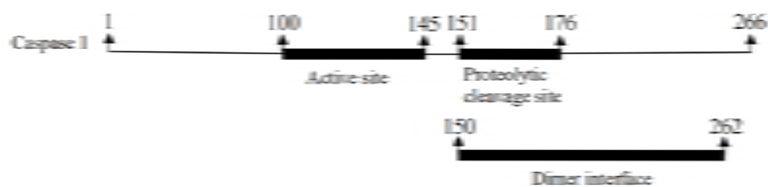

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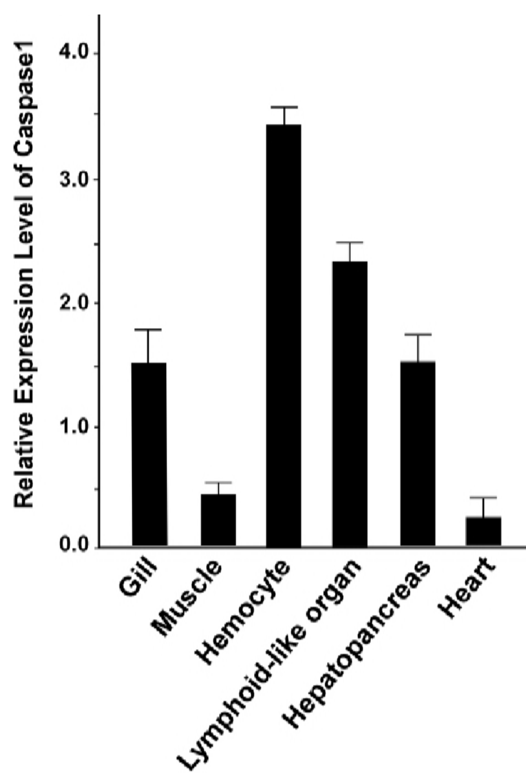

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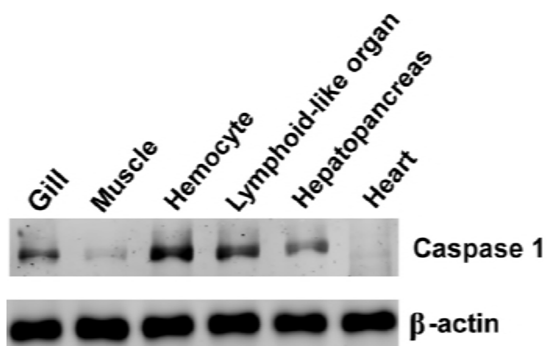

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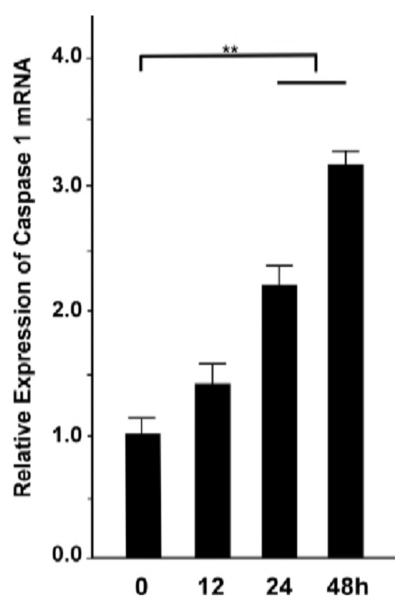

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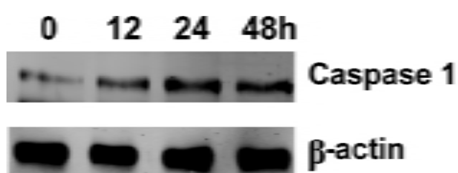

