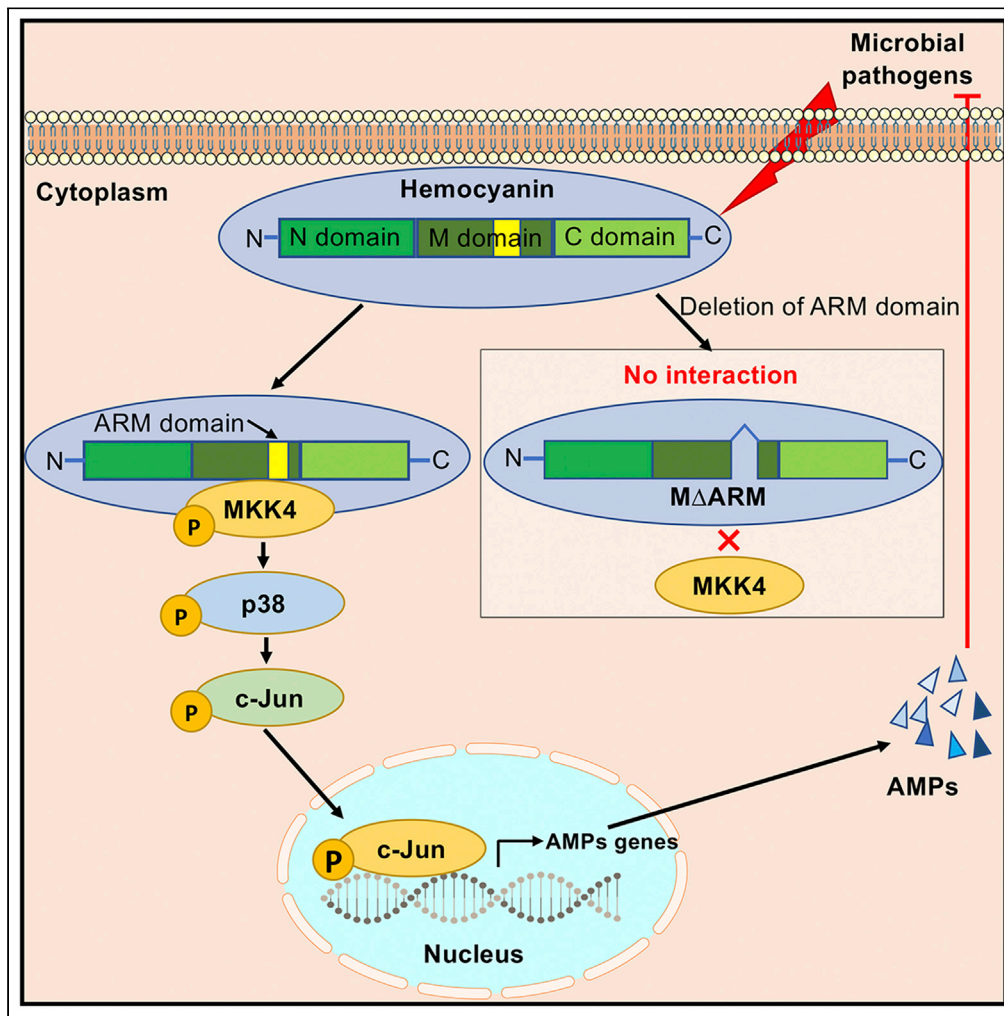


Article

The ARM repeat domain of hemocyanin interacts with MKK4 to modulate antimicrobial peptides expression



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Highlights

Pathogens induce hemocyanin, MKK4-p38-c-Jun proteins, and antimicrobial peptide genes

Hemocyanin modulates MKK4-p38-c-Jun cascade proteins to regulate AMPs gene expression

Hemocyanin interacts with MKK4 to modulate p38 MAPK signaling in penaeid shrimp

Deletion of the ARM repeat domain attenuates the interaction of hemocyanin with MKK4

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Article

The ARM repeat domain of hemocyanin interacts with MKK4 to modulate antimicrobial peptides expression

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SUMMARY

The mitogen-activated protein kinase (MAPK) intracellular signaling pathway mediates numerous biological processes, including antimicrobial immune response by inducing antimicrobial peptides (AMPs) production. Although MAPK signaling cascade proteins have been identified in penaeid shrimp, their modulation via the MKK4-p38-c-Jun cascade and effect on AMPs production is unknown. Here, we show that hemocyanin (PvHMC), antimicrobial peptides (anti-lipopolysaccharide factor, crustin, and penaeidins), and MKK4-p38-c-Jun cascade proteins are simultaneously induced by pathogens (*Vibrio parahaemolyticus*, *Staphylococcus aureus*, and white spot syndrome virus) in *Penaeus vannamei*. Intriguingly, knock-down of PvHMC with or without pathogen challenge attenuated the expression of MKK4-p38-c-Jun cascade proteins and their phosphorylation level, which consequently decreased AMPs expression. Further analysis revealed that PvHMC interacts via its armadillo (ARM) repeat domain with PvMKK4 to modulate the p38 MAPK signaling pathway. Thus, the ARM repeat domain enables penaeid shrimp hemocyanin to modulate AMPs expression during antimicrobial response by activating the p38 MAPK signaling pathway.

INTRODUCTION

The innate immune system is the first line of defense in vertebrates but the only mode of defense in invertebrates (Riera Romo et al., 2016; Syed Musthaq and Kwang, 2014). Unfortunately, the innate immune defense mechanisms in most invertebrates such as penaeid shrimp is not well understood, for which reason it has been the focus of many researchers (Li and Xiang, 2013; Tassanakajon et al., 2013). Given that innate immunity has limited or no memory (Gourbal et al., 2018), the innate immune defense mechanisms have had to evolve or coopt many factors to expand its scale or protection and response repertoire against pathogens and potential pathogens (Gourbal et al., 2018; Netea et al., 2019). Thus, most invertebrates encode immune-related proteins (Aweya et al., 2021), including immunoglobulin-like molecules, that enable them mount direct or indirect immune responses by interacting with other proteins/factors via coordinated immune pathways (Halaby and Mornon, 1998). For instance, the fibrinogen-related protein (FREPs) that comprises a C-terminal fibrinogen (FBG) domain and upstream immunoglobulin domains (Adema, 2015) plays important innate immune functions in bay scallop *Argopecten irradians* (Zhang et al., 2009a), mosquito *Anopheles gambiae* (Dong and Dimopoulos, 2009), and sea cucumber *Apostichopus japonicus* (Jiang et al., 2018) due to the presence of an NF- κ B binding motif on FREP promoter region, which can be activated by pathogens (Jiang et al., 2019). Similarly, the Down syndrome cell adhesion molecule (Dscam), which consists of more than 18,000 isoforms of the Ig-superfamily receptors (Watson et al., 2005), binds to pathogenic bacteria and regulates AMPs expression in *Eriocheir sinensis* (Li et al., 2019b).

Antimicrobial peptides (AMPs) are fundamental effector molecules used by both vertebrate and invertebrates in their innate immune defense against pathogens (Barreto et al., 2018; Destoumieux et al., 1997; Matos et al., 2018; Nguyen et al., 2011). These effector molecules (i.e., AMPs) are generally constitutively expressed but their expression is augmented during immune challenge or microbial infections via various signal transduction pathways including the NF- κ B/Toll and immune deficiency (IMD) (Li et al., 2018, 2019a), MAPK signaling pathways (He et al., 2013; Li et al., 2015, 2016; Wang et al., 2016, 2018), JAK-STAT signaling

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pathway (Sun et al., 2017), etc. In crustaceans, information on the factors or proteins that induce AMPs expression through activation of these signaling pathways is currently limited.

The respiratory glycoprotein hemocyanin also possess many immune-related functions (Coates and Talbot, 2018; Jiang et al., 2007; Laino et al., 2015; Nagai et al., 2001; Siddiqui et al., 2006; Yao et al., 2019) and can act as a pattern recognition receptor (PRR) to recognize pathogen-associated molecular patterns (PAMPs) in antibacterial response (Zhang et al., 2017). Moreover, hemocyanin undergoes proteolytic degradation to generate antimicrobial peptides in response to different pathogenic microorganisms such as viruses, bacteria, and fungi (Destoumieux-Garzon et al., 2001; Lee et al., 2003; Wen et al., 2016; Zhan et al., 2019; Zhang et al., 2004a). Most importantly, the C-terminal domain of hemocyanin contains an Ig-like domain (Zhang et al., 2004b, 2006, 2017) that could account for its numerous immune-related functions. Besides its direct immune effector functions, hemocyanin interacts with TGase to affect hemolymph clotting in penaeid shrimp (Yao et al., 2019), activate the NF- κ B pathway via extracellular signal-regulated kinase (ERK) in the mollusk *Megathura crenulata* (Yasuda and Ushio, 2016), and interact with ERK1/2 in penaeid shrimp to induce an antiviral response (Havanapan et al., 2009). Our preliminary studies revealed that infection of penaeid shrimp with Gram-negative and Gram-positive bacteria and white spot syndrome virus (WSSV) induces an elevated expression of hemocyanin and AMPs.

