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WSSV–host interaction: Host response and immune evasion

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ABSTRACT

As invertebrates, shrimps rely on multiple innate defense reactions, including humoral immunity and cellular immunity to recognize and eliminate various invaders, such as viruses. White spot syndrome virus (WSSV) causes the most prevalent and devastating viral disease in penaeid shrimps, which are the most widely cultured species in the coastal waters worldwide. In the last couple of decades, studies about WSSV implicate a dual role of the immune system in protecting shrimps against the infection; these studies also explore on the pathogenesis of WSSV infection. Herein, we review our current knowledge of the innate immune responses of shrimps to WSSV, as well as the molecular mechanisms used by this virus to evade host immune responses or actively subvert them for its own benefit. Deciphering the interactions between WSSV and the shrimp host is paramount to understanding the mechanisms that regulate the balance between immune-mediated protection and pathogenesis during viral infection and to the development of a safe and effective WSSV defensive strategy.

1. Introduction

The rapid development of shrimp farming in many Asian countries such as China, Malaysia, and Thailand is attributed to improvements in culture technology, such as intensive farming, by a large percentage of semi-intensive and intensive farming systems in the last 20 years [1]. Over 30 species of shrimp have been cultured in ponds. Recently, only a few species, including *Litopenaeus vannamei*, *Fenneropenaeus chinensis*, *Penaeus monodon*, and *Marsupenaeus japonicas* are of utmost importance in terms of large-scale commercial production. The giant tiger shrimp (*P. monodon*), with its large size and rapid growth, and the Pacific white shrimp (*L. vannamei*), with its high-density tolerance and rapid growth are the most commonly cultured species and account for approximately 75% of the global shrimp production (United Nations Food & Agriculture Organization). In China, the current annual production of these shrimps is over 1.5 million metric tons, which is equivalent to about one-third of the total shrimp worldwide (National shrimp and crab industry technology system in China). However, the rapid growth of shrimp farming has led to a remarkable increase in outbreaks of various diseases associated with several protozoal, fungal, bacterial, and viral agents in recent years [2]. Viral diseases provoke the greatest losses, especially the white spot syndrome virus (WSSV), which causes white spot disease (WSS) [3]. Among viral diseases, the WSS has become the greatest threat to global crustacean aquaculture industry. WSSV contains a double-stranded circular DNA genome (~300 kbp) that encodes at least 181 predicted proteins, most of which show no homology to known proteins [4]. Based on its unique morphological and genetic features, the International Committee on Taxonomy of Viruses (ICTV) assigns WSSV as the only member of the genus *Whispovirus* of the virus family *Nimaviridae* [5]. Although the host range of WSSV includes many decapod and non-decapod species, including shrimps, crabs, lobsters, crayfishes, and copepods, WSSV appears to exhibit high pathogenicity and virulence only on penaeid shrimps [6]. WSSV can lead to a cumulative mortality of up to 100% within 3–10 days in cultured shrimp and thus, causes serious economic consequences to shrimp farming [5]. Economic drivers have focused the research on WSS in farmed shrimp. Understanding the molecular basis of host–pathogen interactions will contribute significantly to the management of this pathogen.

As invertebrates, shrimps generally lack lymphocytes or an antibody-based adaptive immunity. Thus, they depend on innate immunity to combat infection. The innate immune system consists of a cellular arm and a humoral arm. Specifically, the humoral defenses include the production of antimicrobial peptides (AMPs), reactive intermediates of oxygen or nitrogen, and the complex enzymatic cascade that regulate clotting or melaninization of hemolymph [7]. By contrast, a cellular immune response that involves different types of hemocytes, which participate in pathogen clearance by phagocytosing microorganisms or

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by the encapsulation of larger microorganisms, cytotoxic reactions, as well as RNA interference (RNAi), are also triggered [7]. Recent studies identify hemocytes as important sources of several humoral effector molecules, (e.g. AMPs), which are required in killing different foreign invaders in shrimps [8,9]. Like other aquatic crustaceans, shrimps are in intimate contact with their environment, where they frequently encounter different viral pathogens, including WSSV. Therefore, shrimps have evolved with sensitive mechanisms for threat recognition and an array of strategies to protect themselves from WSSV infection. In fact, accumulating evidence indicate that host innate immune responses, such as RNAi and apoptosis play key roles in the defense against WSSV [10,11]. Similarly, common mechanisms used by WSSV to evade innate immune responses or to actively subvert innate immune signaling pathways for their own benefit are emerging. Thus, the major topics of discussion in this review are as follows: (1) the virus–host interactions related to humoral immune response, which involves evolutionarily conserved innate immune signaling pathways, including two NF-κB related pathways (Toll pathway and IMD pathway), JAK/STAT pathway and proPO system-mediated melaninization; and (2) cellular immune responses that involve RNA interference (RNAi) and apoptosis. A thorough understanding of the shrimp immune response to WSSV is paramount in developing urgently needed WSSV prevention strategies.