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541 **Fig 2**

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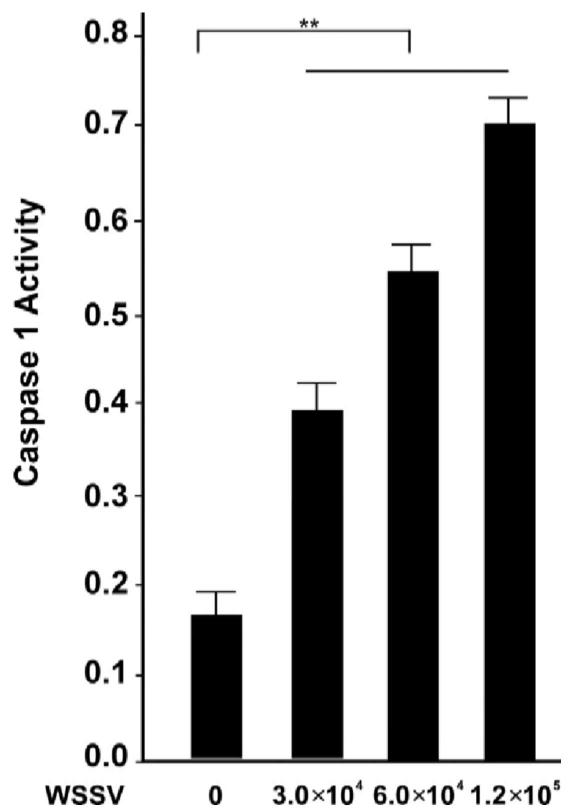

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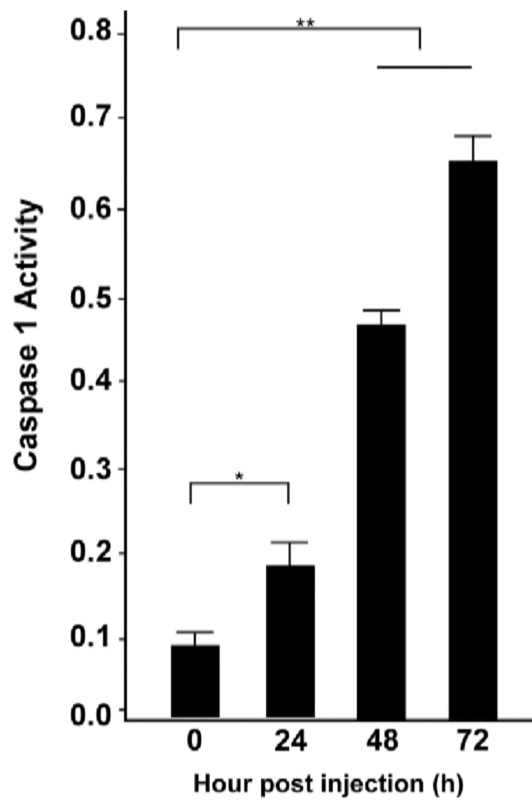

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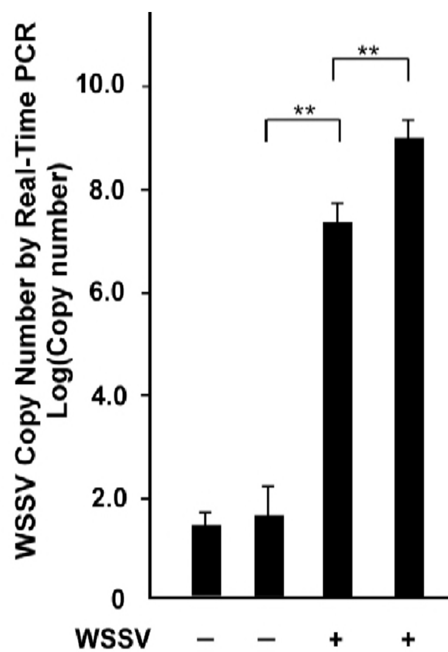

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558 **Fig 3**

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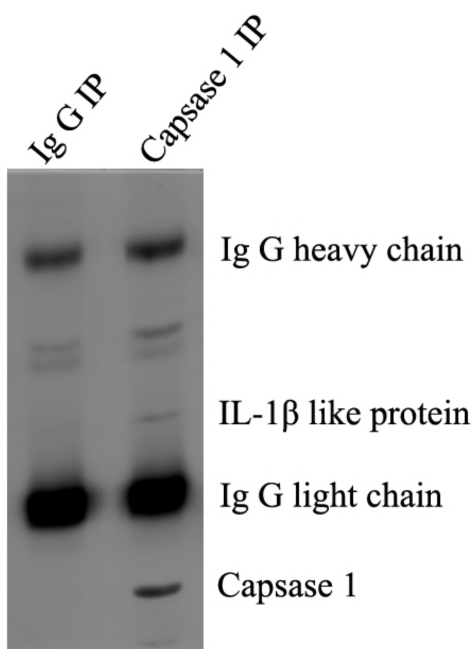