In the current study, we examined the mechanisms by which hemocyanin regulates AMPs expression in shrimp. We observed that penaeid shrimp hemocyanin (PvHMC), especially the armadillo (ARM) repeat domain region, regulates the p38 MAPK signaling pathway by interacting with MKK4 to affect AMPs expression during an immune challenge. Thus, we propose that hemocyanin, through its ARM domain, modulates AMP genes expression during antimicrobial immune response by activating the p38 MAPK signaling pathway in penaeid shrimp.

RESULTS

Hemocyanin and antimicrobial peptides are induced by microbial pathogens

When penaeid shrimp (*Penaeus vannamei*) were challenged with *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and WSSV, transcript levels of genes encoding hemocyanin (PvHMC) and various antimicrobial peptides (i.e., anti-lipopolysaccharide factor (ALF), crustin (CRU), and penaeidins (PEN)) were significantly induced to various degrees in the hepatopancreas compared with control (Figures 1A–1L). For instance, the three pathogens induced mRNA levels of PvHMC by 3- to 4-fold (Figures 1A–1C), while mRNA levels of ALF were induced by 1- to 5-fold (Figures 1D–1F). Similarly, the mRNA levels of CRU were induced by 1- to 15-fold (Figures 1G–1I), while that of PEN were induced by 1- to 7-fold (Figures 1J–1L) by the three pathogens. Among the three microbial pathogens, *V. parahaemolyticus* induced the highest expression of these genes comparatively (Figures 1A, 1D, 1G, and 1J). These are quite intriguing results because hemocyanin can also undergo proteolytic cleavage to generate peptides with antimicrobial activity upon pathogen challenge (Wen et al., 2016; Zhan et al., 2019). Thus, the simultaneous induction of PvHMC, ALF, CRU, and PEN expression by these microbial pathogens suggests a link between these genes or a regulatory role played by PvHMC during pathogen challenge.

Hemocyanin is required for AMPs expression

To examine the relationship between PvHMC and AMPs expression with and without immune stimulation, RNAi was used followed by pathogen challenge. Knockdown of PvHMC (Figure 2A) significantly attenuated the transcript levels of ALF (Figure 2B) and PEN (Figure 2D) but not CRU (Figure 2C). Next, PvHMC silenced shrimp were challenged with *V. Parahaemolyticus*, the bacteria that induced the highest transcript levels. However, knockdown of PvHMC followed by *V. Parahaemolyticus* could not induce the mRNA levels of PvHMC (Figure 2E), ALF (Figure 2F), CRU (Figure 2G), and PEN (Figure 2H) because their expression levels remained significantly downregulated compared with control. These results suggest that PvHMC regulates AMPs expression in penaeid shrimp.

Hemocyanin and MKK4-p38-c-Jun cascade proteins are simultaneously induced by microbial pathogens

In the highly conserved p38 MAPK signaling pathway, p38 is activated by its upstream kinase MKK4 to phosphorylate downstream transcription factors (e.g., c-Jun) during an immune response (Humar et al., 2007; Wang et al., 2018). Given that the MKK4-p38-c-Jun cascade has not been well delineated in penaeid

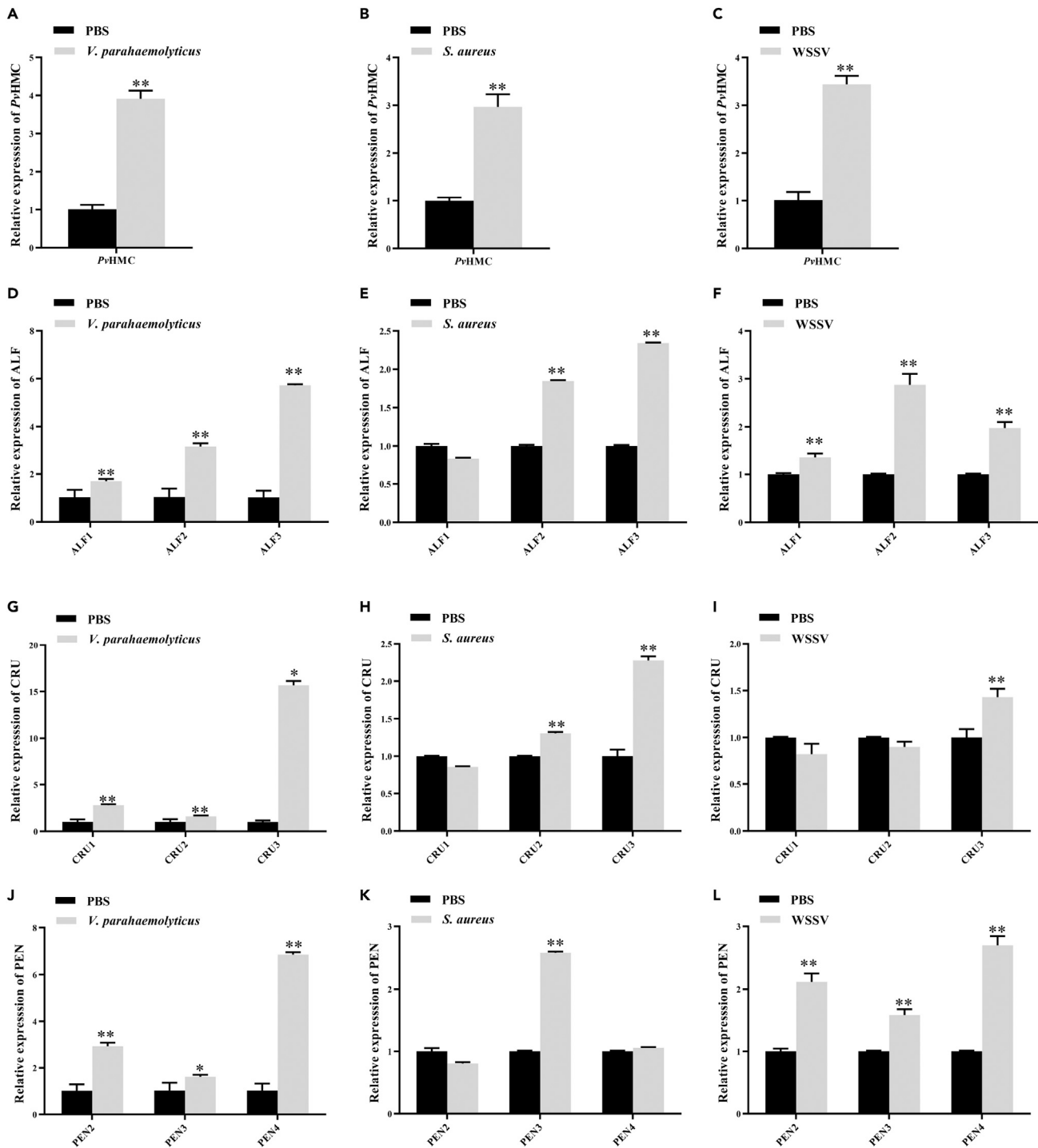


Figure 1. Microbial pathogens induce both hemocyanin and antimicrobial peptides expression

(A–C) PvHMC mRNA levels, (D–F) ALF mRNA levels, (G–I) CRU mRNA levels, and (J–L) PEN mRNA levels in *Penaeus vannamei* hepatopancreas after challenge with *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and WSSV, respectively. mRNA levels of the indicated genes were quantified by qRT-PCR, and normalized to those of EF1 α mRNA. Results reported as mean \pm SEM (n = 3). *p < 0.05, **p < 0.01 vs. control (PBS). PvHMC; hemocyanin; ALF, anti-lipopolysaccharide factor; CRU, crustin; PEN, penaeidin; WSSV, white spot syndrome virus.

