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Review

Antiviral defense in shrimp: From innate immunity to viral infection

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ABSTRACT

The culture of penaeid shrimp is rapidly developing as a major business endeavor worldwide. However, viral diseases have caused huge economic loss in penaeid shrimp culture industries. Knowledge of shrimp innate immunity and antiviral responses has made important progress in recent years, allowing the design of better strategies for the prevention and control of shrimp diseases. In this study, we have updated information on shrimp antiviral immunity and interactions between shrimp hosts and viral pathogens. Current knowledge and recent progress in immune signaling pathways (e.g., Toll/IMD-NF-κB and JAK–STAT signaling pathways), RNAi, phagocytosis, and apoptosis in shrimp antiviral immunity are discussed. The mechanism of viral infection in shrimp hosts and the interactions between viruses and shrimp innate immune systems are also analyzed.

Contents

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1. Introduction

Penaeid shrimp are valuable commercial and aqua-cultured marine species. The Pacific white shrimp *Litopenaeus vannamei* and the black tiger shrimp *Penaeus monodon* are the mainly cultured penaeid shrimps worldwide (Tassanakajon et al., 2013). However, various diseases caused by shrimp pathogens, such as the white spot syndrome virus (WSSV), yellow head virus (YHV), Taura syndrome virus (TSV), and bacteria from the genus *Vibrio*, have led to massive mortality and great loss in the shrimp cultivation industry (Lightner, 2011; Tassanakajon et al., 2013). WSSV, YHV, and TSV are serious shrimp viruses that cause serious economic losses (Lightner, 2011; Tassanakajon et al., 2013). Viral diseases are the main causes of shrimp culture industry losses. Thus, viral diseases have prompted researchers to study the antiviral immunity of shrimp and interactions between shrimp and viral pathogens.

As arthropod species, shrimp mainly rely on the innate immune system, which consists of humoral and cellular responses against viral infections (Li and Xiang, 2013). The direct or indirect recognition of pathogens or pathogen-associated molecular patterns (PAMPs) by germline-encoded proteins called pattern recognition receptors (PRRs) (e.g., Toll receptors) leads to rapid humoral and cellular immune responses (Li and Xiang, 2013). The shrimp innate immune system and diseases caused by shrimp pathogens were recently studied by other groups (Li and Xiang, 2013; Tassanakajon et al., 2013). Our review will discuss current knowledge and recent progress in the study of shrimp antiviral immunity, shrimp–virus interactions, and viral infection and replication mechanisms.

2. Immune signaling pathways

As an arthropod, the basic framework of the shrimp immune system shows high similarities to that of insects (Li and Xiang, 2013; Tassanakajon et al., 2013). The innate immune system of *Drosophila* was well-studied (Lemaitre and Hoffmann, 2007). Most of the immune-related genes are believed to be regulated by the Toll and IMD pathways (De Gregorio et al., 2002). In *Drosophila* and other insects, the Toll and IMD pathways have important functions in antiviral immunity (Kemp and Imler, 2009; Liu et al., 2009a; Sabin et al., 2010; Xi et al., 2008; Xu and Cherry, 2014; Zambon et al., 2005). The JAK–STAT pathway is also required for *Drosophila* antiviral responses (Dostert et al., 2005). The RNA interference (RNAi) pathway is believed to be the main player in insect antiviral immunity (Kemp and Imler, 2009; Sabin et al., 2010; Xu and Cherry, 2014). These signaling pathways have been characterized in recent years, and their functions in shrimp antiviral responses have been studied.

2.1. Toll/IMD-NF-κB signaling pathway

The function of Toll receptor in innate immunity was first characterized in *Drosophila*. Eventually, more Toll-like receptors (TLRs) that can function as PRRs were reported in mammals (Akira et al., 2006; Rämet and Hultmark, 2014). Vertebrate TLRs can directly recognize PAMPs; however, invertebrate TLRs, such as *Drosophila* Tolls, do not directly recognize pathogens but through the binding to cytokine-like ligand Spätzle (Lemaitre and Hoffmann, 2007; Li and Xiang, 2013; Wang et al., 2012). After microbial infections, a proteolytic cascade cleaves the endogenous Toll ligand Spätzle to generate a mature form. This mature ligand binds to the Toll receptor, activating the NF-κB family protein Dif/Dorsal (Lemaitre and Hoffmann, 2007; Li and Xiang, 2013; Wang et al., 2012). Activated Dorsal is translocated to the nucleus and promotes the expression of immune-related genes, such as antimicrobial peptide genes (AMPs) (Lemaitre and Hoffmann, 2007). However, *Drosophila* Toll-7 can directly interact with vesicular stomatitis virus as a PRR similar to mammalian TLRs, and subsequently induce antiviral autophagy (Wang and Wang, 2013a).

A recent study reported that CpG ODNs can directly interact with shrimp Tolls and induce hemocytic immune responses (Sun et al., 2013). Although no components of the *Drosophila* Toll pathway has been identified as the direct detector of viruses, certain viruses, such as *Drosophila* X virus still can activate the Toll-Dif/Dorsal pathway and induce AMP expression. The mutants of NF-κB family protein Dif are more sensitive to viral infection (Ramirez and Dimopoulos, 2010; Sabin et al., 2010; Zambon et al., 2005). Similar effects were also observed in *Aedes aegypti* mosquitoes infected with the dengue virus (Xi et al., 2008).

The Spätzle/Tolls/MyD88/Pelle/TRAF6/Dorsal signaling pathway has been characterized in shrimp (Li and Xiang, 2013; Wang et al., 2011a,b, 2012, 2013b,d; Wang and Wang, 2013a). This signaling pathway can activate both *Drosophila* and shrimp AMP promoters in *Drosophila* S2 cells (Wang et al., 2011a,b, 2012). Using double-stranded RNA (dsRNA)-mediated gene silencing, researchers demonstrated that this signaling pathway can regulate the expression of shrimp AMPs, including penaeidins (PENs), crustins, anti-lipopolysaccharide factors (ALFs), lysozymes, *Vibrio penaeicidae*-induced cysteine and proline-rich peptides in vivo (Wang et al., 2013b,d).

Three types of shrimp Tolls have been reported in the literature, namely, Toll1, Toll2, and Toll3. *L. vannamei* Toll1 (LvToll1) and LvToll3 were localized in the membrane and cytoplasm, whereas LvToll2 was only confined to the cytoplasm (Wang et al., 2012). LvToll2 can significantly activate the promoters of AMPs, whereas LvToll1 and LvToll3 have no effect on them (Wang et al., 2012). In the gill, LvToll1, LvToll2, and LvToll3 were upregulated after WSSV challenge (Wang et al., 2012). LvToll2 can also be upregulated by TSV infection (Sookruksawong et al., 2013). Whether the cytoplasmic localized LvToll2 can detect viruses in cells via the endocytosis pathway, similar to the mechanism in mammals, should be studied. Shrimp Spätzles were also upregulated in the gill after WSSV infection. Injecting the recombinant protein of the C-terminal active domain of shrimp Spätzle into crayfish upregulates the expression of AMPs, suggesting functional Spätzle–Toll signaling in shrimp (Li and Xiang, 2013; Wang and Wang, 2013a).

The immune deficiency (IMD) pathway in *Drosophila* is similar to the vertebrate TNFR pathway. The TNFR pathway functions parallel to the Toll pathway to activate immune responses to Gram-negative bacteria (Lemaitre and Hoffmann, 2007). Some viruses, such as Sindbis and cricket paralysis viruses, also activate the IMD pathway and induce AMP expression (Avadhanula et al., 2009; Costa et al., 2009). Shrimp have a similar IMD pathway and regulate AMP expression. Components of the shrimp IMD pathway, such as LvIKKs, IMD (including LvIMD, FcIMD, and PcIMD), and LvRelish, have been characterized (Lan et al., 2013; Li and Xiang, 2013; Wang et al., 2013a). When LvIMD was overexpressed in *Drosophila* S2 cell, the AMP promoters were activated (Wang et al., 2009). When shrimp IMD was silenced, the expression levels of AMPs, including PENs, crustins, ALFs, and lysozymes, dramatically decreased (Lan et al., 2013).

