NLRP3-like protein negatively regulates the expression of antimicrobial peptides in *Penaeus vannamei* hemocytes

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**ABSTRACT**

Keywords: NLRP3-like, Penaeus vannamei, Regulate, Antimicrobial peptides

NACHT, LRR and PYD domains-containing protein 3 (NLRP3) protein is the core actor involved in inflammasome formation, and plays a pivotal role in the innate immune response. However, whether NLRP3 participates in the regulation of innate immunity in invertebrates is still unknown. In the present study, we characterized a NLRP3 ortholog in *Penaeus vannamei* (designated PvNLRP3-like) with 2514 bp length of open reading frame (ORF) encoding a putative protein of 837 amino acids. Sequence analysis revealed that PvNLRP3-like contained only NACHT domain and shared closely homology with other invertebrates, but the topological structure of NACHT domain of PvNLRP3-like is similar with that in human NLRP3. PvNLRP3-like was ubiquitously expressed in tissues and induced in hemocytes by *Vibrio parahaemolyticus*, *Streptococcus iniae* and White Spot Syndrome Virus (WSSV) challenge, suggesting that PvNLRP3-like participated in the immune responses to pathogens. Furthermore, silencing of PvNLRP3-like followed by *V. parahaemolyticus* stimulation negatively regulated the transcripts of antimicrobial peptides (AMPs) including Lysozyme (LYZ) 3, Crustin (CRU) 2, Anti-lipopolysaccharide factor (ALF) 2/3 and Penaeidins (PEN) 3/4. This study enriches our current knowledge on shrimp innate immunity, and provides novel perspective to understand the immune regulation role of PvNLRP3-like.

1. Introduction

Inflammation underlined a serious of physiological and pathological processes in mammals, which trigger immune system defenses against internal or external threats [1,2]. Pathogen induced inflammatory response relies on pattern-recognition receptors (PRRs) to recognize pathogenic microbes, these germline-encoded receptors include Toll-like receptors (TLRs), RIG-I-like RNA helicases (RLHs), nucleotide-binding domain leucine-rich repeat repeat containing receptors (NLRs) and C-type lectin receptors (CLRs), which are specific for evolutionary conserved molecules related with cellular damage (danger associated molecular patterns; DAMPs) or invasive organisms (pathogen associated molecular patterns; PAMPs) [3]. Inflammasome is a newly identified PRR, which included NACHT, LRR, and PYD domains-containing proteins (NLRP) 1-3, NLR family CARD domain-containing protein 4 (NLRC4) and double-stranded DNA (dsDNA) sensors absent in melanoma 2 (AIM2) [4].

NLRP3 inflammasome is well characterized in mammals, the oligomerization of activated NLRP3 participated in the formation of inflammasome by recruiting apoptosis-associated speck-like protein (ASC) and caspase 1[5], which play a critical role in the release of inflammatory cytokines like interleukin 1 beta (IL-1β) and IL-18, and the induction of pyroptosis [6]. NLRP3 was mainly expressed in the cytosol of several immunocytes like granulocytes, monocytes, dendritic cells, T and B cells [7], which was induced by TLR stimulation in a NF-xB dependent manner [8], suggesting its important role in the primary defense against microbial threats. Increasing evidences supports that NLRP3 not merely participate in inflammasome formation, but also have a regulatory role by their modulation of innate and adaptive immunity, apoptosis, differentiation, and the gut microbiota [9]. For instance, NLRP3 deficiency caused abdominal polymicrobial sepsis in mice and failed to promote bacterial clearance in disseminated bacterial infection [10]. Similarly,
inhibition of the NLRP3 in human enterocytes blocked the activation of AMPs and viral killing [11].

Although invertebrate present a variety of immune or stress responses for external stimulation, and descriptions of inflammation by morphological analysis in some marine invertebrates have been mentioned [12,13]. Molecular pattern involved in inflammation activation and the related elements are not explicit. P. vannamei, as an economic aquatic arthropod, played a special role in evolution of immunology, and gained extensive attention by immunologists. Understanding inflammatory response-related elements can clarify the inflammatory reaction in invertebrate, which is a critical step for disease control in aquaculture. In the current study, a full-length of NLRP3 ORF from P. vannamei (PvNLRP3-like) was cloned and its spatial and time-course expression patterns was investigated to interpret its possible role in response to V. parahaemolyticus challenge. Moreover, the regulatory role of PvNLRP3-like in AMPs responses was also explored by RNA interference. This study provides new insight for understanding inflammatory responses in invertebrates.