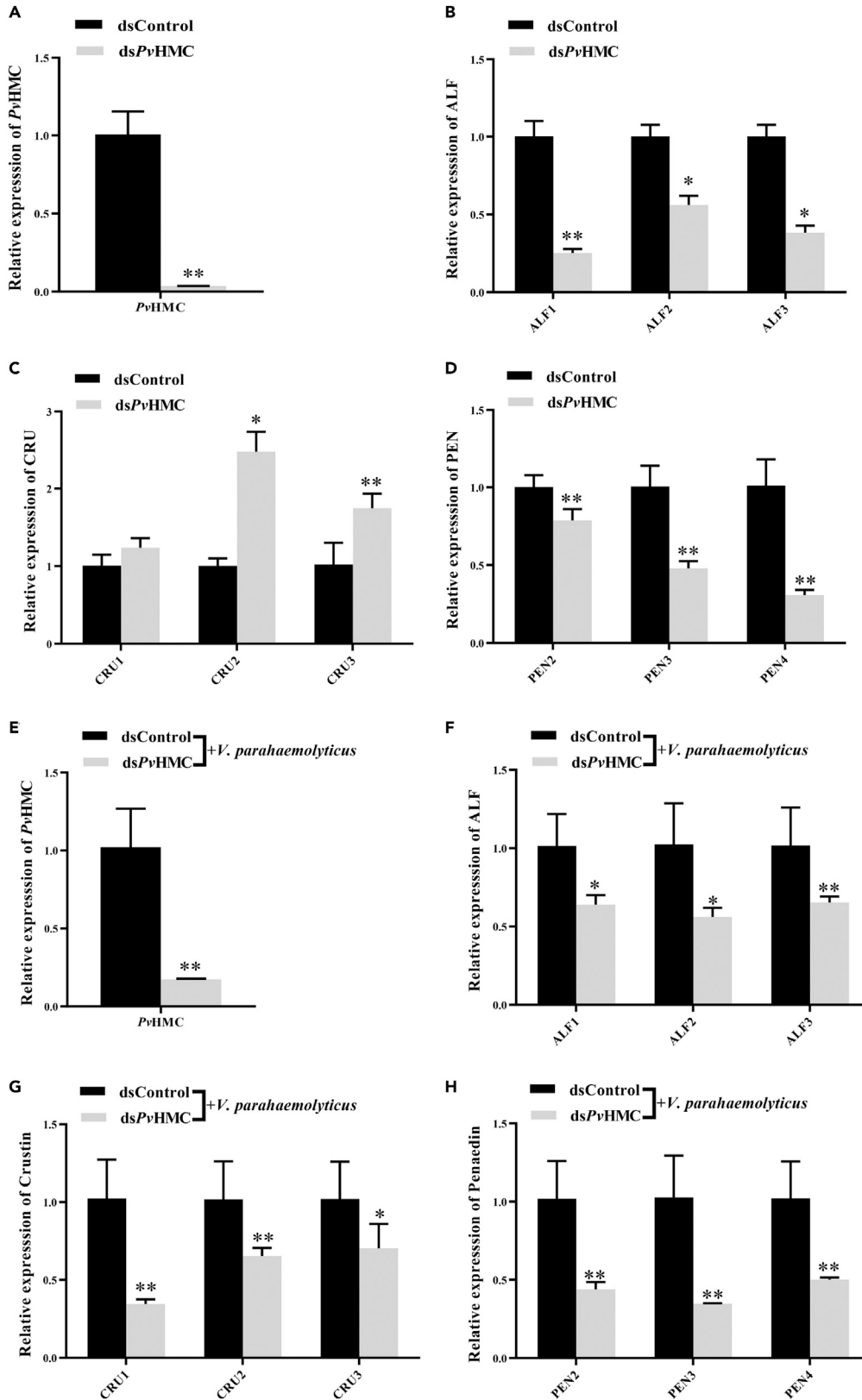


Figure 2. Hemocyanin modulates antimicrobial peptides expression

Relative mRNA expression levels of (A) *PvHMC*, (B) *ALF*, (C) *CRU*, and (D) *PEN* in *Penaeus vannamei* hepatopancreas after RNAi-mediated knockdown of *PvHMC* gene. Relative mRNA expression levels of (E) *PvHMC*, (F) *ALF*, (G) *CRU*, and (H) *PEN* in *Penaeus vannamei* hepatopancreas after *PvHMC* gene knockdown followed by *Vibrio parahaemolyticus* challenge. mRNA levels of the indicated genes were quantified by qRT-PCR, and normalized to those of *EF1 α* mRNA. Results reported as mean \pm SEM (n = 3). *p < 0.05, **p < 0.01 vs. control. *PvHMC*; hemocyanin; *ALF*, anti-lipopolysaccharide factor; *CRU*, crustin; *PEN*, penaeidin.

shrimp, first we went about to ascertain whether p38 is the upstream kinase of c-Jun. Thus, using pull-down assay, we showed the interaction between *P. vannamei* p38 (*Pvp38*) and c-Jun (*Pvc-Jun*) (Figure S1A). Besides, we observed that knockdown of *PvMKK4* or *Pvp38* significantly decreased transcript levels of *Pvc-Jun* (Figures S1B and S1C), which confirms the existence of the *MKK4*-p38-c-Jun cascade in penaeid shrimp.

Next, when shrimp were challenged with *V. parahaemolyticus*, *S. aureus*, and WSSV, transcript levels of *PvHMC* and p38 MAPK cascade proteins (i.e., *MKK4*, p38, and c-Jun) were significantly induced albeit to various levels (Figure 3). For instance, the mRNA levels of *PvHMC* were induced at different time points post challenge with *V. parahaemolyticus* (Figure 3A), *S. aureus* (Figure 3B), and WSSV (Figure 3C). Similarly, transcript levels of *PvMKK4* (Figures 3D–3F), *Pvp38* (Figures 3G–3I), and *Pvc-Jun* (Figures 3J–3L) were all induced respectively upon challenge with *V. parahaemolyticus*, *S. aureus*, and WSSV. Besides, the phosphorylation levels of *PvMKK4* and *Pvp38* (*Pvc-Jun*) proteins increased after challenge with *V. parahaemolyticus* (Figure 3N), *S. aureus* (Figure 3O), and WSSV (Figure 3P) compared with control (Figure 3M). Moreover, the mRNA expression levels of *PvHMC* and that of *PvMKK4*, *Pvp38*, and *Pvc-Jun* had similar expression pattern post challenge with *V. parahaemolyticus* (Figure S2A), *S. aureus* (Figure S2B), and WSSV (Figure S2C).

Hemocyanin modulates p38 MAPK signaling during immune challenge

Given that keyhole limpet hemocyanin (KLH) of *M. crenulata* is reported to activate the NF- κ B pathway via ERK (Yasuda and Ushio, 2016), we went on to explore the relationship between penaeid shrimp hemocyanin (*PvHMC*) and the *MKK4*-p38-c-Jun cascade. Using RNAi, we found that *PvHMC* knockdown (Figure 4A) significantly decreased the mRNA expression levels of *PvMKK4* (Figure 4B), *Pvp38* (Figure 4C), and *Pvc-Jun* (Figure 4E), but not *PvATF2* (Figure 4D). Similarly, knockdown of *PvHMC* resulted in a decrease in the phosphorylation levels of *PvMKK4* (Figure 4B bottom), *Pvp38* (Figure 4C bottom), and *PvATF2* (Figure 4D bottom). On the other hand, transient expression of *PvHMC* in *Drosophila* S2 cells increased the phosphorylation levels of *PvMKK4* and *Pvp38* (Figures S3A and S3B).

When *PvHMC* was silenced followed by *V. Parahaemolyticus* challenge, both mRNA and protein levels of *PvHMC* (Figure 4F), *PvMKK4* (Figure 4G), and *Pvp38* (Figure 4H), and transcript levels *Pvc-Jun* (Figure 4I) were significantly decreased compared with control. These results suggest that knockdown of *PvHMC* with or without microbial challenge attenuates the expression and activation of *MKK4*-p38-c-Jun cascade proteins in penaeid shrimp.

Next, we examined the consequence of silencing penaeid shrimp *PvMKK4* and *Pvp38*, key members of the *MKK4*-p38-c-Jun cascade on AMPs expression. After *PvMKK4* knockdown (Figure 4J), transcript levels of *ALF* (Figure 4K), *CRU* (Figure 4L), and *PEN* (Figure 4M) were significantly downregulated. Similarly, silencing of *Pvp38* (Figure 4N) resulted in significant decrease in the mRNA levels of *ALF* (Figure 4O), *CRU* (Figure 4P), and *PEN* (Figure 4Q) compared with control. These results indicate that both *PvHMC* and *MKK4*-p38-c-Jun cascade proteins modulate the expression of the AMPs *ALF*, *CRU*, and *PEN* in penaeid shrimp.