In *Drosophila*, IMD signaling bifurcates downstream of TAK1, activating both the JNK–cJUN and IKK–Relish signaling pathways (Lemaitre and Hoffmann, 2007) (Fig. 1). In *Drosophila* S2 cells, JNK-dependent regulation of immune genes, including AMPs and cytoskeleton remodeling proteins, has a key function in hemocyte activation (Lemaitre and Hoffmann, 2007). In shrimp, JNK has been cloned and characterized. In the early stage of WSSV infection, LvJNK expression/phosphorylation increases, suggesting that

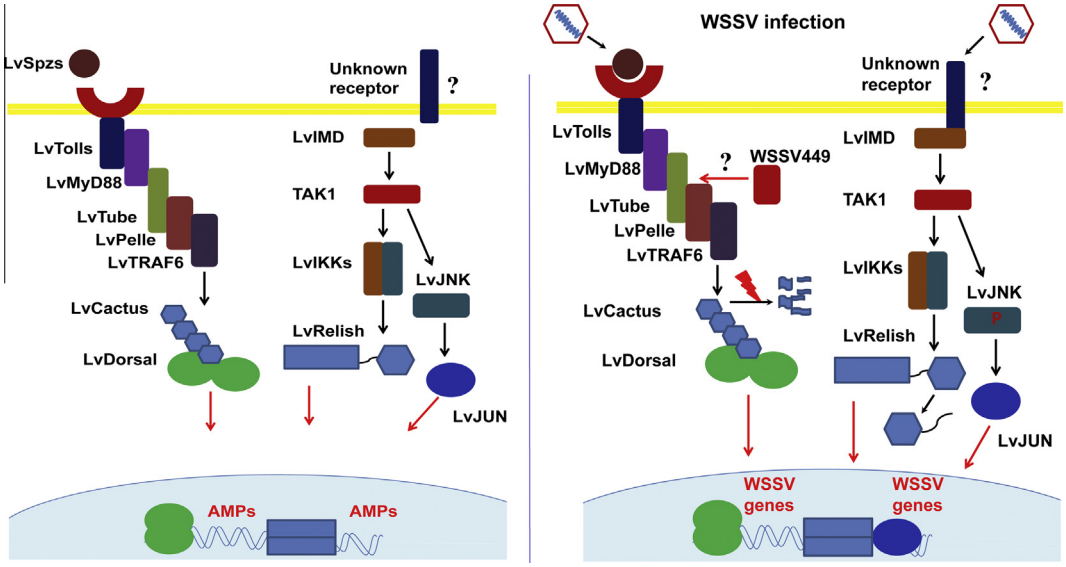

Fig. 1. Toll/IMD-NF-κB signaling pathways in AMP regulation and their interactions with WSSV infection. Shrimp Toll/IMD-NF-κB signaling pathways regulate the expression levels of immune-related genes, such as AMPs. When Toll or IMD was silenced using RNAi, the expression of many AMPs decreased. Stimulated by WSSV infection, shrimp NF-κB factor LvDorsal was activated and translocated to the nucleus, and shrimp NF-κB factor LvRelish could bind to the NF-κB sites of *ie1* promoter and induce *ie1* expression. In a screening of 147 WSSV promoters, WSSV051, WSSV059, WSSV069 (ie1), WSSV083, WSSV090, WSSV107, WSSV244, WSSV303, WSSV371, and WSSV445 are activated by shrimp IKK-NF-κB signaling (Wang et al., 2013a). To modulate the shrimp NF-κB system, WSSV encoded a shrimp Pelle-like protein WSSV449, which shows similarity to insect tubes and may be involved in shrimp Toll-NF-κB signaling pathway activation (Wang et al., 2011a). The Toll/IMD-NF-κB signaling pathways are activated in response to WSSV infection, and the replication of WSSV genes can benefit from their activation.

WSSV infection can activate the IMD–JNK signaling pathway (Shi et al., 2012). Some shrimp AMPs regulated by Toll/IMD pathways possess direct antiviral activities (Liu et al., 2006; Tharntada et al., 2009; Woramongkolchai et al., 2011). Therefore, shrimp Toll and IMD are activated after WSSV and TSV infections, and are involved in innate antiviral responses.

2.2. Interaction between Toll/IMD pathways and WSSV infection

In *Drosophila*, some viral infections can activate the Toll/IMD pathways and induce the expression of immune-related genes. Some virus have developed strategies to block the Toll/IMD-NF-κB signaling pathway at the NF-κB level, which indicates that that these signaling pathways are important in insect antiviral responses (Bitra et al., 2012). The immune signaling pathways are common targets of various viruses to enhance viral replication (Huang et al., 2010; Wang et al., 2011a, 2013b). Viruses, such as HIV-1, hijack and stimulate the host NF-κB signaling pathway as part of their life cycle, and divert NF-κB immune regulatory functions to favor viral replication. NF-κB-binding sites are present in the promoter regions of different classes of genes in viruses, such as HIV-1, xenotropic murine leukemia virus-related virus (XMRV), cytomegalovirus (CMV), herpes virus, hepatitis B virus (HBV), and Epstein–Barr virus (EBV) (Wang et al., 2011a). In HIV-1-infected cells, the activation of NF-κB signaling promotes long terminal repeat (LTR)-driven viral transcription. NF-κB-binding sites during the HIV-1 life cycle are required for HIV transcription in some cell types (Wang et al., 2011a). NF-κB activation can also markedly increase XMRV replication through the NF-κB-binding sites in the LTR of XMRV (Wang et al., 2011a).

Similar to the aforementioned viruses, a successful WSSV infection relies on the effective activation of the NF-κB pathway. When the components of the Toll/IMD-NF-κB signaling pathways, such as LvIKKs or LvJNK, are silenced or inhibited, WSSV replication is reduced and shrimp mortality is decreased (Shi et al., 2012; Wang et al., 2013b). Knock down of *LvIKKβ* and *LvIKKε* using dsRNA-mediated gene silencing in shrimp can delay the outbreak time of WSSV infection. Stimulated by WSSV infection, shrimp NF-κB family protein Dorsal is activated and translocated into the nucleus (Wang et al., 2011a). Another study showed that the activation of LvJNK occurs after WSSV infection (Shi et al., 2012). LvJNK silencing mediated by specific dsRNA in shrimp significantly inhibits WSSV proliferation. Moreover, inhibition of the shrimp JNK signaling pathway by a specific inhibitor results in the reduction of WSSV replication and delay of WSSV gene transcription (Shi et al., 2012). These results indicated that the shrimp NF-κB and JNK pathways are activated by WSSV infection, and WSSV replication can benefit from the activation of the said pathways (Shi et al., 2012).

WSSV may have learned how to benefit from the activation of host Toll and IMD signaling pathways. Several studies reported that the promoters of some WSSV genes contain binding sites for NF-κB and AP1 (or cJUN), which are downstream transcription factors of Toll/IMD pathways (Wang et al., 2011a, 2013b). Electrophoresis mobility shift assays (EMSAs) revealed that shrimp NF-κB factor Relish can bind to the NF-κB sites of WSSV immediately early 1 (ie1) promoter and induce its expression (Huang et al., 2010). NF-κB activation is an early fundamental step of immune activation. The upregulation of WSSV ie1 promoter activity by NF-κB can increase ie1 gene expression, which may further activate other viral genes and be advantageous to the virus infection cycle (Huang et al., 2010). Activation of Toll/IMD pathways in *Drosophila* S2 cells can induce the promoter activities of many WSSV genes, such as WSSV069 (ie1). In the screening of 147 viral gene promoters, WSSV051, WSSV059, WSSV069, WSSV083, WSSV090, WSSV107, WSSV244, WSSV303, WSSV371, and WSSV445 were found to be activated by shrimp IKK-NF-κB signaling pathways (Wang et al., 2013b).

WSSV can actively modulate the expression levels of shrimp Toll/IMD pathway members. In the early stage of infection, some host genes, such as LvTRAF6 and LvJNK, are upregulated to activate Toll and IMD pathways (Shi et al., 2012; Wang et al., 2011b). By contrast, some studies found that after *Vibrio alginolyticus* injection, expression levels of Toll/IMD pathway members decrease in the early stage of infection. *V. alginolyticus* possibly suppresses the activities of Toll/IMD pathways to attenuate host innate