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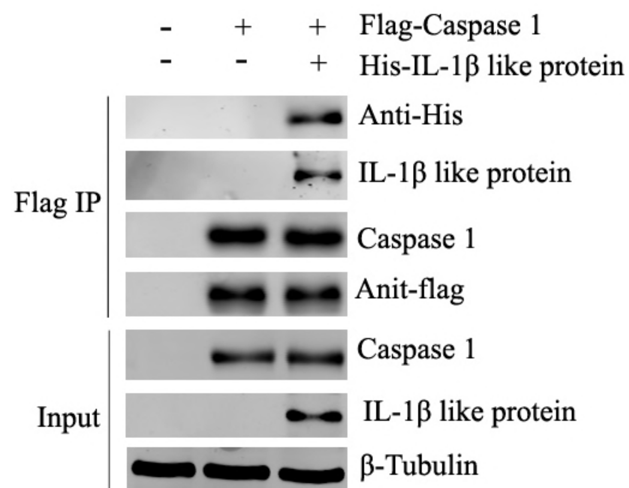

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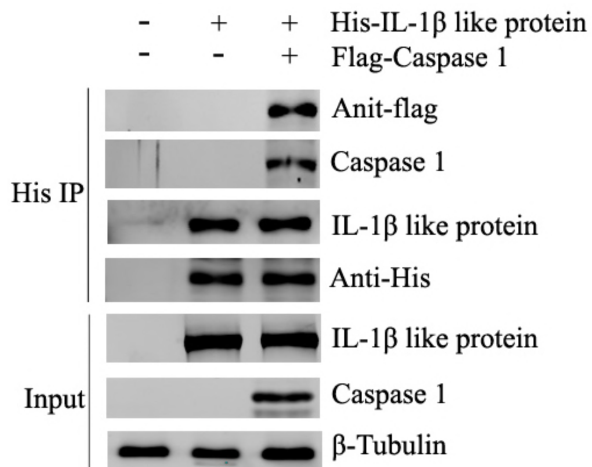

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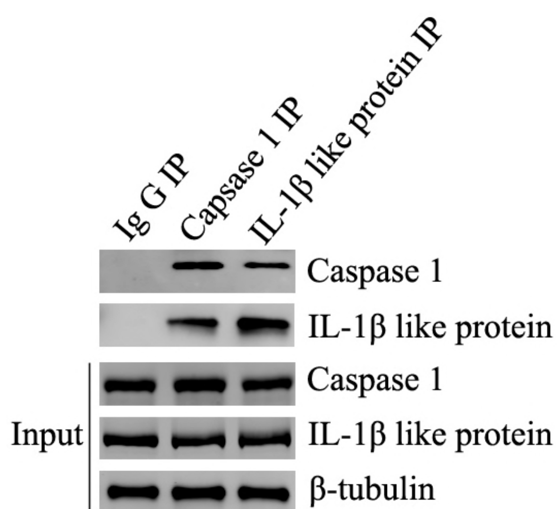