Hemocyanin interacts with MKK4 to modulate p38 MAPK signaling

To examine the relationship between hemocyanin and the p38 MAPK signaling pathway in penaeid shrimp, we explored the potential interaction between *PvHMC* and *MKK4*-p38-c-Jun cascade proteins (i.e., *PvMKK4*, *Pvp38*, *PvERK*, and *PvJNK*). Using pull-down assay, *PvHMC* was found to interact with *PvMKK4* (Figure 5A), *Pvp38* (Figure 5B), and *PvERK* (Figure S4A), but not *PvJNK* (Figure S4B). The interaction of *PvHMC* with *PvMKK4*, *Pvp38*, *PvERK*, and *PvJNK* was further ascertained using co-immunoprecipitation assay after co-transfection of *Drosophila* S2 cells with plasmids expressing these proteins. The results revealed that *PvMKK4*-V5 (Figure 5C), *Pvp38*-V5 (Figure 5D), and *PvERK*-V5 (Figure S4C) all co-precipitated

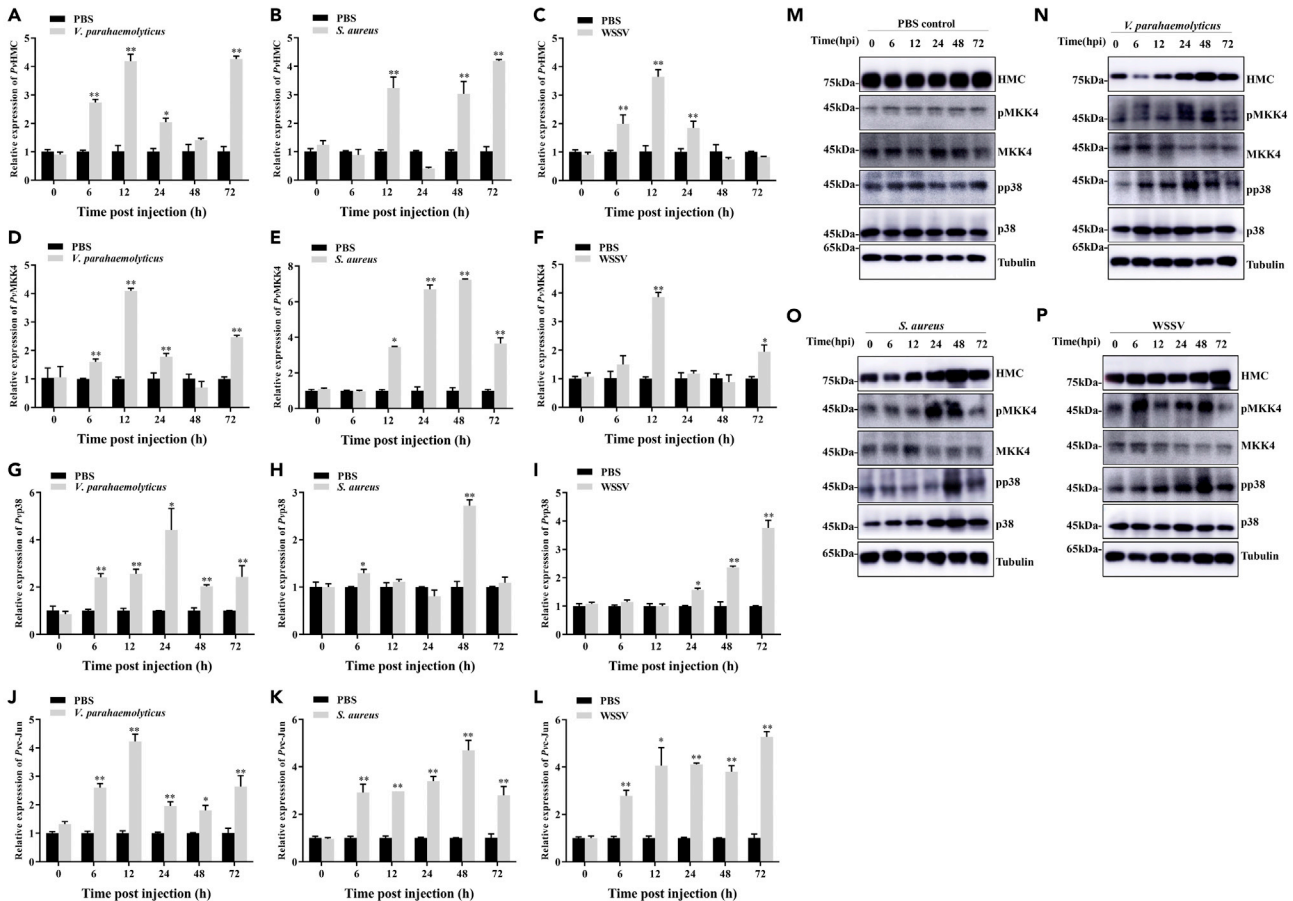


Figure 3. Microbial pathogens induce the expression of PvHMC and p38 MAPK cascade genes
Relative mRNA expression levels of (A–C) PvHMC, (D–F), PvMKK4, (G–I) Pvp38, and (J–L) Pvc-Jun in *Penaeus vannamei* hepatopancreas at the indicated time points post challenge with *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and WSSV, respectively. mRNA levels of the indicated genes were quantified by qRT-PCR, and normalized to those of EF1 α mRNA. Results reported as mean \pm SEM (n = 3). *p < 0.05, **p < 0.01 vs. control. (M–P) Western blot analysis of PvHMC, PvMKK4, Pvp38, and tubulin proteins and phosphorylation levels in *Penaeus vannamei* hepatopancreas after injection with PBS (control), *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and WSSV, respectively. Protein and phosphorylation levels were determined by western blot. The immunoblots shown are representative of at least two independent experiments. PvHMC; hemocyanin; PvMKK4, mitogen-activated protein kinase kinase four; Pvp38, p38 mitogen-activated protein kinase; Pvc-Jun, *Penaeus vannamei* transcription factor c-Jun; WSSV, white spot syndrome virus.

with FLAG-PvHMC, but not PvJNK (Figure S4D). Moreover, PvMKK4 displayed the strongest interaction with PvHMC.

Next, we then went on to explore which of the three domains (Figure 5E) of hemocyanin (i.e., N-terminal α -helical domain, middle M-terminal copper ion-binding domain also containing the ARM repeat domain, and C-terminal Ig-like domain) interacts specifically with PvMKK4. Thus, these four domains of PvHMC (designated PvHMC-N, PvHMC-M, PvHMC-M- Δ ARM, and PvHMC-C) were cloned, expressed, and their interaction with PvMKK4 examined using pull-down assays. Interestingly, only PvHMC-M (Figure 5F: lane 2) could interact with PvMKK4 but not PvHMC-N (Figure 5F: lane 1), PvHMC-M- Δ ARM (Figure 5F: lane 3), or PvHMC-C (Figure 5F: lane 4). These results indicate that PvHMC modulates the p38 MAPK signaling pathway in penaeid shrimp by interacting with PvMKK4 via its ARM repeat domain region (M-domain).

DISCUSSION

The conserved p38 MAPK signaling pathway is activated in response to various environmental and cellular stresses and pathogen infections (He et al., 2013; Roy et al., 2018), and therefore play crucial roles in regulating cell proliferation, differentiation, apoptosis, and immune response (Arthur and Ley, 2013; Zarubin and Han, 2005). A growing number of factors including Dscam (Li et al., 2019b), nucleotide oligomerization