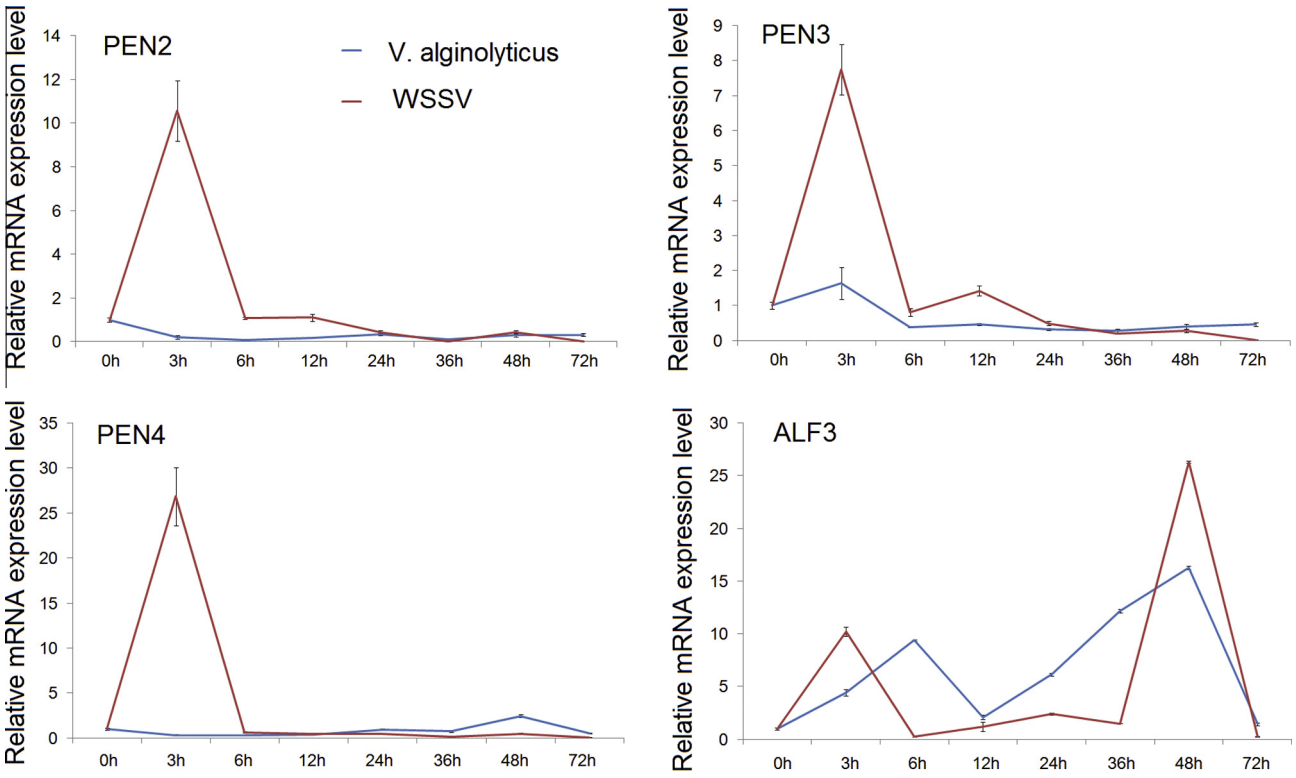

Fig. 2. Expression levels of *L. vannamei* PEN2, PEN3, and PEN4 after WSSV and *V. alginolyticus* infections in hemocytes. The samples were prepared as previously described (Wang et al., 2013a). The relative standard curve method was used to analyze target gene expression by qPCR. Expression values were normalized to those of LvEF-1α. Gene expression level in hemocytes at 0 h was set to 1.0. At the early stage of WSSV infection, shrimp AMPs *PEN2*, *PEN3*, and *PEN4* controlled by the Toll/IMD-NF-κB signaling pathways were upregulated, and their expression levels were later inhibited. Another shrimp AMP *ALF3*, which may not be directly regulated by shrimp Toll/IMD-NF-κB signaling pathways, showed a different expression pattern. This phenomenon was not observed in *V. alginolyticus*-infected shrimp. WSSV may learn how to turn on or off shrimp Toll/IMD-NF-κB signaling pathways to benefit its replication.

immune responses. To manipulate shrimp NF-κB activation, WSSV encodes a shrimp Tube-like protein WSSV449, which may be involved in shrimp Toll pathway activation (Wang et al., 2011a). WSSV449 can function similar to shrimp Tube to activate NF-κB signaling and induce AMP promoter activities. In this study, we detected the expression levels of shrimp *PEN2-4* regulated by Toll/IMD-NF-κB signaling pathways in hemocytes after exposure to WSSV and *V. alginolyticus* infection. We found that *PEN2-4* was greatly induced at the early stage (3 h post-infection) of WSSV infection and suppressed 6 h post-infection (Fig. 2). Therefore, in the early stage of WSSV infection, shrimp Toll/IMD-NF-κB signaling pathways were activated.

After WSSV infection, Toll and IMD pathways in shrimp are activated and AMPs are induced. Some AMPs and other immune-related genes, such as ALFs, PENs, and PmAV, possess direct antiviral activities, including anti-WSSV activity (Liu et al., 2006; Tharntada et al., 2009; Wang and Wang, 2013b; Woramongkolchai et al., 2011). Researchers question how WSSV balances replication and NF-κB activation-induced AMP upregulation. Several studies found that at the early stage of WSSV infection, the Toll/IMD-NF-κB signaling pathways are activated (Fig. 2). The hallmark of NF-κB activation is the induced expression of AMPs (PENs) and some viral i.e., genes, which are targets of the Toll/IMD-NF-κB signaling pathways. The expression of viral i.e., genes can be initiated through NF-κB activation. The i.e., genes may further activate the expression of other viral genes, and may be advantageous to the WSSV infection cycle. At 6 h post-infection, the expression of shrimp AMPs decreased (Fig. 2), suggesting the shutdown of Toll/IMD-NF-κB signaling pathways.

The suppression mechanism of Toll/IMD-NF-κB signaling pathways can be explained by the protective mechanism of the host immune system, which prevents WSSV viral gene expression, and actively inhibiting the Toll/IMD-NF-κB signaling pathways by WSSV to reduce the expression of shrimp AMPs, which can damage replication. Similar to other viruses, such as HIV-1, XMRV, CMV, HBV, and EBV, WSSV has learned how to activate (by WSSV449/other unknown factors) and inhibit (by unknown viral factors) shrimp Toll/IMD-NF-κB signaling pathways to benefit viral replication. We believe that at the early stage of WSSV infection, immune signaling pathways such as the Toll/IMD-NF-κB signaling pathways are activated. Simultaneously, viral early genes (e.g., *ie1*) are expressed, which then regulate the expression of other viral genes, including some genes that can inhibit immune signaling pathways. Thus, the activated NF-κB pathway, which can induce AMPs and other immune genes that are harmful to viruses, will be shut down by later expressed viral genes.

2.3. JAK–STAT signaling pathway

In vertebrates, viral infections detected by PRRs (e.g., TLRs) will trigger inflammation and type I interferon (IFN) responses, which are hallmarks of the host innate immune system in defending against viral infections. Type I IFNs can activate JAK–STAT signaling pathways via a type I IFN receptor, leading to the production of interferon-stimulated genes (ISGs) (de Veer et al., 2001; Sadler and Williams, 2008). ISGs, such as IRFs, dsRNA-dependent protein kinase PKR, ADAR (adenosine deaminase, RNA-specific), and 2′5′-oligoadenylate synthase, mediate the inhibition of viral replication, clearance of virus-infected cells, and induction of non-specific antiviral responses (de Veer et al., 2001; Sadler and Williams, 2008).

In *Drosophila*, the JAK–STAT signaling pathway is also required for host antiviral responses (Dostert et al., 2005). Cytokine activa-

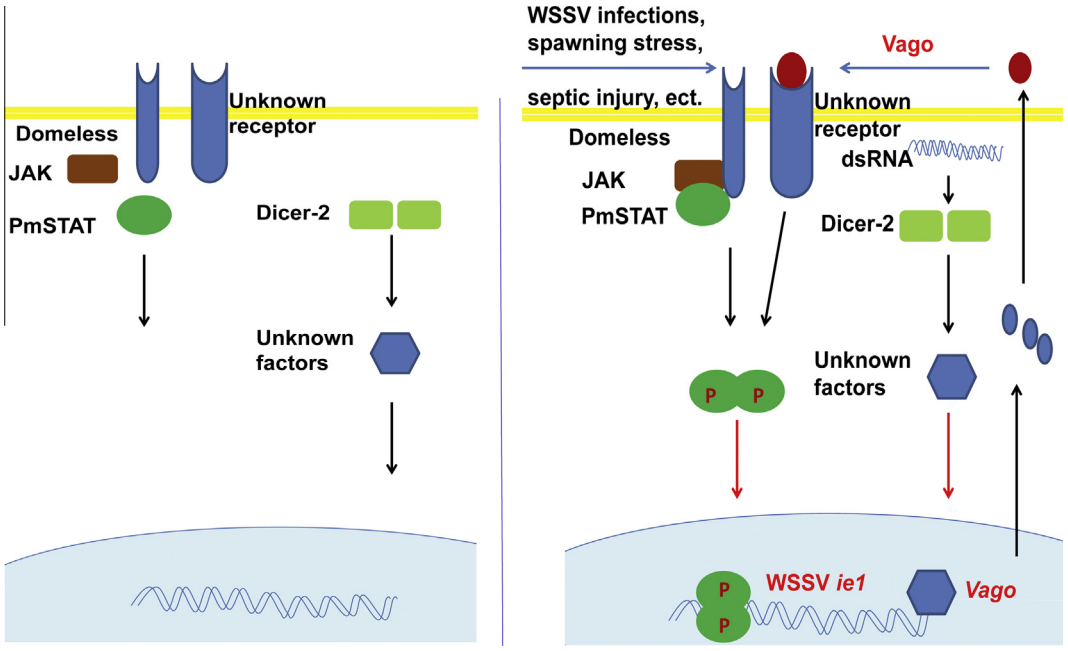

Fig. 3. Activation of shrimp JAK/STAT pathway and its contribution to WSSV infection. After WSSV infection, the phosphorylation level of PmSTAT increased, and the activated STAT was translocated from the cytoplasm to the nucleus. The activated STAT can bind to the promoter region of WSSV *ie1* through the STAT-binding motif, thereby enhancing its transcription (Liu et al., 2007). PmSTAT directly transactivates WSSV *ie1* gene expression and contributes to its high promoter activity. WSSV successfully annexed the shrimp JAK/STAT pathway to enhance the expression of viral *ie1*.