2. Materials and methods

2.1. Animal, tissue collection and pathogen challenge

Healthy penaeid shrimp (6–8 g) obtained from a local shrimp farm in Shantou, Guangdong province, China, were maintained at 25 °C in recirculating aquaculture system contained aerated seawater (with 10 % salinity) and fed with nutrition-balanced commercial diet twice daily. After three days of acclimatization under these standard condition, hemolymph was collected through abdominal sinus of healthy shrimps with a sterile needle and syringe into an equal volume of precooled anticoagulant buffer (22.80 g/L glucose, 7.95 g/L sodium citrate, 19.65 g/L NaCl and 3.35 g/L EDTA-Na2, pH 7.0), and hemocytes were collected by centrifuging hemolymph at 800 ×g for 5 min at 4 °C. Other tissues like hepatopancreas, heart, gill, intestine, stomach, eyestalk and nerve were dissected on ice with sterile scissors and grinded in liquid nitrogen. All animal experiments complied with the guide and approval of the Animal Research and Ethics Committees of Shantou University, China. Totally 200 healthy shrimps were randomly selected for pathogen challenge experiment, every 50 shrimps of each group were intramuscularly injected with 100 μL Vibrio parahaemolyticus (1 × 10^8 CFU/mL), Streptococcus iniae (1 × 10^8 CFU/mL), or White Spot Syndrome Virus (WSSV) (1 × 10^9 copies/mL) in 0.65 % sterile normal saline, or an equal volume of normal saline as control. Hemocytes samples of five individuals in each group were harvested at 0, 6, 12, 24, 36, 48 and 72 h post infection. Subsequently, total RNA was extracted for Real-time quantitative polymerase chain reaction (qPCR) to monitor the transcriptional level of PvNLRP3-like during pathogen stimulation.

2.2. Total RNA extraction and cDNA synthesis

Total RNA from different shrimp tissues or pathogen stimulated hemocytes were extracted using the RNAFAST 200 kit (FeiJie, Shanghai, China). First-strand cDNA synthesis was carried out with 1 μg total RNA through EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China).

2.3. Sequence and bioinformatics analysis

The full length cDNA sequence encoded putative PvNLRP3-like was retrieved from our previous published transcriptome data [14]. The open reading frame (ORF) of PvNLRP3-like was predicted using ORF finder (http://www.ncbi.nlm.nih.gov/orf /orf.html). Gene specific primers (designed by Primer premier 5 software) targeting to the ORF of PvNLRP3-like (Table 1) were used for gene amplification by PCR. After sequencing by Beijing Genomics Institute (BGI, Beijing, China), EXPASY (Expert Protein Analysis System, http://www.expasy.org) was used to translate PvNLRP3-like nucleotide sequence into amino acid sequence, then simple modular architecture research tool (SMART) (http://smart. embl-heidelberg.de) was employed to annotate the functional domains of PvNLRP3-like. The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) was utilized to find regions of similarity between the putative PvNLRP3-like nucleotide sequence with the NLRP3 sequences of other species. Multiple protein sequence alignment was performed using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/), while the phylogenetic tree (neighbor-joining) was constructed based on the full-length amino acid sequences of NLRP3 orthologous sequences of P. vannamei and other species by using the MEGA 7.0 software (boot-strapped for 1000 times). Three-dimensional (3D) protein structures of PvNLRP3 was modeled by SWISS-MODEL (http://swissmodel.expasy.org) and visualized using molecular visualization software PyMOL.