2. Cellular immunity and WSSV

2.1. RNA interference (RNAi) is a major antiviral mechanism in shrimps

RNA interference (RNAi) is an evolutionarily conserved mechanism of post transcriptional gene regulation among plants, arthropods, and mammals. It is the control of expression of target genes through mRNA destabilization and translation repression or gene transcription via chromatin modification [12,13]. In general terms, RNAi pathways involve the production of small ncRNAs (non-coding RNAs), and their biogenesis and function are based on two proteins, namely, Dicer (Dcr) and Argonaute (Ago) [14]. The two major classes of small silencing RNAs are microRNAs (miRNAs) and small interfering RNAs (siRNAs); these RNAs are extensively studied in shrimps.

In the canonical miRNA biogenesis pathway, miRNA biogenesis is a sequential process initiated in the nucleus and continued in the cytoplasm [15]. Primary miRNAs (pri-miRNAs) are transcribed from intracellular pathogens (e.g. virus) or host genomes and form a hairpin structure, which are mainly processed by the nuclear RNase III enzyme Drosha and its co-factor Pasha. This initial processing step leads to the formation of precursor miRNAs (pre-miRNAs), which are exported into the cytoplasm. A second RNase III enzyme, Dicer1, further processes pre-miRNAs into mature miRNA duplexes that are ~20 nucleotides in length. The miRNA duplex is loaded onto the Ago1 protein to form the miRNA programmed RNA-induced silencing complex (miRISC). The mature miRISC goes on to target mRNAs and silence their expression via slicing or more often through a combination of translation repression, mRNA deadenylation, and decay [16]. These key constituent proteins (Drosha, Pasha, Dicer1 and Ago1) implicated with miRNA biogenesis have been identified from different shrimp species, including *L. vannamei*, *P. monodon*, and *M. japonicas* [17–23]. The identification of their biological functions suggests the presence of an intact and functional miRNA biogenesis progression in shrimp (Fig. 1). More specially, 40 distinct viral miRNAs encoded by WSSV have been identified from viral infected cell via miRNA microarray and Northern blot analyses [24]. The biogenesis of these exogenous miRNAs was demonstrated to be dependent on host Drosha and Dicer1, as when the host Drosha or Dicer1 genes were silenced by the sequence-specific siRNAs, the synthesis of a viral miRNA (WSSV-miR197 in this case) was repressed and could not be detected [24]. The essential role of Drosha in miRNA maturation was also supported by the failure of the Drosha-silenced cells to generate several endogenous miRNAs (miR-let7, miR-1, and miR-100) in shrimp [17]. Host Ago1 was further demonstrated to interact with a viral miRNA (WSSV-miR197), suggesting that the host Ago1 is required for the normal functioning of viral miRNA inactive RISC [24]. In summary, the shrimp clearly possesses an intact miRNA biogenesis pathway that is responsible for the production of exogenous viral miRNAs and endogenous host miRNAs.