tion through the JAK–STAT pathway of numerous genes has been suggested in countering viral infections in *Drosophila* (Dostert et al., 2005; Lemaitre and Hoffmann, 2007). In *Drosophila* and mosquitoes, knockdowns and mutations of JAK led to an increase in viral infection, indicating an antiviral response involving the JAK–STAT pathway, presumably as a result of STAT-dependent expression of antiviral effectors, such as *vir-1*. The JAK–STAT signaling pathways are also considered as part of the antiviral response in other insects (Xu and Cherry, 2014). *Vago* was recently reported to function as an IFN-like cytokine in antiviral immune responses through the JAK–STAT signaling pathway. *Vago* is a virus-inducible gene, whose expression relies on Dicer-2. Dicer-2 can activate an unknown signaling pathway in the presence of viral dsRNA to induce *Vago* expression (Deddouche et al., 2008; Takeuchi and Akira, 2008). In *Culex* mosquito, a similar Dicer-2-Vago signaling pathway was also found. The authors showed that Vago is secreted from cells infected with the West Nile virus (WNV) infection, and induces an antiviral state in uninfected cells. Secreted Vago restricts WNV infection in *Culex* cells by activating the JAK–STAT pathway and upregulating the expression of STAT-dependent virus-inducible gene *vir-1* (Kingsolver and Hardy, 2012; Paradkar et al., 2012).

A similar JAK–STAT signaling pathway was also characterized in shrimp (Chen et al., 2008; Lin et al., 2012; Okugawa et al., 2013) (Fig. 3). The expression of the members of the JAK–STAT signaling pathway, such as SOCS and STAT, is induced by WSSV or poly(I:C) (Okugawa et al., 2013). After WSSV infection, an increased level of phosphorylated (activated) STAT in the lymphoid organ of shrimp was detected (Chen et al., 2008) (Fig. 3). In WSSV-infected shrimp, activated STAT was translocated from the cytoplasm to the nucleus in the primary culture cells of shrimp. Shrimp STAT is activated in response to WSSV infection (Chen et al., 2008). However, instead of inhibiting or disrupting STAT activation, WSSV exploits the host STAT by using it to bind to the promoter region of WSSV *ie1* to enhance *ie1* transcription (Liu et al., 2007). An element containing a STAT-binding motif of *ie1* promoter is important for promoter activity. Using EMSA, a specific shrimp STAT-DNA complex was detected. Levels of activated STAT were higher in WSSV-infected *P. monodon* than those in WSSV-free *P. monodon*.

A transactivation assay of the WSSV *ie1* promoter demonstrated that increasing the level of shrimp STAT leads to dose-dependent increases of *ie1* promoter activity (Liu et al., 2007). Therefore, shrimp STAT directly transactivates *ie1* expression and contributes to high promoter activity. WSSV ie1 protein also exhibits transactivation, dimerization, and DNA-binding activity, which may contribute to the replication of WSSV in host cells (Liu et al., 2009a). A recent study reported that stress increases activated STAT, which subsequently triggers WSSV replication in WSSV-infected brooders (Lin et al., 2012). This result may explain why WSSV is easier to outbreak in pawning stress. All these studies indicated that WSSV successfully annexes a putative shrimp defense mechanism via the JAK–STAT pathway to enhance the expression of *ie1*, and contributes to viral replication.

3. RNAi pathway

RNAi, mediated by small RNAs that are 21–30 nucleotides in length, has emerged as a key regulator in invertebrate antiviral immunity (Bartel, 2004). Numerous studies have revealed that the administration of artificial dsRNA/siRNA to shrimp can provide an effective protection against virus invasion, suggesting that an intact RNAi pathway in crustacean organisms has an important function in the immune response to viral infection (Tirasophon et al., 2005; Xu et al., 2007; Yodmuang et al., 2006). Although small noncoding RNAs from some crustaceans have been sequenced using high-throughput sequencing (Huang et al., 2012a; Yang et al., 2012), their functions remain poorly understood. Understanding the molecular mechanisms underlying RNAi in crustaceans will help researchers apply RNAi-based techniques to control various viral diseases in crustacean species in marine aquaculture.

Recent research efforts have focused on the identification and functional characterization of shrimp RNAi pathway components, such as Dicer1, Dicer-2, HIV-1 transactivating response RNA-binding protein, eukaryotic initiation factor 6, and several members of the Argonaute family (Labreuche and Warr, 2013). Although con-

siderable progress has been made in the characterization of RNAi machinery in penaeid shrimps, many issues still need to be addressed (Labreuche and Warr, 2013).

3.1. siRNA-directed antiviral defense in shrimp

RNAi mediated by siRNA or long dsRNA is an evolutionarily conserved mechanism in eukaryotes that has proven to be a natural antiviral mechanism in plants, invertebrates, and mammalian cells (Ding and Voinnet, 2007). The RNAi technique is an alternative and more effective approach to counteract viral infections in shrimp (Xu et al., 2007). Administration of dsRNA or siRNA to virus genes can inhibit virus replication in vivo, as well as disease progression. These effects were confirmed in different viruses, including YHV, IMNV, TSV, PmDNV, and WSSV. In WSSV, virus replication is efficiently suppressed with the injection of sequence-specific dsRNA/siRNA targeting VP19, VP28, or VP281 in shrimp (Kim et al., 2007; Xu et al., 2007). The dsRNA targeting PmDNV ns1 (nonstructural 1) or vp gene (structural gene) suppresses virus replication in *P. monodon* (Attasart et al., 2011). The inhibition of YHV replication is observed by cognate dsRNA, and contributes to lower mortality in black tiger shrimp (Tirasophon et al., 2007; Yodmuang et al., 2006). Although these experiments strongly suggested the important function of RNAi in the antiviral protection in shrimp, whether RNAi is employed as a natural antiviral immune strategy of animals to defend the infection of different viruses remains uncertain.

The dsRNA intermediates are likely formed during the genome replication of positive-strand RNA viruses (e.g., TSV and YHV) and DNA viruses (e.g., WSSV). The dsRNA precursors are presumably generated from intramolecular interactions within virus transcripts or bi-directional transcripts. These dsRNAs will potentially engage the shrimp RNAi pathway and trigger effective antiviral responses. An antiviral siRNA (vp28-siRNA) accumulated in the organs and cells of WSSV-infected shrimp. The results reveal that the biogenesis and function of vp28-siRNA were dependent on shrimp Dicer2 and Ago2 proteins, respectively. The inhibition of vp28-siRNA production by knockdown of Dicer2 expression or blocking of vp28-siRNA action with antisense oligonucleotide results in a significant increase in WSSV genomic copies at 24–48 h post-infection (Huang and Zhang, 2013). The present study highlights a novel aspect of the siRNA pathway in the immune response of animals against the attack of DNA viruses.

3.2. Antiviral miRNAs in crustaceans

Given that miRNAs lin-4 and let-7 were first identified in *Caenorhabditis elegans* as potential regulators of animal development, a total of 24,521 miRNAs from various organisms have been deposited in miRBase (Release 20.0, June 2013), including mammals, plants, insects, nematodes, and viruses (Carthew and Sontheimer, 2009; Ding and Voinnet, 2007). Among the deposited miRNAs, 55 are crustacean miRNAs. High-throughput sequencing of the small RNAs of WSSV-infected shrimp at various times post-infection revealed 63 host miRNAs, of which 48 are highly conserved in animals (Huang et al., 2012a). Compared with miRNAs in virus-free shrimp, 31 miRNAs displayed differential expressions in response to WSSV infection. Our results indicate that 25 miRNAs were up-regulated and six miRNAs were down-regulated in shrimp with WSSV challenge. These miRNAs can mediate immune signaling pathways (Huang et al., 2012a). In addition, a set of shrimp miRNAs involved in innate immune systems, including apoptosis, phagocytosis, and pro-phenoloxidase system, was identified by high-throughput sequencing and Northern blot (Yang et al., 2012). A total of 24 miRNAs are proposed to have significant effects on phagocytosis, apoptosis, and the pro-phenoloxidase system (Yang et al., 2012).

Although increasing evidence from mammals reveals that miRNAs have key functions in the antiviral immune response, the function of crustacean miRNAs in response to viral infection remains elusive. Shrimp miR-7 targets the 3′-untranslated region (3′UTR) of the WSSV early gene wsv477 involved in early DNA replication and virus proliferation, and inhibit the expression of wsv477 in vitro and in vivo (Huang and Zhang, 2012). The injection of synthesized miR-7 into shrimp leads to a 1000-fold decrease in WSSV genomic copies compared with that of the control WSSV in the late stage of viral infection (Huang and Zhang, 2012). Therefore, our study was the first to demonstrate how crustacean miRNA functions in host antiviral responses by targeting a viral transcript.