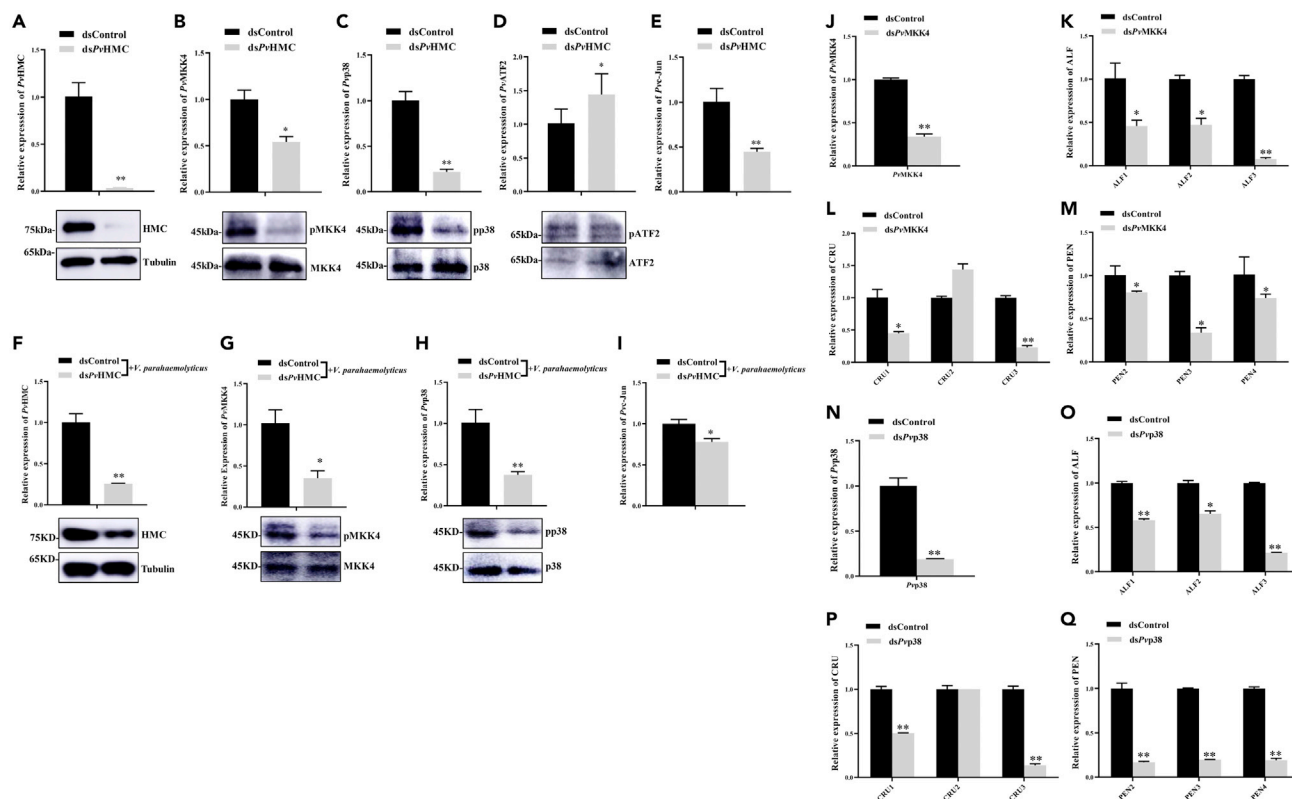


Figure 4. Hemocyanin modulates p38 MAPK signaling to induce antimicrobial peptides expression in shrimp hepatopancreas

(A) PvHMC mRNA and protein levels, (B) PvMKK4 mRNA, protein, and phosphorylation levels, (C) Pvp38 mRNA, protein, and phosphorylation levels, (D) PvATF2 mRNA, protein, and phosphorylation levels, and (E) Pvc-Jun mRNA levels, after shrimp were injected with dsControl or dsPvHMC. (F) PvHMC mRNA and protein levels, (G) PvMKK4 mRNA, protein, and phosphorylation levels, (H) Pvp38 mRNA, protein, and phosphorylation levels, and (I) Pvc-Jun mRNA levels, after shrimp were injected with dsControl or dsPvHMC followed by *Vibrio parahaemolyticus* challenge. Relative mRNA expression levels of (J) PvMKK4, (K) ALF, (L) CRU, and (M) PEN genes in shrimp hepatopancreas after PvMKK4 knockdown. Relative mRNA expression levels of (N) Pvp38, (O) ALF, (P) CRU, and (Q) PEN genes in shrimp hepatopancreas after Pvp38 knockdown. mRNA levels of the indicated genes were quantified by qRT-PCR, and normalized to those of EF1 α mRNA, whereas protein and phosphorylation levels were determined by Western blot. Results reported as mean \pm SEM (n = 3). *p < 0.05, **p < 0.01 vs. control. The immunoblots shown are representative of at least two independent experiments.

domain 2 (NOD2) (Ren et al., 2019), chicken avian β -defensin 8 (AvBD8) (Hong et al., 2020), interleukin-21 (IL-21) (Long et al., 2019), etc., can modulate the MAPK signaling pathway during immune response. Here, we reveal that the armadillo (ARM) domain region also referred to as the M-domain of penaeid shrimp hemocyanin interacts with MKK4 to modulate p38 MAPK signaling during immune response. Although the N- and C-terminal domains of hemocyanin have previously been implicated in various immune-related functions (Destoumieux-Garzon et al., 2001; Fan et al., 2019; Zhang et al., 2017), our current data show that only the middle ARM domain region (M-domain) of hemocyanin interacts specifically with MKK4 in the MKK4-p38-c-Jun cascade to induce AMPs expression.

Hemocyanin, the respiratory copper-containing glycoprotein in mollusks and arthropods, has been implicated in numerous immune-related functions (see recent reviews by (Aweya et al., 2021; Coates and Costa-Paiva, 2020)). As an important immune response protein, hemocyanin is induced by immune challenge (Zhao et al., 2016; Zheng et al., 2018) and undergoes proteolytic degradation to generate peptides with antimicrobial activity (Destoumieux-Garzon et al., 2001; Wen et al., 2016; Zhan et al., 2019). It was therefore intriguing that the expression of hemocyanin (PvHMC), antimicrobial peptides (i.e., ALF, CRU, and PEN), and MKK4-p38-c-Jun cascade proteins (i.e., MKK4, p38, and c-Jun) were all induced by *V. parahaemolyticus* (Gram-negative), *S. aureus* (Gram-positive), and WSSV (Figure 1). Moreover, the phosphorylation levels of PvMKK4 and Pvp38 proteins increased when that of PvHMC increased (Figures 3M–3P). Interestingly, these pathogens also induced some changes in the corresponding background proteins, which indicate that these proteins (PvMKK4 and Pvp38) could directly or indirectly be involved in immune

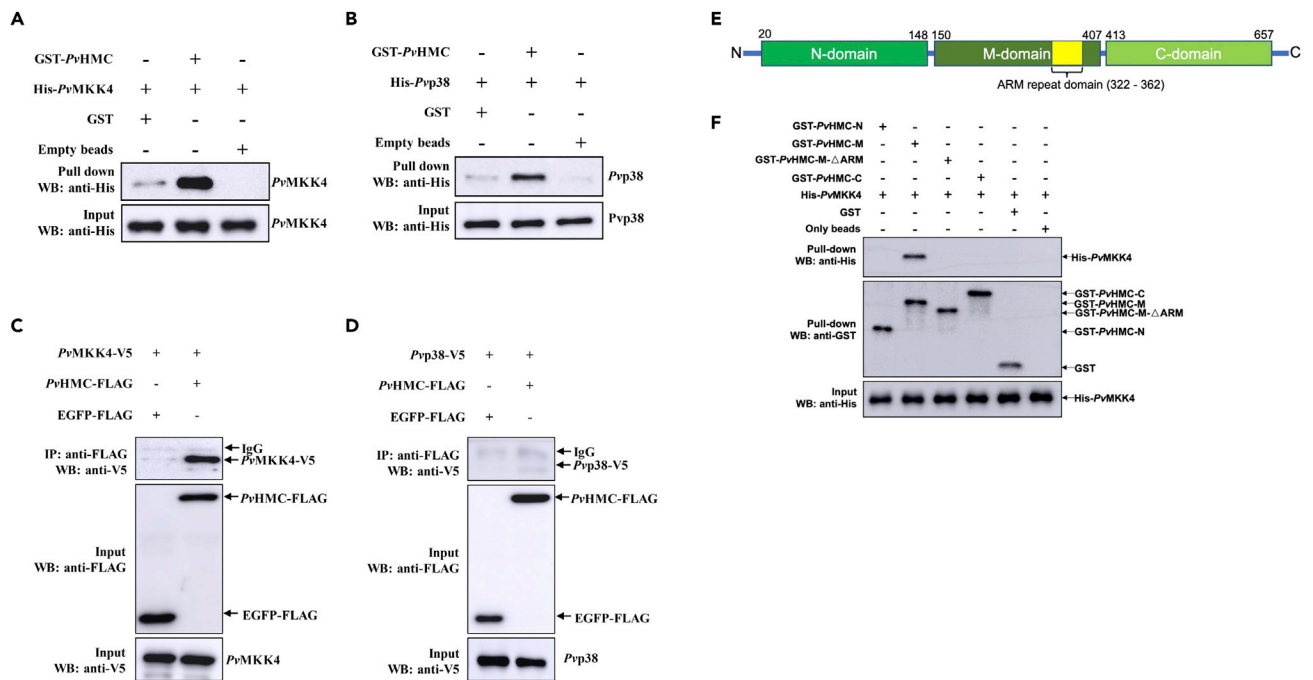


Figure 5. Hemocyanin interacts with p38 MAPK signaling proteins

(A and B) Immunoblots of protein-protein interaction analysis between GST-PvHMC and His-PvMKK4 or GST-PvHMC and His-Pvp38. (C and D) Co-immunoprecipitation and immunoblot analysis of the interaction between PvHMC-FLAG and PvMKK4-V5 or PvHMC-FLAG and Pvp38-V5. (E) Protein domain structure of *Penaeus vannamei* hemocyanin showing the N-domain, M-domain (with the ARM repeat domain motif), and C-domain. (F) Immunoblots of protein-protein interaction analysis between GST (control), GST-PvHMC-N, GST-PvHMC-M, GST-PvHMC-M-ΔARM, and GST-PvHMC-C with His-PvMKK4. Results reported are representative of at least two independent experiments.