| Table 1 | Primers sequence used in this study. |
|---------|-------------------------------------|
| **Primers for PCR** | **Sequence (5′-3′)** |
| PvNLRP3-αF | ATGAACTCGTCCACGACCA |
| PvNLRP3-αR | TCTGGCTGCTGCTGCTGCT |
| T7F | GGATCCGCTGCTGCTGCTGCT |
| T7R | GGATCCGCTGCTGCTGCTGCT |
| **Primers for dsRNA** | **Sequence** |
| dsPvNLRP3-αT7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPvNLRP3-αT7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsEGFP-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsEGFP-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN4-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN4-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN3-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN3-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN2-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN2-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsCRU2-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsCRU2-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsLYZ4-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsLYZ4-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsLYZ3-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsLYZ3-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsLYZ2-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsLYZ2-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsLYZ1-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsLYZ1-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsALF3-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsALF3-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN4-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN4-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN3-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN3-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN2-T7R | GGATCCTAATGAGACTCCTAATGAGACT |
| dsPEN2-T7F | GGATCCTAATGAGACTCCTAATGAGACT |
Fig. 1. Sequence and structural features of PvNLRP3-like. (A) The open reading frame (ORF) sequence of *P. vannamei* NLRP3 (Genbank accession number: GETZ01048109.1) with deduced amino acid sequence shown with one-letter codon. Nucleotides and amino acids are numbered on the left of the sequences. The initiation codon (ATG) and stop codon (TGA) are bold. The NACHT domain is shaded. (B) The predicted functional domain of NLRP3 protein in different species. Pyrin domain was abbreviated as PYD; Nucleotide-binding and oligomerization domain was abbreviated as NACHT; Ligand binding leucine-rich repeat domain was abbreviated as LRR. (C) Multiple sequence alignment in NACHT domain of NLRP3 protein of different species. The NLRP3 proteins used for the analysis were derived from *P. vannamei* (ROT76171.1), *P. trituberculatus* (MPC60931.1), *A. japonicus* (PIK60310.1), *D. rerio* (XP_021330446.1), *M. musculus* (NP_001346567.1) and *H. sapiens* (NP_004886.3), respectively. Identical amino acid residues are shaded in black and similar residues in gray. (D) The neighbor-joining phylogenetic tree...
based on the sequences of NLRP3 proteins from *P. vannamei* (ROT76171.1), *P. trituberculatus* (MPC60931.1; MPC09300.1), Hyalella azteca (XP_018026711.1; XP_018007488.1), *Trinorchesia longiramus* (KAF2354093.1), *Acropora millepora* (XP_029183657.1; XP_029210808.1), *Acropora digitifera* (XP_015770834.1; XP_015772621.1); *H. sapiens* (NP_004886.3) and *M. musculus* (NP_001346567.1), respectively. Letters below the tree branches represent the bootstrap values. The location of PvNLRP3-like is indicated by a black filled circle.

**Fig. 1.** (continued).
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(Version 2.5.2), the template was selected on a strict criterion of >30% sequence identity and >80% target coverage.

2.4. Realtime quantitative polymerase chain reaction (qPCR)

The SYBR® Green I-based qPCR reaction was performed in 96-well optical plates on Roche Light-Cycler 480 system (Roche, Switzerland). The relative expression level of PvNLRP3-like and antimicrobial peptides including LYZ 1/2/3/4, CRU 1/2/3 and PEN 2/3/4 were evaluated by comparative ΔCt method (2^-ΔΔCt method) and used elongation factor 1 alpha gene of P. vannamei (PvEF1α) as an internal control. The specific primers used for qPCR were listed in Table 1.

2.5. RNA interference (RNAi) experiments

Double strain RNA (dsRNA) mediated PvNLRP3-like knockdown was performed using two pairs of dsRNA (designated as dsNLRP3-1 and dsNLRP3-2), which target to a 471 bp (704–1174) and a 460 bp (1898–2357) fragment of the ORF sequence of PvNLRP3-like, respectively. Similarity, a 429 bp length of dsRNA target to enhanced green fluorescent protein (EGFP) gene (designated as dsEGFP) was used as negative control. All the dsRNA were synthesized following the protocol of the T7 RiboMAX™ Express RNAi System (Promega, USA). Primers used for dsRNA synthesis are listed in Table 1.

For RNAi assay, 100 μL of 10 μg dsNLRP3-1 or dsNLRP3-2 was introduced by intramuscular injection at the second or third abdominal segment of shrimp, while the control group was each injected with equivalent amount of dsEGFP. At 72 h post dsRNA injection, shrimps were challenged with 100 μL(1 × 10^7 CFU) of V. parahaemolyticus. Shrimp hemocytes were collected from experiment group and control group at 48, 72 and 96 hpi, followed by RNA extraction, cDNA synthesis and qPCR analysis to determine knockdown efficiency. The relative expression level of antimicrobial peptides including LYZ 1/2/3/4, CRU 1/2/3, ALF 1/2/3 and PEN 2/3/4 were measured by qPCR.

2.6. Statistics analysis

The data from the experiments were expressed as the means ± standard deviations (means ± SDs). p < 0.05 was considered statistically significant, all statistical analyses were performed using SPSS 25.0 software.