3.3. Interplay between viral infection and the RNAi system

To counteract RNAi pathway-mediated antiviral immunity, viruses produce viral suppressors of RNAi (VSRs) to block various stages of the silencing process (Ding and Voinnet, 2007; Li and Ding, 2006). Some VSRs have been reported to suppress siRNA production, whereas others act by either sequestering siRNAs to prevent their incorporation into the RISC or prevent short- and long-distance spread of the RNA silencing effect. VSRs are proposed to alter the function of host miRNAs (Labreuche and Warr, 2013). RNAi is an important antiviral defense mechanism in shrimp. Considering the outcome of selective pressures that host and virus have imposed on each other over evolutionary time, shrimp viruses may similarly encode VSRs to counteract the host antiviral responses. One study found that WSSV can inhibit shrimp RNAi responses to benefit viral replication. In the hepatopancreas of shrimp infected with WSSV, dsRNA-induced gene silencing failed, suggesting the existence of an RNAi suppression mechanism by WSSV; but, RNAi suppression by WSSV was not detected in the gills (Robalino et al., 2007). WSSV infection can impair RNAi-mediated antiviral responses of shrimp, which supports the idea that RNAi has a natural antiviral function in shrimp as it does in insects. However, the viral gene with the ability to suppress RNAi has not been identified. The identification and characterization of this viral gene will provide an opportunity to address the relevance of anti-RNAi functions in the interactions between WSSV and the shrimp host.

4. Nucleic acid induces non-specific antiviral immunity

In vertebrates, virus-derived nucleic acids, such as single-stranded RNA (ssRNA), dsRNA, and unmethylated CpG-DNA, can be recognized by TLRs and retinoic acid-inducible gene-I-like receptors (RLRs) and subsequently activate the immune system (Akira et al., 2006). ssRNA is detected by TLR7 and TLR8, DNA is detected by TLR9, and dsRNA is detected by TLR3 and RLRs. Detection of ssRNA, DNA, and dsRNA results in the activation of IRF3/7 pathways and induction of type I IFNs (Akira et al., 2006). Type I IFNs can activate multiple intracellular antiviral pathways, such as JAK–STAT pathway, and induce ISGs that can interfere with many steps in the viral life cycle, thereby limiting the amplification and speed of the virus and attenuating infection (Liu et al., 2009a; Wang et al., 2013e).

Until recently, the IFN system was generally thought to be absent from invertebrates because of the lack of IFM core elements in several fully sequenced invertebrate genomes (Green and Montagnani, 2013; Robalino et al., 2004, 2007; Wang et al., 2013e). In invertebrates, RNAi is believed to be the main player in antiviral responses in a sequence-dependent manner (Kemp and Imler, 2009; Kingsolver et al., 2013; Sabin et al., 2010). Intrigu-

ingly, the injection of nucleic acid mimics, such as dsRNA, poly(I:C), poly(C:G), CL097, poly C, and CpG-DNA, can also induce non-specific antiviral immunity in shrimp and crab, which is similar to the responses of the vertebrate IFN system (Labreuche and Warr, 2013; Labreuche et al., 2010; Liu et al., 2009a; Robalino et al., 2004, 2007; Wang et al., 2013e). Among these mimics, dsRNA and poly(I:C) stimulate the strongest non-specific antiviral immunity. Injection of dsRNAs of diverse length, sequence, and base composition is capable of protecting shrimp from mortality caused by TSV or WSSV infection (Robalino et al., 2007). Transfection with dsRNA into *P. monodon* primary cells can attenuate replication and pathogenicity of YHV and WSSV (Robalino et al., 2007). Another study showed that dsRNA-induced non-specific antiviral immunity is length-dependent in shrimp, and suggested that the existence of immune mechanisms is analogous, to some extent, to those described in vertebrates (Labreuche et al., 2010). Therefore, shrimp possibly have receptors for dsRNA, ssRNA, and DNA, and possess nucleic acid-induced non-specific antiviral immunity similar to vertebrates.dsRNA-induced non-specific antiviral immunity is thought to be the second arm of host antiviral defense in shrimp, besides the RNAi-mediated sequence-specific antiviral response (Labreuche et al., 2010; Labreuche and Warr, 2013; Robalino et al., 2007). However, the recognition mechanism of dsRNA, which is responsive to the non-specific antiviral immunity of shrimp, is still unclear. dsRNAs are regarded as important stimulators of immune responses in vertebrates and invertebrates. In vertebrates, the dsRNA sensors include TLR3 and DExD/H-box helicases, such as RLRs. However, whether shrimp Tolls and DExD/H-box helicases Dicer1 and Dicer2 also contribute to the dsRNA-induced non-specific antiviral immunity is still elusive. In *Drosophila*, dsRNA is detected by Dicer2 to trigger an unidentified signal that leads to the inducible expression of the gene Vago, which controls the viral load in the fat body (Deddouche et al., 2008). A recent study reported that secreted Vago can restrict WNV infection in *Culex* mosquito cells by activating the JAK–STAT signaling pathway (Paradkar et al., 2012). Vago can also function as an IFN-like antiviral cytokine in mosquitoes (Paradkar et al., 2012). Genes of this antiviral Dicer2-Vago signaling pathway have been identified in shrimp (Chen et al., 2011). Vago may function as a cytokine-like IFN in nucleic acid-induced non-specific antiviral immunity in shrimp. The shrimp Dicer2-Vago pathway is a potential candidate that may contribute to dsRNA-induced non-specific antiviral immunity. The CL097, poly C, and CpG-DNA-induced non-specific antiviral responses have not been reported in other invertebrates, and the mechanism is completely unknown.

A recent study reported that CpG-ODN can directly interact with shrimp Tolls and induce hemocytic immune responses (Sun et al., 2014). However, whether shrimp Toll-mediated CpG ODN recognition contributes to CpG-DNA-induced non-specific antiviral immunity still needs further investigation. In insects and shrimp, viral infections can activate the Toll and IMD pathways. The target genes of these pathways have been involved in antiviral responses like AMPs. Nucleic acids from viruses may also activate the Toll and IMD pathway and induce AMP to inhibit viral infection (Liu et al., 2006; Tharntada et al., 2009; Wang and Wang, 2013b; Woramongkolchai et al., 2011).

The findings on nucleic acid-induced non-specific antiviral immunity in shrimp suggest a possible evolutionary link of nucleic acid recognition in innate antiviral immunity between invertebrates and vertebrates. These results may reveal new paradigms of how eukaryotic cells resist viruses, especially dsRNA-induced antiviral immunity in shrimp. Invertebrate immune responses are truly more complex than previously envisaged. Based on the antiviral activity induced by dsRNA in shrimp, the possible function, potency, and application of dsRNA in shrimp antiviral immunity should also be investigated.

5. Cell-mediated antiviral mechanisms

In invertebrates such as insects, the cellular response to infection involves phagocytosis, apoptosis, nodule formation, and encapsulation of pathogens. Phagocytosis and apoptosis have an important function in the shrimp response to WSSV infection (Wang and Zhang, 2008).

5.1. Phagocytosis

Phagocytosis is a highly conserved process representing an important component of the innate immune system in multicellular organisms (Stuart and Ezekowitz, 2008). The cascades of phagocytosis start with particle recognition and binding of particles to cell surface receptors, which activate diverse signaling pathways. These signals coordinate an orderly progression of cellular changes, including reorganization of the plasma membrane and cortical cytoskeleton, which results in phagosome formation. The phagosome undergoes fission and limited fusion events with endosomes and lysosomes, resulting in a mature phagolysosome that can destroy pathogens by low pH, hydrolysis, and radical formation (Stuart and Ezekowitz, 2008). In shrimp, several molecules implicated in the regulation of phagocytosis have been identified to have essential functions in the host antiviral responses.

5.1.1. Rab proteins

Increasing studies have shown that small G proteins have a key function in membrane trafficking and phagocytosis pathways (Samaj et al., 2006; Stuart and Ezekowitz, 2005). Based on their structural features, small G proteins are subdivided into five families, namely, Rab, Rho, Ras, Sar1/Arf, and Ran families (Faruqi et al., 2001; Schwartz et al., 2007). Rab family proteins consist of more than 60 members, and function in endocytic and exocytic membrane trafficking, and induction of effector proteins into specific membrane subdomains in eukaryotic cells (Armstrong, 2000). Some evolutionarily conserved members of the Rab family, including Rab5, Rab6, Rab7, and Rab11, are also involved in phagocytosis, but their molecular mechanisms in phagocytosis against viral infections remain elusive (Kitano et al., 2008; Niedergang and Chavrier, 2004; Wu et al., 2008). The Rab gene from *Peneaus japonicus* shrimp, called Pjrab, showed high similarity with Rab 6 homolog of other species. The PjRab protein had GTP-binding activity, and contained characteristic signatures of Rab proteins with six GTP-binding domains and five Rab specific domains. The expression of PjRab gene was upregulated in WSSV-resistant shrimp, indicating that the PjRab protein may be part of the shrimp antiviral response (Wu and Zhang, 2007).