response (Roos-Engstrand et al., 2005). Although, some members of the MAPK pathway proteins including ERK1/2 (Havanapan et al., 2009) have been reported to interact with and phosphorylate hemocyanin to affect its functions, there has never been a report on hemocyanin modulating the MAPK signaling pathway to induce AMPs expression in penaeid shrimp. We were therefore intrigued to observe simultaneous induced expression of PvHMC and AMPs by microbial pathogens, although PvHMC is capable of generating peptides with antimicrobial activity through proteolytic cleavage (Wen et al., 2016; Zhan et al., 2019). Interestingly, both *PvHMC* and *CRU* genes are also induced by stress (de la Vega et al., 2007), thus, although PvHMC is required for AMPs expression, *CRU* shows a different expression pattern, when PvHMC is depleted or depleted followed by pathogen challenge, which we contend is because of its response to stress or enigmatic expression upon bacterial challenge (Smith et al., 2008). Thus, we reasoned that PvHMC could have an effect on the expression of other AMPs via the p38 MAPK signaling pathway. Indeed, the expression of both antimicrobial peptides (i.e., ALF, CRU, and PEN) and MKK4-p38-c-Jun cascade proteins (i.e., MKK4, p38, and c-Jun) was not induced after PvHMC depletion with or without pathogen challenge.

Generally, activation of the p38 MAPK pathway induces AMPs expression (He et al., 2013; Li et al., 2015; Wang et al., 2018), hence, knockdown of PvMKK4 or Pvp38 in penaeid shrimp attenuated the expression levels of ALF, CRU, and PEN (Figures 4J–4Q), an observation synonymous to PvHMC silencing with or without immune challenge (Figure 2). These data indicate a relationship between PvHMC and the MKK4-p38-c-Jun cascade because knockdown of *PvHMC* decreased the mRNA and protein levels of MKK4, p38, and c-Jun, coupled with decreased phosphorylation levels of MKK4 and p38. The consequence of *PvHMC* depletion on the p38 MAPK pathway was further manifested in reduced AMPs expression. Our data are similar to previous studies, including *Dscam* in the Chinese mitten crab (*E. sinensis*), which interacts with ERK to enhance AMPs production (Li et al., 2019b), and *P. vannamei* CDC42, a small GTPase of the Ras superfamily that modulates multiple intracellular signal transduction pathways including MAPK signaling pathway-related genes i.e., ERK, JNK, and p38 (Peng et al., 2015). Although no prior reports have implicated shrimp hemocyanin (PvHMC) in the induction of AMPs expression via p38 MAPK pathway

modulation, the fact that both *PvHMC* silencing and overexpression in *Drosophila* S2 cells affected p38 MAPK activation (Figures 4 and S3) clearly shows their relationship. Afterall, avian β -defensin 8 (AvBD8), which itself is an AMP, induces the expression of proinflammatory cytokines and chemokines in chicken macrophage cell line by activating the MAPK signaling pathway via ERK1/2 and p38 (Hong et al., 2020). Thus, hemocyanin, like other immune response proteins, can modulate the p38 MAPK pathway during penaeid shrimp immune response.

In both vertebrates and invertebrates, armadillo (ARM) repeat domain-containing proteins have been shown to interact with the MAPK-JNK-ERK1/2-mTOR pathway (Lim et al., 2019). Moreover, ARM repeat domain proteins are functionally very versatile (Tewari et al., 2010) and have been implicated in various innate and adaptive immune functions (Hu et al., 2017; Wang et al., 2013; Zhang et al., 2016, 2020). Thus, given that the MKK4-p38-c-Jun cascade is mainly modulated through protein-protein interactions (Kim et al., 2015; Roy et al., 2018), we reasoned that *PvHMC* might interact with the MKK4-p38-c-Jun cascade proteins through one of its multi-domains (i.e., N-, M-, and C-terminal domain). As anticipated, a direct interaction between the ARM repeat domain region (M-domain) of *PvHMC* and *PvMCK4* was observed, while deletion of the ARM repeat domain (*PvHMC-M-ΔARM*) attenuated this interaction (Figure 5). This is quite intriguing because in previous studies of shrimp hemocyanin protein, only the N- and C-domains have been linked to immune functions (Naresh et al., 2015; Qin et al., 2018; Zhang et al., 2017). Given that in penaeid shrimp, MKK4 (MAPKKs) is the upstream kinase that interacts with and phosphorylates p38 (MAPKs) (Wang et al., 2018), the interaction between *PvHMC* and *PvMCK4* and between *Pvp38* and *Pvc-Jun* indicates a regulatory role of *PvHMC* on the *PvMCK4-Pvp38-Pvc-Jun* cascade in penaeid shrimp. Afterall, the MAPK signaling pathway can be modulated by several factors to induce AMPs expression (He et al., 2013; Li et al., 2015; Wang et al., 2018); hence, *PvHMC* could be a previously unidentified factor that modulates the p38 MAPK pathway during antimicrobial immune response in penaeid shrimp.

From the foregoing, it is conceivable that the versatility of the ARM repeat domain endows *PvHMC* the ability to modulate the p38 MAPK signaling pathway to induce AMPs expression in penaeid shrimp. No similar findings have hitherto been reported in crustaceans, and therefore indicates that the multifunctionality of hemocyanin could be due to its ability to expand its functional repertoire through various mechanisms, including direct antimicrobial activity (Yan et al., 2011; Zhang et al., 2006, 2009b), generation of peptides with antimicrobial activity (Destoumieux-Garzon et al., 2001; Lee et al., 2003; Wen et al., 2016; Zhan et al., 2019; Zhang et al., 2004a), immunomodulatory activity (Havanapan et al., 2009; Yao et al., 2019; Yasuda and Ushio, 2016), and inducing AMPs production via the p38 MAPK signaling pathway (current study). Most importantly, our findings show that all domains of hemocyanin have immune modulatory activity in penaeid shrimp.

Collectively, our current data reveal that hemocyanin (*PvHMC*) interacts with *PvMCK4* via its ARM repeat domain to modulate the p38 MAPK signaling pathway to induce AMPs expression (Figure 6). These findings highlight the significance of hemocyanin as a crucial immune effector protein that expands its functional mechanisms by exerting direct or indirect antimicrobial activity through modulation of other immune response pathways in crustaceans.