Growing evidence suggest that the principal components of shrimp canonical miRNA biogenesis pathway play an important role in several viral infections, including WSSV in some cases. In particular, PmAgo, a *P. monodon* Ago1 protein harboring over 80% sequence identity with insect Ago1 homologs, was induced to a twofold to threefold increase during the early period of infection with yellow head virus (YHV) [19]. LvDcr1 mRNA from *L. vannamei* was upregulated in the hemocytes and gills after Taura syndrome virus (TSV) infection [22]. The knockdown of PmDcr1 from *P. monodon* resulted in more rapid mortalities and higher viral loads (gill-associated virus), demonstrating that Dicer1 is involved in the antiviral defense in shrimp [21]. A more detailed contribution of *M. japonicas* Ago1 to the shrimp antiviral defense has been demonstrated [23]. The authors identified three Ago1 isoforms, namely, Ago1A, Ago1B, and Ago1C, in *M. japonicas* shrimp, of which isoforms Ago1A and Ago1B containing an insertion sequence in the PIWI domain were significantly upregulated in lymphoid organs in response to WSSV challenge. Silencing Ago1A or/and Ago1B but not Ago1C with sequence-specific siRNAs led to a significant increase in WSSV loads, indicating that Ago1A and Ago1B isoforms are involved in shrimp antiviral immunity [23]. RNase III Drosha is a key component of miRNA maturation. Drosha from *M. japonicas* shrimp was shown to be significantly up regulated in lymphoid organs in response to WSSV challenge [17]. The knockdown of Drosha *in vivo* led to defect in miRNA maturation and subsequent higher virus loads after WSSV infection in shrimp [17]. Notably, some synthetized viral miRNAs, such as WSSV-miR211 and WSSV-miR212 have been shown to be required for successful WSSV infection, although the exact action of these viral miRNAs during WSSV infection is still unclear [24]. Other WSSV encoded miRNAs have been predicted to target many virus self genes, of which some can encode structural proteins that are essential for the assembly of virion and receptor recognition during virus infection [24]. More importantly, WSSV can express miRNAs such as WSSV-miR-N24 so that it could escape the host's antiviral immune response, which will be described in detail elsewhere. In addition, it is worth noting that WSSV miRNAs also target virus genes and further promote the virus infection. He et al. demonstrated that WSSV-miR-66 targeted wsv094 and wsv177 genes, and that WSSV-miR-68 targeted wsv248 and wsv309 genes at the early stage of WSSV infection [25]. It was revealed that the four target genes play negative roles in the WSSV infection. The targeting of the four virus genes by WSSV-miR-66 and WSSV-miR-68 led to the promotion of virus infection. Conversely, WSSV genes are targeted by host-encoded miRNAs. For instance, shrimp miR-7 from *M. japonicas* can target the 3′-untranslated region (3′ UTR) of the WSSV early gene wsv477. The administration of synthesized miR-7 *in vivo* rendered shrimps to contain lower than 1000-fold viral loads in gills. On the contrary, blocking endogenous miR-7 by AMO-miR-7 *in vivo* led to about a 10-fold increase in viral loads in gills [26]. These results suggest that host-encoded miRNAs play a key role in defense against WSSV.

Double-stranded RNA (dsRNA) from exogenous and endogenous sources can trigger a second RNAi mechanism, which is the small interfering RNA (siRNA or common called RNAi) pathway (Fig. 1). dsRNA is sensed by Dicer2, which processes it into duplex siRNAs of 21–24 nucleotides. These siRNAs are subsequently loaded onto the Ago2 protein to form a pre-RISC complex. The passenger strand of the duplex is then ejected, leading to the formation of a mature siRISC complex. Then, the mature siRISC complex uses the remaining guide strand to recognize and cleave a complementary mRNA [27]. In the past ten years, progress has been made in the identification and functional characterization of the core components of RNAi pathway in shrimps. The first report indicated that the shrimp has an intact RNAi machinery, as manifested by the potent sequence-specific silencing of hemocyanin