3. Results

3.1. Sequence characteristics of PvNLRP3-like

The complete coding sequence of PvNLRP3-like (GenBank accession number: ROT76171.1) contained 2514 nucleotides, which encodes a protein of 837 amino acids (Fig. 1A). Functional protein domain analysis by SMART program showed that PvNLRP3-like have typical NACHT domain but lack of PYRIN and LRR domains, which are similar in other invertebrates like Portunus trituberculatus and Apostichopus japonicus (Fig. 1B). Consequently, multiple sequence alignment was performed using the amino acids of NACHT domain of NLRP3 in different species. The results indicated that NACHT domain of PvNLRP3-like shared 33.12%, 21.97%, 26.01%, 26.14% and 26.86% sequence identity with that of Portunus trituberculatus (MPC60931.1), Apostichopus japonicus (PIK60310.1), Danio rerio (XP_021330446.1), Mus musculus (NP_001346567.1) and Homo sapiens (NP_004886.3), respectively (Fig. 1C). The phylogenetic tree was built using the amino acid sequence of PvNLRP3-like and 11 homologues of other species acquired from the NCBI database. The phylogenetic analysis showed that PvNLRP3-like
shares evolutionary relationship with other invertebrates, which formed a separate cluster with two NLRP3 homologues of \( P. \) trituberculatus (Fig. 1D). Although the NACHT domain of \( P. \) vannamei-like shared low sequence homology with that in \( H. \) sapiens, ramachandran plots presented the similar protein secondary structure elements of NACHT domain of NLRP3 in \( P. \) vannamei and \( H. \) sapiens, which shared common patterns on their potential energy surface and global minima (Fig. 2A). Furthermore, both of their predicted 3D protein structures were also topologically conserved (Fig. 2B).

3.2. Tissue specific and pathogens inducible expression profiles of \( P. \) vannamei NLRP3-like

The mRNA expression levels of \( P. \) vannamei NLRP3-like in different shrimp tissues were determined by qPCR. \( P. \) vannamei NLRP3-like was ubiquitously expressed in all detected tissues, including hemocytes, hepatopancreas, heart, gill, intestine, stomach, eyestalk and nerve. Among these tissues, transcripts of \( P. \) vannamei NLRP3-like were most abundance in nerve and eyestalk, moderate in stomach, intestine, gill, heart, and with relative low expression in hepatopancreas and hemocytes (Fig. 3A). Since NLRP3 is the sensor that relied by innate immune cells, which process an appropriate immune response against microbial infection [15]. Several shrimp pathogens like \( V. \) parahaemolyticus, \( S. \) iniae and WSSV were used to challenge shrimp, and the transcripts of \( P. \) vannamei NLRP3-like in hemocytes (immune cells) were measured post different time points infection. Compared with the control group, the respond pattern of \( P. \) vannamei NLRP3-like was different upon the challenge of \( V. \) parahaemolyticus (Gram-negative bacteria), \( S. \) iniae (Gram-positive bacteria) and WSSV (virus). A
significant increase of PvNLRP3-like could be detected by *V. parahaemolyticus* challenge at 6, 12, 24 and 36 hpi (Fig. 3B). While the mRNA level of PvNLRP3-like only significantly increased at 6 hpi but decreased at 24 hpi after *S. iniae* challenge (Fig. 3C). In response to WSSV, the PvNLRP3-like transcript significantly decreased at 12 and 24 hpi (Fig. 3D).

### 3.3. *PvNLRP3-like interference inhibits *V. parahaemolyticus* induced AMPs expression

To further explore the function of PvNLRP3-like in response to *V. parahaemolyticus* challenge, two dsRNA (dsNLRP3-1 and dsNLRP3-2) targeting different regions of PvNLRP3-like were designed to knockdown PvNLRP3-like in shrimp hemocytes. As shown in Fig. 4A, a continuous interference effect could be detected using dsNLRP3-2 injection, which significantly decrease 84.4% and 35.3% of PvNLRP3-like mRNA level during 72–96 hpi, respectively, while dsNLRP3-1 only significantly decreased 34.2% of PvNLRP3-like transcripts but increased 98% at 72 and 96 hpi, respectively.