Limitations of the study

This study demonstrates that penaeid shrimp hemocyanin (*PvHMC*) interacts specifically with MKK4 via its ARM repeat domain to modulate the p38 MAPK signaling pathway, thereby inducing the expression of antimicrobial peptides. While the effect of hemocyanin on the MAPK cascade proteins and AMPs expression was demonstrated using *in vivo* knockdown, the *in vitro* experiments were performed using *Drosophila* Schneider S2 cells, due to the absence of commercial shrimp cell lines. Given that *Drosophila* does not express hemocyanin constitutively, it would therefore be important to replicate this work when suitable shrimp cell lines become available. Similarly, some of the MAPK cascade proteins could not be determined due to the absence of suitable antibodies. Future work should therefore consider including all these proteins and performing the *in vitro* experiments with shrimp cell lines.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE

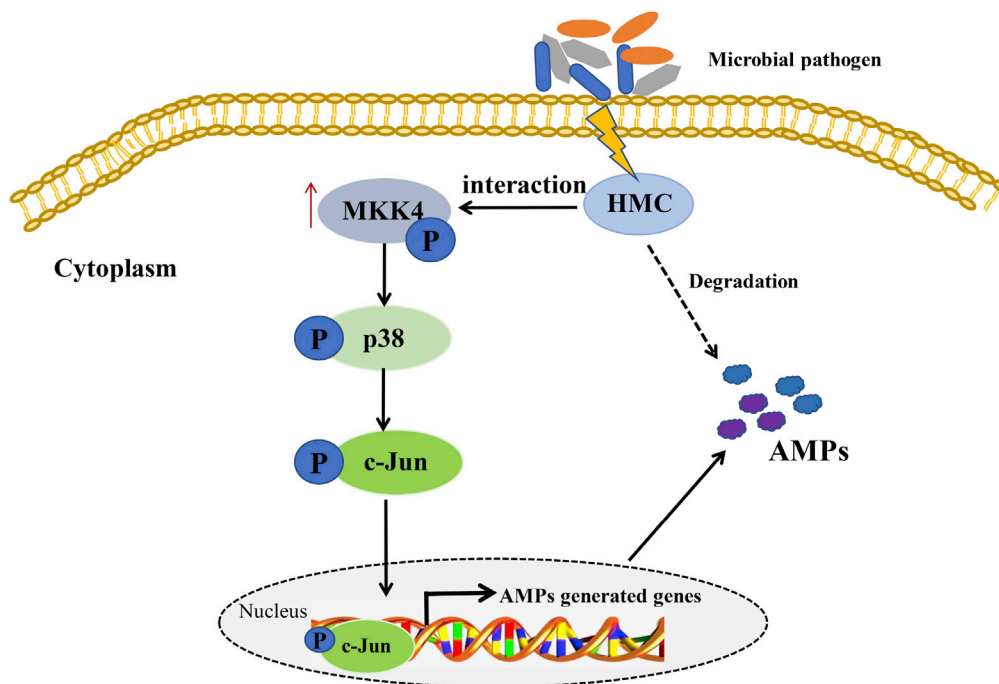


Figure 6. Diagrammatic summary of proposed mechanism by which hemocyanin (PvHMC) modulates p38 MAPK signaling during antimicrobial response in penaeid shrimp

Microbial pathogens induce PvHMC expression, promoting binding via its armadillo (ARM) repeat domain with MKK4 to activate (phosphorylate) p38 and c-Jun, which allows phosphorylated c-Jun to enter the nucleus to promote the transcription of antimicrobial peptide genes. PvHMC can also undergo proteolytic degradation in response to microbial pathogens to generate peptides with antimicrobial activity.

● **RESOURCE AVAILABILITY**

- Lead contact
- Materials availability
- Data and code availability

● **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

- Animals
- Challenge experiments

● **METHOD DETAILS**

- Total RNA extraction and cDNA synthesis
- qPCR analysis
- RNA interference experiments
- Plasmid constructions and *in silico* analysis of PvHMC sequence
- GST pull-down
- Cell culture and transfections
- Co-immunoprecipitation
- SDS-PAGE and Western blotting
- Statistical analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2022.103958>.

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AUTHOR CONTRIBUTIONS

Y. Z. and J. J. A. conceived and designed the experiments; Y. Z. and J. J. A. acquired funding; K. Z., J. F., and L. Y. performed the experiments; D. Y., F. W., X. C., S. L., and H. M. contributed reagents and analytic tools; Y. Z., J. J. A., F. W., and S. L. supervised the work; K. Z. and J. J. A. wrote the original draft; J. J. A., K. Z., and Y. Z. reviewed and edited the paper. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects. We worked to ensure diversity in experimental samples through the selection of the cell lines. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-tubulin antibody	Sigma-Aldrich	Cat# T6074; RRID:AB_477582
Mouse anti-FLAG antibody	Sangon Biotech	Cat# D110005
Rabbit anti-Phospho-p38 MAPK (Thr180/Tyr182) antibody	Cell Signaling	Cat# 9211; RRID:AB_331641
Rabbit anti-p38 antibody	This paper	N/A
Rabbit polyclonal anti-shrimp hemocyanin	Zhang et al. (2017)	N/A
Rabbit anti-Phospho-c-Jun (Ser73) (D47G9) antibody	Cell Signaling	Cat# 3270; RRID:AB_2129575
Rabbit anti-c-Jun antibody	This paper	N/A
Rabbit anti-Phospho-SEK1/MKK4(Ser257/Thr261) antibody	Cell Signaling	Cat# 9156; RRID:AB_2297420
Rabbit anti-SEK1/MKK4 antibody	Cell Signaling	Cat# 9152; RRID:AB_330905
Rabbit anti-His antibody	TransGen Biotech	Cat# HT501-01
Mouse anti-GST antibody	TransGen Biotech	Cat# HT601-01
Rabbit anti-V5 antibody	Sangon Biotech	Cat# D191104
Horse radish peroxidase (HRP)-linked goat anti-rabbit and goat anti-mouse	Thermo Fisher	Cat# 31460; RRID:AB_228341
Bacterial and virus strains		
<i>Vibrio parahaemolyticus</i>	Marine Culture Collection of China	MCCC 1A02609
<i>Staphylococcus aureus</i>	Lab isolated strain	N/A
White spot syndrome virus, WSSV	Lab isolated	N/A
Biological samples		
Penaeid shrimp (<i>Penaeus vannamei</i>)	Shantou Huaxun Aquatic Product Corporation (Guangdong, China)	N/A
Chemicals, peptides, and recombinant proteins		
Phenylmethylsulfonyl fluoride, PMSF	Beyotime Biotechnology	Cat# ST505
Phosphatase inhibitor cocktail	Roche	Cat# M7528
RealStar Green Power Mix	GenStar	Cat# A311-01
FuGENE HD Transfection Reagent	Promega	Cat# E2311
Protease inhibitor cocktail	Thermo Fisher	Cat# 87785
Critical commercial assays		
T7 RiboMAX™ Express RNAi System	Promega	Cat# P1700
RNAFast 200 kit	Feijie	Cat# 220011
EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit	TransGen Biotech	Cat# AT311
Experimental models: Cell lines		
<i>Drosophila Schneider</i> S2 cells, cell line	Laboratory of Jianguo He, Sun Yat-sen University, China	N/A
Oligonucleotides		
Primers for <i>in vitro</i> dsRNA synthesis, see Table S1	This paper	N/A
Primers for gene verification, see Table S1	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/
GraphPad Prism Version 5 for Windows	GraphPad Software	https://www.graphpad.com
Adobe Photoshop	Adobe	https://www.adobe.com

RESOURCE AVAILABILITY**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jude Juventus Aweya (jjaweya@stu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Original western blot images reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Animals**

Healthy *Penaeus vannamei* (size 6–8 g) obtained from Shantou Huaxun Aquatic Product Corporation (Shantou, Guangdong, China), were cultured at room temperature (~24°C) in recirculating water tanks filled with aerated seawater (1% salinity) and fed twice daily with commercial feed. Shrimp were acclimatized to laboratory conditions for at least three days, after which only healthy shrimp (i.e., active, well fed, had no high mortality without challenge or treatment, and with no unusual marks or spots on their body) were used for the experiments. All animal experiments were carried out per the guidelines and approval of the Animal Research and Ethics Committees of Shantou University, China.

Challenge experiments

Healthy 120 shrimp, randomly divided into four groups (n = 30), were injected intramuscularly via the third abdominal segment with 100 µL white spot syndrome virus WSSV (1×10^5 copies), *Vibrio parahaemolyticus* (1.0×10^5 CFU/g) or *Staphylococcus aureus* (1.0×10^5 CFU/g). Control group shrimps were injected with sterile PBS (100 µL). For challenge experiments after knockdown (72 h), shrimp were injected with 100 µL PBS with or with *V. parahaemolyticus* (1.0×10^5 CFU/g). At specific time points post-injection (i.e., 0, 12, 24, and 48 h), hepatopancreas samples were removed from five randomly selected shrimp per group for RNA extraction and cDNA synthesis. Hepatopancreas samples were also collected and processed for SDS-PAGE and Western blot analysis as previously described (Aweya et al., 2020). Briefly, hepatopancreas samples from 5 randomly selected shrimp in each group were homogenized in pre-cooled PBS containing 4x phenylmethylsulfonyl fluoride (PMSF) (Cat# ST505, Beyotime Biotechnology, Shanghai, China). Next, samples were centrifuged at 100 g for 7 min at 4°C to collect the cells, followed by washing five times with PBS before being lysed with IP lysis buffer containing protease and phosphatase inhibitor cocktail (Cat# M7528, Roche, IN, USA) and 2x PMSF. Cell lysates were centrifuged at 20,000 g for 20 min at 4°C to collect the supernatant, which were mixed with 5x loading buffer (42 mmol/L Tris-HCl, containing 100 mL/L glycerol, 23 g/L SDS, 50 g/L 2-mercaptoethanol and 0.02 g/L bromophenol blue), and boiled for 10 min before use.