Further investigation revealed that the PjRab protein could regulate shrimp hemocytic phagocytosis through a protein complex consisting of PjRab, β-actin, tropomyosin, and envelope protein VP466 of WSSV. Silence of PjRab by a specific siRNA led to significant increase in WSSV genomic copies, suggesting that PjRab may have an essential function in shrimp antiviral responses (Wu et al., 2008). Furthermore, PjRab was able to interact with the viral protein VP466, which could enhance GTPase activity of PjRab in vivo and in vitro. This result suggests that VP466 may function as a GTPase-activating protein of Rab6. In the VP466-Rab-actin pathway, the increase in Rab6 activity induced rearrangements of the actin cytoskeleton, resulting in the formation of actin stress fibers that promoted phagocytosis against the virus (Ye et al., 2012a). These findings revealed that PjRab GTPase, within this tetramer complex, may have a function in host response against viral invasion and trigger downstream phagocytic responses against virus. By contrast, VP466 could be employed by the host to initiate

immunity, thereby representing a novel molecular mechanism of small G proteins underlying the virus–host interaction.

Although small G proteins were revealed to be fundamentally important in phagocytosis in animals by organization of the actin cytoskeleton, the process involved in the regulation of phagocytosis through actin reorganization by small G proteins in shrimp remains unclear. Ye et al. investigated the molecular mechanism of Rab6 in phagocytosis against viral infection in both *Marsupenaeus japonicus* shrimp and *Drosophila melanogaster* (Ye et al., 2012a, 2012b). Their results showed that the shrimp Rab6 binds with actin to regulate shrimp hemocyte phagocytosis through the induction of actin rearrangement against WSSV infection. The same molecular mechanism was revealed in the Rab6 protein of *D. melanogaster*. The Rab6 protein of *D. melanogaster* had the same conserved function in phagocytosis as shrimp Rab6, suggesting that the phagocytic function of rab6 was conserved in invertebrates.

Compared with the early marker (Rab5) and late marker (LAMP1) of phagosomes, fly Rab6 was critically involved in the regulation of actin organization throughout the entire phagocytic process (Ye et al., 2012a, 2012b). These studies presented novel molecular events in the regulation of phagocytosis by small G proteins. Rab6 proteins are evolutionarily conserved in invertebrates and vertebrates. Therefore, further study of mammalian cells is necessary to help researchers better understand the molecular events of small G proteins in immune response against viral infections in animals.

5.1.2. Ran protein

A Ras-related nuclear protein, Ran, emerged as an essential player in nucleocytoplasmic transport (Allen et al., 2000; Dasso, 2001). Ran is essential for such cellular activities as centrosome duplication, microtubule dynamics, chromosome alignment, kinetochore attachment of microtubules, nuclear-envelope dynamics and phagocytosis (Ribbeck and Görlich, 2002; Rout et al., 2003). The Ran gene, with the full-length cDNA of 645 bp, has been characterized in shrimp. Like PjRab6, the PjRan protein also demonstrated GTP-binding activity. PjRan was revealed to be expressed in different tissues of shrimp, and upregulated in WSSV-resistant and WSSV-infected shrimp at 4 h post-infection. These results suggest that Ran proteins may have a key function in shrimp immunity against viral infection (Han and Zhang, 2007).

Further studies showed that PjRan interacts and forms a complex with myosin, which is an important protein in the phagocytic process. Loss-of-function or gain-of-function assays revealed that PjRan regulated shrimp hemocytic phagocytosis. Depletion of PjRan by sequence-specific siRNA led to a significant increase in virus copies, whereas the overexpression of PjRan caused a significant decrease in virus copies, suggesting the involvement of PjRan in antiviral immunity via regulated phagocytosis (Liu et al., 2009b). These findings show a direct interaction between Ran GTPase and the cytoskeleton protein, and present a novel pathway concerning antiviral immunity that will help us to better understand the molecular events in the immune response against viral infection in invertebrates.

Interestingly, the promoter region within PjRan gene contained a typical TATA box. Based on luciferase assays, the transcription of PjRan gene was upregulated by Ran protein through a direct interaction between the Ran promoter and PjRan protein, thereby indicating the existence of feedback regulation in Ran gene expression (Zhao et al., 2011b). Based on the aforementioned findings, PjRan protein, required in shrimp antiviral phagocytosis, was used to screen for immunostimulants. GTPase activity assays showed that the injection of IL-4 and lysophosphatidylcholine molecules into shrimp resulted in an increase in Ran protein activity, which suggests that the two molecules may improve phagocytic activity through the activation of the Ran protein. The enhancement of phagocytic activity could effectively inhibit WSSV infection in shrimp, resulting in a significant decrease in mortality. The decreased mortality enhances hemocytic phagocytosis, which protects shrimp from WSSV infection (Zhao et al., 2011a). Our study presented a novel strategy for the screening of immunostimulants. We used key proteins in the immune responses of aquatic organisms as the target proteins, which proved to be helpful in the development of efficient approaches to prevent pathogenic infections in aquatic organisms.

5.1.3. ADP ribosylation factor (Arf)

Arfs are a family of small GTPase-binding proteins that are involved in the regulation of actin cytoskeleton organization and membrane dynamics (Myers and Casanova, 2008). Arf6 regulates membrane trafficking in the actin cytoskeleton, and functions as a regulatory molecule of phagocytosis (Beemiller et al., 2006). In shrimp, a novel class II Arf (designated as MjArf4) was cloned and characterized from *M. japonicus*. Similar to other Arfs, MjArf4 contains an N-terminal myristoylated site, p loop, switch regions, and interswitch region. Studies on High Five cells showed that MjArf4 dispersed into the entire cell when in GDP-bound form, whereas GTP binding promoted the formation of a punctuate Golgi-like structure, suggesting that the subcellular distribution of MjArf4 was dependent on GDP/GTP binding. WSSV challenge triggered MjArf4 to be expressed at a high level. Therefore, the Arf family may be involved in phagocytosis during WSSV infection (Zhang et al., 2010).

5.1.4. Recognition proteins

A family of transcriptionally variant Down syndrome cell adhesion molecule (Dscam) receptors was first identified in *D. melanogaster* (Schmucker et al., 2000), and identified in some crustacean organisms, such as *Daphnia* spp., *Penaeus vannamei*, *P. monodon*, and *Pacifastacus leniusculus* (Watthanasurorot et al., 2011). In *D. melanogaster*, Dscam was detected in the fat bodies of insects and implicated in the pathways of neuronal development during ontogeny (Schmucker et al., 2000). Furthermore, *D. melanogaster* Dscam mutants have impaired phagocytosis, suggesting that this variable receptor family may have a function in PAMP detection (Watson et al., 2005). Dscam receptors have been characterized in shrimp *P. monodon* and *P. vannamei*. The Dscam receptor was found in *P. vannamei* as a soluble form in plasma (Chou et al., 2009), whereas the Dscam receptor in *P. monodon* has both cell surface and soluble forms (Chou et al., 2011).

Within the *Arthropoda*, the domains Ig2, Ig3, and Ig7 demonstrate extreme exon splice variation; thus, more than 220,000 various isoforms of the *Pa. leniusculus* Dscam, 13,000 different isoforms in *Daphnia* spp., and 31,000 different isoforms are predicted in the mosquito *Anopheles gambiae* (Brites et al., 2008; Chou et al., 2009, 2011; Watthanasurorot et al., 2011). The diverse set of alternative splice forms enable mosquito-specific recognition and defense against a broad spectrum of pathogens (Dong et al., 2006). However, researchers have yet to determine whether Dscam receptors in crustaceans function in phagocytosis or contribute to defense against various pathogens.

C-type lectins have important functions in pathogen recognition, innate immunity, and cell–cell interactions (Wang and Wang, 2013b). The first shrimp C-type lectin (named PmLec) was characterized from *P. monodon*. PmLec has strong hemagglutinating activity, bacterial-agglutinating activity, and an opsonic effect that enhances hemocyte phagocytosis. After preincubation with purified protein, the phagocytic activity of hemocytes against *Escherichia coli* increase in a dose-dependent manner (Luo et al., 2006). Another C-type lectin (called LvCTL1) was identified from *L. vannamei* (LvCTL1) (Zhao et al., 2009). LvCTL1 is a 156-residue polypep-

tide containing a C-type carbohydrate recognition domain (CRD) with an EPN (Glu99-Pro100-Asn101) motif, which has predicted ligand-binding specificity for mannose. Reverse transcription-PCR analysis revealed that the expression of LvCTL1 was specific in the hepatopancreas of *L. vannamei*. Recombinant LvCTL1 (rLvCTL1) demonstrated hemagglutinating activity and ligand-binding specificity for mannose and glucose. Furthermore, rLvCTL1 also showed strong affinity for WSSV, and interacted with several envelope proteins of WSSV. The binding of rLvCTL1 to WSSV protected shrimps from viral infection, and prolonged the survival of shrimps against WSSV infection (Zhao et al., 2009). These results suggest that LvCTL1 could bind to envelope proteins of WSSV and exhibit antiviral activity.