Since NLRP3 and AMPs were induced in NF-κB dependent manner [8,16,17], the association of PvNLRP3-like and different AMPs in shrimp was further explored. After successfully knockdown of PvNLRP3-like by dsNLRP3-2 injection at 72 hpi, a 24 h of *V. parahaemolyticus* stimulation was carried out (Fig. 4B), and four types of AMPs (LYZ, CRU, ALF, PEN) expression induced by *V. parahaemolyticus* after PvNLRP3-like silencing were examined by qPCR. The results indicated that depleted of PvNLRP3-like followed by *V. parahaemolyticus* could significantly increase the transcripts of PvLYZ3, PvCRU2, PvALF2, PvALF3, PEN3 and PEN4 (Fig. 4C–F), suggesting that NLRP3 might negatively regulate the expression of antimicrobial peptides.

### 4. Discussion

Inflammatory response plays a pivotal role in regulating a wide
range of physiological and pathological processes, such as cell proliferation [18], apoptosis [19], necrosis [20]. As a key effector in inflammation, NLRP3 has received extensive attention in mammalian models [21,22]. However, the study on the characteristics and function of NLRP3 in invertebrates, especially in aquatic animal like shrimp is not sufficient. The very few NLRP3 homologs identified in invertebrates

Fig. 4. Effects of PvNLRP3-like knockdown on *V. parahaemolyticus* induced AMPs expression. (A) Knockdown efficiency of PvNLRP3-like was analyzed by qPCR. Shrimp hemocytes were collected at 48, 72 and 96 h post-injection with dsNLRP3-1, dsNLRP3-2 or dsEGFP as control. The transcripts of PvNLRP3-like in dsEGFP injected groups was set to 1.0 for which the others were normalized. (B) Schematic representation of the dsRNA injection and *V. parahaemolyticus* challenge. Penaeid shrimps (*n* = 5) were injected with dsRNA followed by *V. parahaemolyticus* challenge at 72 h post-dsRNA injection. (C-F) Transcript levels of (C) PvLYZ 1-4, (D) PvCRU 1-3, (E) PvALF 1-3 and (F) PvPEN 2-4 were determined by qPCR after PvNLRP3-like interference. The transcript level of different AMPs for control group (normal saline) was set to 1.0. Data represent mean ± SD (*n* = 5) for three independent experiments. Statistical analysis was performed by unpaired Student’s t-test and indicated by an asterisk (*p* < 0.05 and **p* < 0.01).
have restricted research of invertebrate inflammatory response. In this study, the NLRP3 homolog in *P. vannamei* (designated PvNLRP3-like) was first characterized and the immunoregulation role of PvNLRP3-like on AMPs expression were investigated.

Sequence analysis revealed the evolutionally conserved of PvNLRP3-like in invertebrates. As intracellular receptors, NLRP3 protein families have similar structures with a pyrin domain (PYD) at the amino terminus, a central nucleotide-binding and oligomerization domain (NACTH) and a ligand binding leucine-rich repeat domain (LRR) at the carboxy terminus [23]. In the absence of immune stimulation, an internal interaction occurs between the NACTH and LRR domains, which suppressed the interaction between NLRP3 and ASC, consequently preventing assembly of the inflammasome [24]. Distinctively, functional domain prediction indicated that PvNLRP3-like contained only NACHT domain (Fig. 1B), as well as that in *P. trituberculatus* and *A. japonicus*, which might affect the transformation of inactivated NLRP3 to activated NLRP3 through the dissociation between NACTH and LRR domain [6]. However, despite the amino acids of NACTH domain of PvNLRP3-like shared low identity with that in *H. sapiens*, (Fig. 1c), they presented similar topological protein structure (Fig. 2), which might indicate the conserved function of NACTH domain from invertebrate to vertebrate.