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578 **Fig 4**

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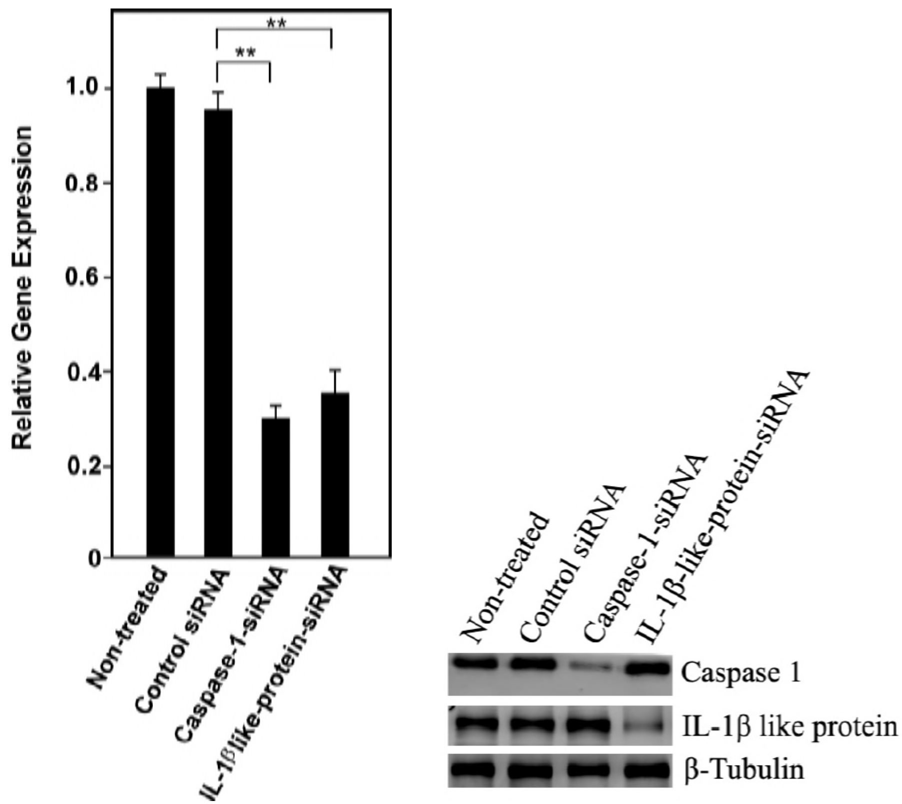

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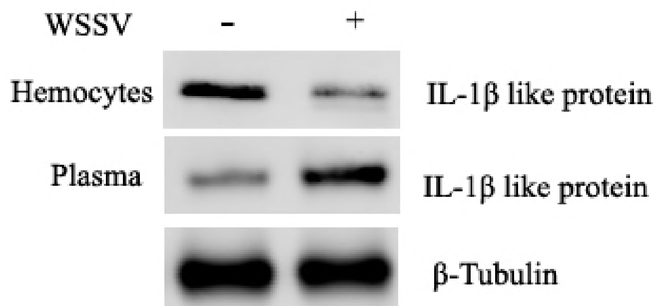

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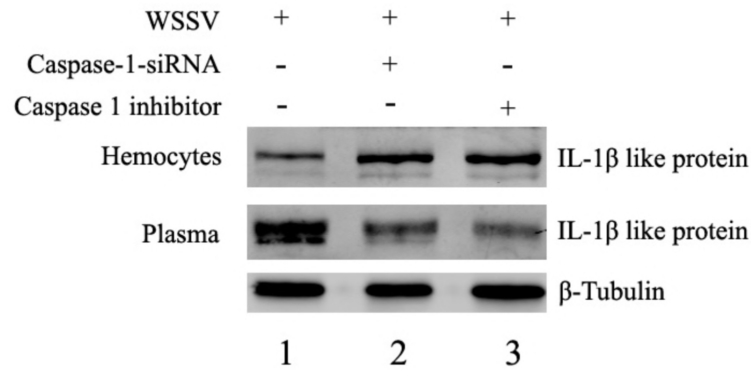

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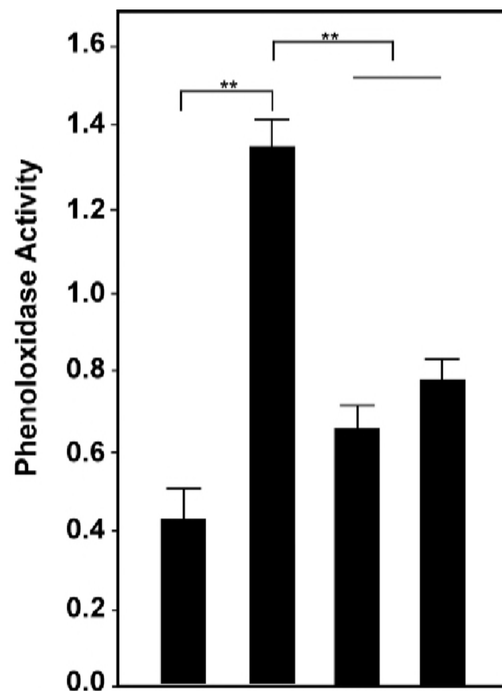