A C-type lectin, PcLT, was recently obtained from *Procambarus clarkii* (Chen et al., 2013). PcLT contains a CRD, which can bind to *V. alginolyticus* and WSSV. RT-PCR and qRT-PCR analyses showed that PcLT was specifically expressed in the hepatopancreas, and mRNA was markedly upregulated by *V. alginolyticus* and WSSV challenge. Moreover, rPcLT enhanced phagocytosis, facilitated the subsequent clearance of *V. alginolyticus*, and prolonged the survival of WSSV-infected shrimp. These findings suggest that PcLT not only served as a PRR, but also functioned as a phagocytosis modulator, participating in host defense against invaders (Chen et al., 2013).

Overall, phagocytosis is a complex process that includes membrane invagination, coated vesicle formation, directed vesicle trafficking, formation of the phagocytic cup, and engulfment of particles (Stuart and Ezekowitz, 2008). Increasing evidence reveals that phagocytosis has a critical function in the crustacean immune response to pathogen infection through the uptake and degradation of infectious pathogens. Although more knowledge about phagocytosis in crustacean antiviral immunity has been obtained, the molecular events that form this process are largely unknown because the molecular events involved in the regulation of phagocytosis and biological systems of phagocytosis are extremely complicated.

5.2. Apoptosis

Apoptosis can participate in innate antiviral responses by eliminating harmful cells (Hardwick, 2001). When viral infections are detected, apoptosis is triggered to eliminate virus-infected cells, preventing the virus from diffusing and infecting adjacent cells. Thus, apoptosis can significantly improve host antiviral responses. However, viruses have developed distinct strategies to escape or retard apoptosis. Viruses can block apoptosis to prevent the premature death of infected cells, maximizing the viral progeny from a lytic infection or facilitating a persistent infection. Viruses can also actively promote apoptosis to spread viral progeny to neighboring cells. Viruses can perform both pro- and anti-apoptotic functions to facilitate different stages of infections (Galluzzi et al., 2008; Hardwick, 2001).

5.2.1. Viral infection induces apoptosis in shrimp

Apoptosis has an important function in WSSV pathogenesis. WSSV infection can induce obvious characteristic signs of apoptosis (i.e., nuclear disassembly, chromosomal DNA fragmentation into a ladder, and increased caspase-3 activity) in many tissues, such as abdominal epithelium, stomach epithelium, hepatopancreatic interstitial cells, gills, and muscles (Leu et al., 2013). Among the examined tissues, apoptosis was detected as early as 6 h post-WSSV infection in the subcuticular epithelium, and increased apoptosis severity was observed with the progress of infection. Interestingly, infection induces apoptosis in bystander cells that are free of WSSV virions, whereas virion-containing cells are non-apoptotic (Leu et al., 2013). Thus, WSSV infection can inhibit the apoptosis of infected cells by viral proteins, such as WSSV449 (ORF390 or AAP-1) and WSSV222. However, some signals from the virus or infected cells can be received by neighboring cells to induce apoptosis.

Caspases, the main proteins in the initiation and execution stages of apoptosis, have vital functions at various stages of the apoptotic process (Wang et al., 2013c). Five types of caspase genes have been reported in penaeid shrimps. The expression levels of these genes are extremely sensitive to WSSV (Leu et al., 2013). Silencing of *PjCaspase*, *Lvcaspase2*, *Lvcaspase3*, and *Lvcaspase5* resulted in increased copy numbers of WSSV, indicating a requirement for shrimp caspases in apoptotic responses against viral infection (Leu et al., 2013; Wang et al., 2008b, 2013c).

The WSSV-resistant shrimp contained a special fragment of *PjCaspase* gene, designated as fragment 3. Downregulation or overexpression of the *PjCaspase* gene containing fragment 3 led to significant inhibition or enhancement of virus-induced apoptosis, but had no effect on bacterium challenge. Silencing or overexpression of *PjCaspase* gene containing fragment 3 led to a 7-fold increase or 11-fold decrease in WSSV copy numbers, respectively. Therefore, the sequence diversification of *PjCaspase* may generate a specifically antiviral defense against WSSV infection (Zhi et al., 2011b). Small molecules IL-2 and evodiamine targeting *PjCaspase* can increase the apoptotic activity of shrimp hemocytes in vivo, and the enhancement of apoptotic activity effectively inhibits WSSV infection with the decrease in mortality of WSSV-infected shrimp (Zhi et al., 2011a). Many studies have indicated that WSSV-induced apoptosis represents an antiviral immune response in shrimp, and inhibition of apoptosis by the inhibitor zVAD-FMK or *PjCaspase* silencing facilitates the multiplication of WSSV (Leu et al., 2013; Wang et al., 2008b; Wang and Zhang, 2008).

Apoptosis is also believed to constitute a defense responsible for eliminating TSV and YHV infections (Leu et al., 2013). The widespread and progressive occurrence of apoptosis in *P. monodon* infected with YHV is a major cause of dysfunction and death of the host. YHV-infected cells show signs of viral-triggered apoptosis, such as nuclear pyknosis and karyorrhexis (Leu et al., 2013). In addition, the transcriptional level of defender against apoptotic death 1 (*DAD1*), a negative regulator of apoptosis, decreased dramatically after YHV challenge in *P. monodon*. This result suggests that DAD1 functions in shrimp apoptosis caused by YHV infection.

Ribophorin I belongs to the oligosaccharyltransferase complex involved in apoptosis. The expression of ribophorin I was upregulated and remained high until the moribund stage in YHV-infected shrimp (Molthathong et al., 2008). Therefore, ribophorin I may be involved in shrimp apoptosis in the defense against YHV infection.

5.2.2. Interactions between shrimp apoptosis and viral replications

To manipulate host apoptosis, WSSV modulates the expression of shrimp apoptosis-related genes, such as *caspases*, *fortilin*, and *VDAC*, and actively promotes apoptosis to spread from virus progeny to neighbor cells. WSSV also encodes viral proteins with anti-apoptotic activities, such as WSSV449 (ORF390 or AAP-1) and WSSV222, to block apoptosis for preventing premature host cell death and maximizing virus progeny (Leu et al., 2010, 2013; Wang et al., 2013c; Yan et al., 2010). WSSV infection induces apoptosis in bystander cells that are free of WSSV virions, but not in virion-containing cells, suggesting that WSSV infection can inhibit apoptosis in virion-containing cells by viral proteins (Leu et al., 2013). WSSV449 inhibited shrimp caspase activity in vivo and in vitro by binding, and was cleaved by shrimp caspase (Leu et al., 2008, 2010). WSSV449 can also modulate NF-κB activity, which may be another way of inhibiting apoptosis during WSSV infection besides direct inhibition of shrimp caspase activity (Leu et al., 2013; Wang et al., 2013c).

WSSV222, an E3 ubiquitin ligase that acts through ubiquitin-mediated degradation, may function as an anti-apoptotic protein in WSSV-infected shrimp via ubiquitin-mediated degradation of a tumor suppressor-like protein (TSL) (He et al., 2006, 2009; Leu et al., 2013). In mammalian cells, TSL expression induces apoptosis, and TSL-induced apoptosis can be blocked by WSV222. WSSV222 silencing inhibited the degradation of TSL in WSSV-challenged shrimp, thereby reducing the cumulative mortalities and delaying the mean time to death in WSSV-infected shrimp (He et al., 2006, 2009; Leu et al., 2013).

In addition, WSSV-encoded ICP11 showed apoptosis-inducing activity (Leu et al., 2013; Wang et al., 2008a). ICP11 bound to histone proteins in the cytosol of WSSV-infected hemocytes and HeLa cells, preventing them from moving into the nucleus to participate in nucleosome assembly. ICP11 could induce apoptosis when expressed in HeLa cells (Wang et al., 2008a). However, the apoptosis-inducing activity of ICP11 was likely to be incidental to its destabilization of nucleosomes. Whether an incidental consequence or not, WSSV presumably benefits from preventing the apoptosis induced by ICP11 (Leu et al., 2013).

In 2013, Watthanasurorot et al. reported that the C1q-binding proteins calreticulin (CRT) and gC1qR can form a complex in the cytoplasm as a response to WSSV infection, resulting in apoptosis prevention (Watthanasurorot et al., 2013). When the peptides of the complex proteins are overexpressed in human cancer cells, the cells will undergo apoptosis, suggesting that this mechanism is conserved from arthropods to humans (Watthanasurorot et al., 2013). In a recent study, they further show that CRT can directly interact with WSSV structural proteins, including VP15 and VP28, during an early stage of virus infection (Watthanasurorot et al., 2014). CRT was detected in the viral envelope of purified WSSV virions. CRT was also important for proper oligomerization of the viral structural proteins VP26 and VP28. When CRT glycosylation was blocked with tunicamycin, both viral replication and assembly was significantly decreased (Watthanasurorot et al., 2014). Taken together, these studies suggest that CRT may be involved in apoptosis during infection and confer several advantages to WSSV from the initial steps of infection, to the assembly of virions. It is another example that WSSV-induced apoptosis may represent an antiviral immune response in shrimp, and that WSSV has learned how to hijack it for its replication cycle.