Investigating the distribution of PvNLRP3-like in immune-related tissue is helpful for us to understand its immune function. In marmals, NLRP3 proteins were primarily presented in immune and inflammatory cells like macrophages, monocytes, dendirct cells, and splenic neutrophils [25,26]. In penaeid shrimp (*P. vannamei*), we found that PvNLRP3-like is constitutively expressed in various tissues and mainly expressed in some nervous and endocrine tissues like nerve and eyestalk, while hemocytes, the important immune cells in shrimp, displayed lowest expression level (Fig. 3A). We considered that the expression of NLRP3 in hemocytes was inducible, and actually different pathogens challenge induced various responsive pattern of PvNLRP3-like expression in hemocytes, *V. parahaemolyticus* stimulation activated the transcript of PvNLRP3-like during 12–36 hpi, while *S. iniae* challenge upregulated the mRNA level of PvNLRP3-like at 6 hpi but downregulated at 24 hpi. A significant decrease of PvNLRP3-like expression was also detected at 12 and 24 h post WSSV infection (Fig. 3B–D). Similarly, NLRP3 is a critical regulator of innate immune response to several pathogens stimulation like mycoplasma [27], fungi [28], bacteria [29] and virus [30] in human. For instance, *Mycobacterium tuberculosis* infection promoted NLRP3 activation and inflammatory cytokines expression at 24 hpi in human acute monocyte leukemia cell line THP-1 [29]. Compared to WSSV induced PvNLRP3-like transcript decrease, after 16 h exposure to SARS-CoV-2 Spike protein, upregulation of Nlrp3 mRNA was measured in very small embryonic-like stem cells of human umbilical cord blood [30]. These cumulative evidences suggest that PvNLRP3 is participated in primary innate immune response of *P. vannamei*.

In penaeid shrimp, some of AMPs, such as penaeidins, crustins and antitilipopolysaccharide factors, were considered as the downstream effectors of activated Toll pathway for defending against bacterial invasion [16,17]. Similarly, NLRP3 transcript was also recognized as TLR activated manner [8]. Interestingly, some immune- regulatory crosstalk between NLRP3 and AMPs have been reported. For instance, *Giardia spp.* promote the production of antimicrobial peptides and attenuate disease severity induced by attaching and invading *Giardia spp.* which might affected the transformation of inactivated NLRP3 to activated NLRP3 during infection with *Pseudomonas aeruginosa* [31], or affected the expression level of several AMPs like LYZ 3, CRU 2, ALF 1/2/3 and PEN 3/4 have been changed after RNAi PvNLRP3-like (Fig. 4C–F). These details and our experimental results indicated possible cooperation mechanism of NLRP3 and AMPs in shrimp, which implied that PvNLRP3-like might negatively regulate the expression of antimicrobial peptides. In spite of this, a significantly decrease of PvALF1 have been found after PvNLRP3-like silencing (Fig. 4E), which indicated a complex regulatory mechanism of NLRP3-like on AMPs expression.

In summary, an evolutionally conserved NLRP3 from penaeid shrimp (*P. vannamei*) was characterized, which was important for the antibacterial immune response via negatively regulating AMPs expression. Since knowledge about the immune regulatory mechanism of inflammation is still limited in shrimp, our studies are helpful for expanding the relative understanding and providing novel perspectives of inflammatory effector in invertebrates.

**Declaration of Competing Interest**

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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**References**

[1] R. Medzhitov, Origin and physiological roles of inflammation, Nature 454 (2008) 426–435, https://doi.org/10.1038/nature07201.

[2] D.R. Neill, S.H. Wong, A. Bellosi, R.J. Flynn, M. Daly, T.K. Langford, C. Bucks, C. M. Kane, P.G. Falton, R. Pannell, Nocystes represent a new innate effector leukocyte that mediates type-2 immunity, Nature 464 (2010) 1367–1370, https://doi.org/10.1038/nature08900.

[3] G. Trinchieri, A. Sher, Cooperation of toll-like receptor signals in innate immune defence, Nat. Rev. Immunol. 7 (2007) 179–190, https://doi.org/10.1038/nri2038.

[4] E. Onuki, M. Campbell, S.L. Doyle, Targeting the NLRP3 inflammasome in chronic inflammatory diseases: current perspectives, J. Inflamm. Res. 8 (2015) 15, https://doi.org/10.2147/JIR.S51250.

[5] S.B. Willingham, I.C. Allen, D.T. Bergstrahl, W.J. Brickley, M.T.H. Huang, D. J. Taxman, J.A. Duncan, J.P.Y. Ting, NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and-independent pathways, J. Immunol. 183 (2009) 2008–2015, https://doi.org/10.4049/jimmunol.0900138.

[6] Y. He, H. Hara, G. Nihira, The mechanism and regulation of NLRP3 inflammasome activation, Trends Biochem. Sci. 41 (2016) 1012–1021, https://doi.org/10.1016/j.tibs.2016.09.002.