METHOD DETAILS

Total RNA extraction and cDNA synthesis

Total RNA was extracted from shrimp hepatopancreas tissues using the RNA Fast 200 kit (Cat# 220011, Fei-Jie, China) according to the manufacturer's instruction. The total RNA concentration was quantified using NanoDrop 2000 spectrophotometer (Model# ND-ONE-W, Nano-drop Technologies, Wilmington, DE, USA), and the RNA quality checked on 1% agarose gel electrophoresis. Next, RNA samples were immediately used for cDNA synthesis with the TransScript™ One-step gDNA removal and cDNA Synthesis Super-Mix kit (Cat# AT311, TransGen Biotech, Beijing, China) following the manufacturer's instructions. The cDNA samples were either used immediately or stored at -20°C in aliquots for later use.

qPCR analysis

The quantitative polymerase chain reaction (qPCR) analysis used the RealStar Green Power Mix (Cat# A311-10, GenStar, Beijing, China) on a qTOWER 3G Real-Time PCR system (Model# 1016-67, Analytik Jena AG, Germany). The qPCR mixture contained 1 μL cDNA, 10 μL of 2x RealStar Green Power Mix, 10 nM of each primer, and ddH₂O to a total volume of 20 μL . The following program was used: one cycle at 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 30 s. Relative gene expression was calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen, 2001) with the EF1 α gene (EF1 α) as the internal control. Triplicate samples were analyzed per treatment for at least three independent experiments. The primer sequences used are listed in Table S1.

RNA interference experiments

Double-stranded RNAs (dsRNAs) targeting shrimp hemocyanin PvHMC (GenBank: X82502.1), PvMKK4 (GenBank: KY693644.1), Pvp38 (GenBank: JX990130.1), and Pvc-Jun (GenBank: KF999956.1) were synthesized by *in vitro* transcription using the T7 RiboMAX™ Express RNAi System kit (Cat# P1700, Promega, Madison, WI, USA) following the manufacturer's protocol. For control, dsRNA targeting the enhanced green fluorescent protein (EGFP) was produced in the same way. The gene-specific primers and amplicons sizes are shown in Table S1. In the RNAi experiments, the experimental group shrimps were injected with 100 μL (2 $\mu\text{g/g}$ shrimp) of the respective dsRNA, while control group shrimps were injected with an equivalent amount of dsEGFP. Hepatopancreas tissues were collected at different time points from five randomly selected shrimps per group for total protein and RNA extraction as described above.

Plasmid constructions and *in silico* analysis of PvHMC sequence

The functional domains of PvHMC were predicted with the online simple modular architecture research tool (SMART) (<http://smart.embl-heidelberg.de>). His-tagged proteins of PvMKK4 (GenBank: KY693644.1), PvERK (GenBank: KC896761.1), PvJNK (GenBank: JN035903), and Pvp38 (GenBank: JN035902.2) were subcloned into the pET-28a (Cat# 69864-3, Novagen, Thermo Fisher Scientific, Waltham, MA, USA), whereas GST-tagged fusion proteins of the N-, M- (i.e., armadillo (ARM) repeat domain region), M-domain with deleted ARM, and C-domains of shrimp hemocyanin PvHMC (GenBank: X82502.1) and Pvc-Jun (GenBank: KF999956.1) were subcloned into the pGEX-6p-1 vector (Cat# 27-4597-01, GE, Boston, MA, USA) for prokaryotic expression. For eukaryotic expression, the open reading frame (ORF) of PvHMC with FLAG-tag at the C-terminal was subcloned into the pIZ/V5-His vector (Cat# V8000-01, Invitrogen, Carlsbad, CA, USA) at KpnI and XhoI restriction sites to generate the pIZ-PvHMC-FLAG plasmid. Similarly, PvERK, PvJNK, Pvp38, and PvMKK4 were subcloned into the pIZ/V5-His vector to generate pIZ-PvERK-V5, pIZ-PvJNK-V5, pIZ-Pvp38-V5, and pIZ-PvMKK4-V5 plasmids that express the respective V5-tagged proteins. All primers used for constructing these expression plasmids are listed in Table S1.

GST pull-down

Pull-down and Western blot analyses were performed as described previously (Yao et al., 2019). Briefly, purified GST, GST-PvHMC, and GST-Pvc-Jun were incubated with His-PvERK, His-PvJNK, His-Pvp38, or His-PvMKK4 at 4°C , respectively for 2 h. Next, samples were washed ten times with 0.01 M PBS (plus 1% Triton) before being analyzed by SDS-PAGE and Western blot. As input control, 5% of the purified proteins were analyzed by Western blot.

Cell culture and transfections

Drosophila Schneider S2 cells (kind gift from Prof. Jianguo He, Sun Yat-sen University, Guangzhou, China) were cultured at 27°C in Schneider's *Drosophila* Medium (Cat# 21720-024, Invitrogen, Carlsbad, CA, USA)

supplemented with 10% fetal bovine serum (Cat# 10091148, Gibco Life Technologies, Grand Island, NY, USA). Confluent cells were seeded onto 24-well plates (Cat# 725021, NEST Biotechnology, Shanghai, China) at a density of 1×10^6 cell/mL per well in a volume of 500 μ L medium. At 60–80% confluence, cells were transfected with 250 ng of pIZ-PvHMCs-EGFP-Flag plasmid or with an equal amount (250 ng) of pIZ-EGFP-Flag using the FuGENE HD Transfection Reagent (Cat# E2311, Promega, Madison, WI, USA) according to the manufacturer's instructions. At 48 h post-transfection, cells were harvested and washed three times with pre-cooled PBS before being lysed in IP Lysis Buffer (Cat# CW2334S, Pierce) containing protease inhibitor cocktail (Cat# 87785, Thermo Fisher Scientific, Waltham, MA, USA). Cell lysates were centrifuged at 20,000 g for 20 min at 4°C to collect the supernatant. Next, lysates were mixed with 5 \times loading buffer, boiled for 10 min before being analyzed by SDS-PAGE and Western blot as previously described (Aweya et al., 2020).

Co-immunoprecipitation

To examine protein-protein interactions between the different proteins, co-immunoprecipitation analysis was performed. Briefly, pIZ-PvERK-V5, pIZ-PvJNK-V5, pIZ-Pvp38-V5, and pIZ-PvMKK4-V5 were each co-transfected with pIZ-PvHMC-FLAG or pIZ-EGFP-FLAG (as a control) into *Drosophila* S2 cells. At 48 h post-transfection, cells were harvested and washed three times with ice-cold PBS before being lysed in IP Lysis Buffer containing protease inhibitor cocktail (Cat# 87785, Thermo Fisher Scientific, Waltham, MA, USA). Next, cell lysates were used for co-immunoprecipitation analysis with anti-FLAG® M2 beads (Cat# M8823, Sigma-Aldrich, St Louis, MO, USA) as described previously (Wang et al., 2018). Samples were then subjected to SDS-PAGE and Western blot analysis, with 5% of each cell lysate used as input control.

SDS-PAGE and Western blotting

Samples prepared as described above were separated on SDS-PAGE before being transferred onto polyvinylidene fluoride (PVDF) membranes (Cat# R0NB30936, Millipore, Billerica, MA, USA) with the Mini Trans-Blot cell wet transfer system (Model# 1658030, Bio-Rad, Richmond, CA, USA) according to the manufacturer's protocol. Next, membranes were blocked for 2 h at room temperature with 5% skimmed milk dissolved in Tris buffer solution with Tween (TBST) (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4), followed by incubation with the respective primary antibodies at 4°C for 12 h. After being washed four times (10 min each) with TBST, membranes were then incubated with the corresponding secondary antibodies for 1 h at room temperature before being washed four times (10 min each) with TBST. Signals were detected by chemiluminescence using enhanced chemiluminescence (ECL) reagent (Cat# WBLUF0100, Millipore, Billerica, MA, USA) and captured on the Amersham Imager 600 (Model# V1.0.0, GE, Boston, MA, USA).

Statistical analysis

Results are presented as mean \pm S.D. The statistical significance between two measurements was determined by Student's t test and the p values indicated by asterisks. Significance was considered at $p < 0.05$.