Numerous studies have shown that apoptosis has an important function in shrimp host defense against various viral infections. Apoptosis is a complex process involving the interaction of many proteins. Some of these proteins have been characterized from shrimp. Therefore, further studies may focus on identifying more proteins involved in apoptosis, and investigate the interactions between apoptosis and viral infections in antiviral immunity of shrimp.

6. Viral infections

In the past decade, many studies have been conducted to better understand how viruses infect their host. These studies aimed to use the gained knowledge to develop better strategies for interfering with viral infections and disease control. One approach is the study of interactions between the host and viral proteins. In particular, virus-binding proteins may have a key function in the viral infection process.

Data on infections of vertebrates suggest that viruses can use their envelope proteins for target cell recognition and entry (Spaan et al., 1988). To isolate and characterize WSSV-binding proteins from shrimp, Sritunyalucksana et al. performed a blot of shrimp hemocyte membrane proteins that was overlaid with a recombinant WSSV envelope protein (rVP28). The reactive bands on the blot were detected using anti-VP28 antibody. Among three membrane-associated molecules identified by liquid chromatography–tandem mass spectrometry, PmRab7, a shrimp small GTPase protein, was found to bind directly to VP28 protein, suggesting that PmRab7 is involved in WSSV infection (Sritunyalucksana et al., 2006). Silencing of PmRab7 through dsRNA-Rab7 injection dramatically inhibited WSSV-VP28 mRNA and protein expression, and repressed WSSV replication in shrimp. These results indicate that PmRab7 was a key cellular factor required for WSSV replication in shrimp. PmRab7 possibly functions in the endosomal trafficking pathway, and silencing of PmRab7 prevented successful viral trafficking necessary for replication. However, the subcellular location of PmRab7 and molecular mechanism of PmRab7 underlying virus transport within the host cells remain unknown (Ongvarrasopone et al., 2008).

Through analysis of the phage display library of the WSSV genome, a WSSV envelope protein VP187 (wsv209) was revealed to interact with cellular surface protein integrin. Either blocking with an integrin-specific antibody or RGD-containing peptide of VP187 or RNA silencing using a specific dsRNA targeting β-integrin could effectively inhibit WSSV infection, which indicates that β-integrin may serve as a cellular receptor for WSSV infection (Li et al., 2007). Recently, cellular surface protein glucose transporter 1 (Glut1) was revealed to interact with WSSV envelope protein, VP53A. Glut1 was highly expressed in almost all organs. In vitro and in vivo neutralization experiments using synthetic peptide included WSSV-binding domain (WBD) of Glut1, and the results reveal that the WBD peptide suppressed WSSV infection and reduced shrimp mortality after WSSV challenge (Huang et al., 2012b).

Although tetraspanins are also considered as possible targets of some viruses, their functional roles in invertebrates remain unknown. Tetraspanin-3 was recently identified from the cDNA library of Chinese shrimp (*Fraxinus chinensis*). FcTetraspanin-3 was constitutively expressed in all examined tissues and increased in hepatopancreas, lymphoid organ, and intestine by WSSV challenge. Neutralization experiments using antibody to the large extracellular loop of FcTetraspanin-3 significantly reduced the mortality of shrimp caused by WSSV. Knockdown of FcTetraspanin-3 led to a decrease in the viral load of WSSV in shrimp (Gui et al., 2012).

Given the lack of stable shrimp cell lines, primary cultured hemocytes were employed as an in vitro model to investigate the process of WSSV entry. By labeling virions and endosomes with fluorescent dyes, we found that WSSV co-localized with early endosomes in shrimp hemocytes. Treatment with an inhibitor of endocytosis, methyl-β-cyclodextrin, could suppress the entry of WSSV in shrimp hemocytes. These findings reveal that the host-cell machinery of caveolae-mediated endocytosis could be usurped by WSSV for replication, thereby revolutionizing our understanding of endocytosis within cells (Huang et al., 2013a,b; Marsh and Helenius, 2006). The interactions between viral and cell-surface proteins may induce conformational changes in the envelope spike and entry of the viral core into the cell cytoplasm (Clapham and McKnight, 2002). Therefore, cell surface receptors have important functions in WSSV entry and their influence on cell tropism.

During viral infection, cytoskeleton and cellular proteins are often recruited by the invading viruses to be taken up into the cytoplasm (Klasse et al., 1998). During TSV infection, actin can bind to the TSV capsid protein (Senapin and Phongdara, 2006). Using the yeast two-hybrid system and in vitro pull-down assays, all three TSV capsid proteins (VP1, VP2, and VP3) were shown to interact with host β-actin and EF1-α. The interaction between host actin and these viral proteins possibly occurs after the virus penetrates the cell and facilitates the transport of viral components in the cytoplasm. EF1-α can interact with the viral matrix (MA) and

nucleocapsid domains of HIV type-1 Gag polyprotein (Cimarelli and Luban, 1999). The interaction between host EF1-α and viral MA may cause an inhibition of viral genome translation, which is required for the packaging in the nascent virion. In addition, TSV-VP1 could also bind to lysozyme and laminin receptor/ribosomal protein p40 (Lamr/p40). Lysozyme has a key function in the destruction of bacterial cell walls and antibacterial defenses in several shrimp species, such as the kuruma shrimp *P. japonicas* (Hikima et al., 2003). However, how lysozyme is also involved in virus–host interaction in shrimp is unknown.

Lamr/p40 protein serves as the mammalian cell receptor for some arthropod-borne flaviviruses, including dengue vand tick-borne encephalitis virus (Protopopova et al., 1997; Thepparit and Smith, 2004). Homolog analysis revealed that the laminin-binding site of human Lamr/p40 was conserved in shrimp protein and other arthropods, indicating that Lamr/p40 may serve as a common viral receptor in both mammals and arthropods. Furthermore, shrimp Lamr/p40 can interact with two other shrimp RNA viruses (IMNV and YHV) (Busayarat et al., 2011). Administration of yeast cell lysate containing recombinant Lamr/p40 protected shrimp from YHV invasion. Attempts to study the mechanism of shrimp Lamr/p40 on viral pathogenesis by gene knockdown have failed because knockdown is important for shrimp survival (Senapin et al., 2010). These findings revealed that Lamr is implicated in other essential cellular processes that might be independent of its shrimp–virus interaction.

Recent progress in molecular biology techniques has enabled the collection of information on the factors, mechanisms, and strategies used by crustacean viruses to infect and replicate in their host cells. However, further research is still needed to fully understand the basic nature of crustacean viruses, their exact life cycle, and mode of infection. These data will expand our knowledge and contribute to developing effective prophylactic or therapeutic measures.

7. Conclusions and future studies

All organisms, including shrimp, require powerful immune systems to protect themselves from viral pathogens. Shrimp innate immunity is similar to the innate immunity of insects and higher vertebrates in pattern recognition of microbial pathogens and subsequent immune signal transduction. Recent progress has highlighted the involvement of innate immune signaling pathways, RNAi, phagocytosis, and apoptosis in shrimp antiviral defense. Many viral-induced molecules have been identified using SSH or next-generation sequencing techniques (Li et al., 2013; Sookruksawong et al., 2013; Wang et al., 2013e). Some viral-induced proteins, such as AMPs, are target genes of the shrimp Toll/IMD pathways (Lan et al., 2013; Li and Xiang, 2013; Wang et al., 2009, 2012, 2013d). However, the functions of most viral-induced molecules and signaling pathways responsible for their regulation are unknown.

Although viral infections activate the shrimp immune system and induce hundreds of genes, such as AMPs (Li et al., 2013; Sookruksawong et al., 2013), studies on the mechanism by which viruses are recognized by the host immune system are limited. The viral PAMPs that activate shrimp immune responses have yet to be identified. Further studies are required to determine how these viral pathogens are sensed and how the appropriate antiviral program is initiated.

For successful replication, viruses encode a myriad of evasion strategies to benefit from host immune responses, many of which are beginning to be elucidated (e.g., the Tube-like viral protein WSSV449 can activate Toll-NF-κB signaling pathways and inhibit shrimp caspase-initiated apoptosis to promote WSSV replication). The discovery of the machinery for small RNA-mediated antiviral silencing in shrimp has begun to revolutionize our understanding of virus–host interactions. Cellular responses involving phagocytosis and apoptosis have key functions in the shrimp response to WSSV invasion. However, the molecular mechanisms underlying these processes are largely elusive. A clearer understanding of immune responses and mechanisms of viral infection and the shrimp immune system will not only aid in our understanding of fundamental aspects of antiviral defense, but may also provide basic knowledge to develop better strategies for controlling viral diseases in shrimp aquaculture.

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