[7] S.H. McCall, M. Sahrabi, A.B. Young, C.S. Worley, J.A. Duncan, J.P.Y. Ting, I. Marr, NACTH and pyrin domain-containing receptor implicated in bacterially induced cell death, J. Bone Mineral Res. 23 (2008) 30–40, https://doi.org/10.1359/jbmr.071002.

[8] Y. Qiao, P. Wang, J. Qi, L. Zhang, C. Gas, TLR-induced NF-κB activation regulates NLRP3 expression in murine macrophages, FEBS Lett. 586 (2012) 1022–1026, https://doi.org/10.1016/j.febslet.2012.02.045.

[9] F. Di Virgilio, The therapeutic potential of modifying inflammasomes and NOD-like receptors, Pharmacol. Rev. 65 (2013) 872–905, https://doi.org/10.1124/pr.112.006171.

[10] Z. Zhong, Y. Lu, X. Yang, Y. Tang, K. Zhao, C. Yuan, X. Zhong, The roles of NLRP3 inflammasome in bacterial infection, Mol. Immunol. 122 (2020) 80–88, https://doi.org/10.1016/j.molimm.2020.02.020.

[11] A. Manko-Prykhoa, T. Allain, J.-P. Motta, J.A. Cotton, T. Feener, A. Oyeyemi, S. Bindra, B.A. Vance, J.L. Wallace, P. Beck, *Giardia spp.* promote the production of antimicrobial peptides and attenuate disease severity induced by attaching and efacing enteropathogens via the induction of the NLRP3 inflammasome, Int. J. Parasitol. 50 (2020) 363–375, https://doi.org/10.1016/j.ijpara.2019.12.011.

[12] G. De Vico, F. Carella, Morphological features of the inflammasome response in molluscs, Res. Vet. Sci. 93 (2012) 1109–1115, https://doi.org/10.1016/j.resves.2012.03.014.

[13] Z. Lv, Z. Wei, Z. Zhang, C. Li, Y. Shao, W. Zhang, X. Zhao, Y. Li, X. Duan, J. Xiong, Characterization of NLRP3-like gene from *Apostichopus japonicus* provides new evidence on inflammation response in invertebrates, Fish Shellfish Immunol. 68 (2017) 114–123, https://doi.org/10.1016/j.fsi.2017.07.024.

[14] Z. Zheng, F. Wang, J.J. Aveya, R. Li, D. Yao, M. Zhong, S. Li, Y. Zhang, Comparative transcriptomic analysis of shrimp hemocytes in response to acute hepatopancreatic necrosis disease (AHPND) causing *Vibrioparahaemolyticus* infection, Fish Shellfish Immunol. 74 (2018) 109–115, https://doi.org/10.1016/j.fsi.2017.12.032.

[15] M. Lamanfani, T.-D. Kangnangi, NLRP3: an immune sensor of cellular stress and infection, Int. J. Biochem. Cell. Biol. 42 (2010) 792–795, https://doi.org/10.1016/j.jbiol.2010.01.008.
[16] F. Li, J. Xiang, Signaling pathways regulating innate immune responses in shrimp, Fish Shellfish Immunol. 34 (2013) 973–980, https://doi.org/10.1016/j.fsi.2012.08.023.

[17] A. Tassanakajon, V. Rimphanitchayakit, S. Visetnan, P. Amparyup, K. Somboonwiwat, W. Charoensapsri, S. Tang, Shrimp humoral responses against pathogens: antimicrobial peptides and melanization, Dev. Comp. Immunol. 80 (2018) 81–93, https://doi.org/10.1016/j.dci.2017.05.009.

[18] H. Du, Y. Wang, Y. Zeng, X. Huang, D. Liu, L. Ye, Y. Li, X. Chen, T. Liu, H. Li, Tanshinone IIA suppresses proliferation and inflammatory cytokine production of synovial fibroblasts from rheumatoid arthritis patients induced by TNF-α and attenuates the inflammatory response in AIA mice, Front. Pharmacol. 11 (2020) 568, https://doi.org/10.3389/fphar.2020.00568.

[19] J. Wang, J. Mao, R. Wang, S. Li, B. Wu, Y. Yuan, Kaempferol protects against cerebral ischemia reperfusion injury through intervening oxidative and inflammatory stress induced apoptosis, Front. Pharmacol. 11 (2020) 424, https://doi.org/10.3389/fphar.2020.00424.