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595 **Fig 5**

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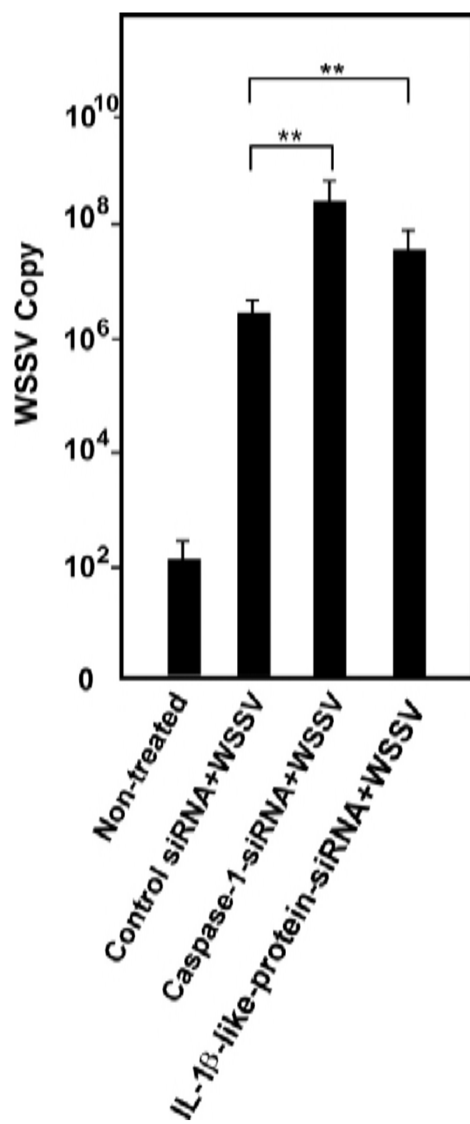

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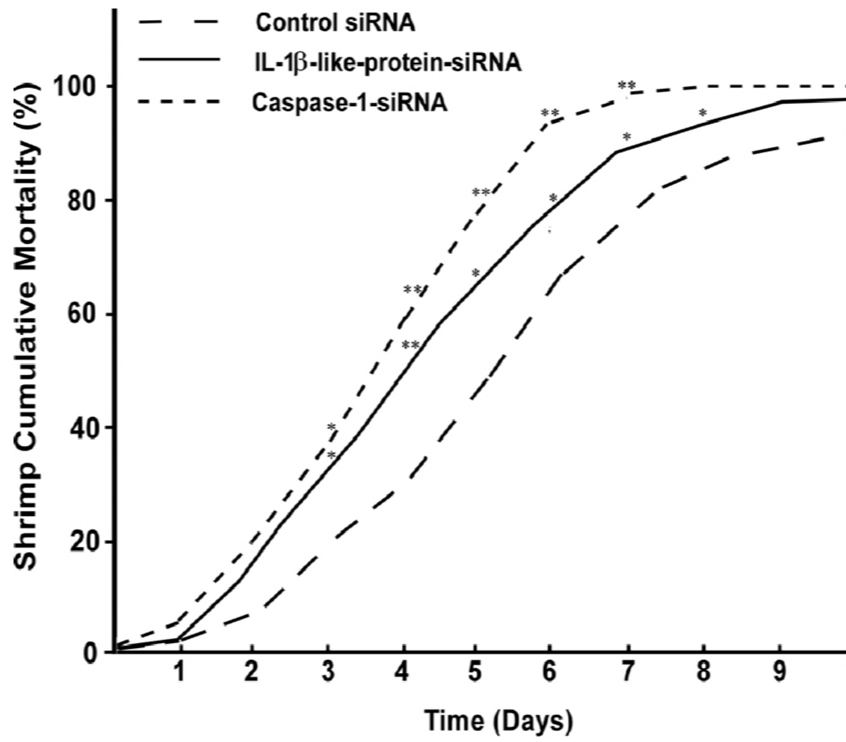

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