[20] L. Li, Z. Bi, Y. Hu, L. Sun, Y. Song, S. Chen, F. Mo, J. Yang, Y. Wei, X. Wei, Silver nanoparticles and silver ions cause inflammatory response through induction of cell necrosis and the release of mitochondria in vivo and in vitro, Cell. Biol. Toxicol. 37 (2021) 177–191, https://doi.org/10.1007/s10565-020-09526-4.

[21] A. Pandey, C. Shen, S. M. Man, Cell biology of inflammasome activation, Trends Cell Biol. 0962-8924 (21) (2021), https://doi.org/10.1016/j.tcb.2021.06.010, 00126-4.

[22] T.Y. Lin, M.C. Tsai, W. Tu, H.C. Yeh, S.C. Wang, S.P. Huang, C.Y. Li, Role of the NLRP3 inflammasome: insights into cancer hallmarks, Front. Immunol. 11 (2021) 3723, https://doi.org/10.3389/fimmu.2020.610492.

[23] S.L. Cassel, S. Joly, F.S. Sutterwala, The NLRP3 inflammasome: a sensor of immune danger signals, Semin. Immunol. 21 (2009) 194–198, https://doi.org/10.1016/j.semiunimm.2009.05.002.

[24] M. Inoue, M.L. Shinohara, Nlrp3 inflammasome and MS/EAE, Autoimmune Dis. (2013), 859145, https://doi.org/10.1155/2013/859145, 2013.

[25] G. Guarda, M. Zenger, A.S. Yazdi, K. Schroder, I. Ferrero, P. Menu, A. Tardivel, C. Mattmann, J. Tschopp, Differential expression of NLRP3 among hematopoietic cells, J. Immunol. 186 (2011) 2529–2534, https://doi.org/10.4049/jimmunol.1002720.

[26] Y. Zhong, A. Kinio, M. Saleh, Functions of NOD-like receptors in human diseases, Front. Immunol. 4 (2013) 335, https://doi.org/10.3389/fimmu.2013.00335.

[27] J.A. Segovia, T.-H. Chang, V.T. Winter, J.J. Coalson, M.P. Cagle, L. Pandrani, S. Bose, J.B. Baseman, T.R. Kannan, NLRP3 is a critical regulator of inflammation and innate immune cell response during Mycoplasma pneumoniae infection, Infect. Immun. 86 (2017), https://doi.org/10.1128/IAI.00548-17 e00548-17.

[28] A.G. Hsie, J. Tomalka, S. Ganenee, K. Patel, B.A. Hall, G.D. Brown, K.A. Fitzgerald, A critical role for the NLRP3 inflammasome in host defense against the human fungal pathogen Candida albicans, Cell Host Microbe 5 (2009) 487–497, https://doi.org/10.1016/j.chom.2009.05.002.

[29] M. Wei, L. Wang, T. Wu, J. Xi, Y. Han, X. Yang, D. Zhang, Q. Fang, B. Tang, NLRP3 activation was regulated by DNA methylation modification during Mycobacterium tuberculosis infection, Biomed. Res. Int. (2016), 4323281, https://doi.org/10.1155/2016/4323281, 2016.

[30] M.Z. Ratajczak, K. Bujko, A. Ciechanowicz, K. Sielatycka, M. Cymer, W. Marlicz, M. Kucia, SARS-CoV-2 entry receptor ACE2 is expressed on very small CD45− precursors of hematopoietic and endothelial cells and in response to virus spike protein activates the Nlrp3 inflammasome, Stem Cell Rev. Rep. 17 (2021) 266–277, https://doi.org/10.1007/s12015-020-10010-z.

[31] B.J. McHugh, R. Wang, H.-N. Li, P.E. Beaumont, R. Kells, H. Stevens, L. Young, A. G. Rossi, R.D. Gray, J.R. Dorin, Cathelicidin is a “fire alarm”, generating protective NLRP3-dependent airway epithelial cell inflammatory responses during infection with Pasteurella multocida, PLoS Pathog. 15 (2019), e1007694, https://doi.org/10.1371/journal.ppat.1007694.

[32] L.H. Li, T.C. Ju, C.Y. Hsieh, W.C. Dong, W.T. Chen, K.F. Hua, W.J. Chen, A synthetic cationic antimicrobial peptide inhibits inflammatory response and the NLRP3 inflammasome by neutralizing LPS and ATP, PLoS One 12 (2017), e0182057, https://doi.org/10.1371/journal.pone.0182057.