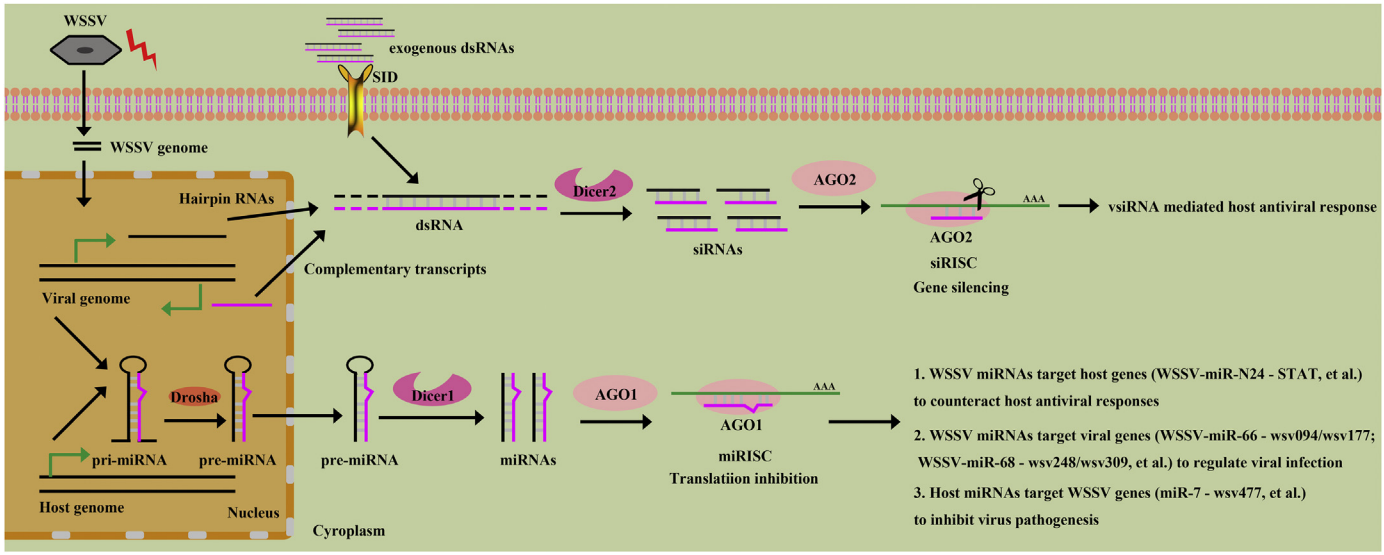

Fig. 1. Schematic representation of the canonical small silencing RNAs biogenesis pathways in shrimps. Two major classes of small silencing RNAs are present: microRNAs (miRNAs) and small interfering RNAs (siRNAs), both of which play key roles in defense against WSSV (See the text for detail).

mRNA in the hepatopancreas of *L. vannamei* after injecting hemocyanin-specific dsRNA into the tail muscle of the shrimp [28]. Several members of the siRNA pathway, including Dicer2 and Ago2, from different shrimps have been demonstrated to be involved in the biogenesis of siRNA or to be responding to viral infection [18,29–31].

RNAi is a natural antiviral immune mechanism in plants and insects, as illustrated by the presence of virus-derived siRNAs (vsiRNAs) in infected cells; vsiRNAs serve as a molecular marker for the induction of natural antiviral RNAi and as specificity determinants of the defense mechanism [32]. In shrimps, several studies demonstrated that the induction of the RNAi pathway by injecting shrimp with exogenous siRNAs or long dsRNAs specific to viral genes can inhibit unrelated viruses, such as WSSV, YHV, TSV, and *P. monodon* densovirus [28,33–37]. From the results of these experiments, whether RNAi was utilized by shrimp as a natural antiviral mechanism against these viruses is not yet decided. The study by Huang and Zhang [38] showed a convincing result of the detection of a WSSV-derived siRNA (vp28-siRNA) in all the virally infected shrimp organs and tissues. The results also indicated that the host siRNA pathway core components Dicer2 and Ago2 proteins are required for the biogenesis and function of vp28-siRNA, respectively, and played major roles in antiviral immunity in shrimp. As a result, following this logic, RNAi has strongly been proven to be a natural antiviral immune mechanism in shrimp, as supported by the presence of vp28-siRNA in virally infected shrimp. Notably, the siRNA-using RNAi system in eukaryotes has been documented as an immune response against the invasion of RNA viruses. WSSV has a double-stranded DNA genome; thus, we can hypothesize that the production of significant amounts of dsRNA during its infectious cycles is presumably attributed to the intramolecular interactions within viral transcripts and bi-directional transcription (Fig. 1). The virus-derived dsRNA then is sensed and processed by the host siRNA machinery (Dicer2) to generate viRNAs, which confer the antiviral defense against virus.

In plants and insects, viral pathogens often develop strategies to bypass or suppress host RNAi response, as their hosts produce vsiRNAs that have direct and specific roles in antiviral defense. One of these strategies is to express diverse viral suppressors of RNAi (VSRs) to target the core components of the siRNA pathway [39–43]. Until now, no VSR has been identified from WSSV, although a repressed phenomenon was observed in shrimp that directed the silencing of endogenous genes hemocyanin. Alternatively, STAT could have been inhibited in the hepatopancreas but not in the gills of WSSV-infected animals [44]. The ability of WSSV to suppress host siRNA-mediated gene silencing in some specific cells attests to (albeit indirect) the importance of this pathway to the control of viral infection.

2.2. Nucleic acid-induced nonspecific antiviral immunity in shrimp

The detection of foreign nucleic acids is an important strategy for the innate immune recognition of pathogens. In vertebrates, pathogen-derived nucleic acids are sensed by several PPRs, particularly TLR9, AIM2, and cGAS, which detect dsDNA, and TLR3, TLR7, TLR8, RIG-I, MDA5, and OAS that detect various forms of RNA [45–47]. The activation of these receptors initiates signaling cascades that lead to the induction of type I interferons (IFNs) and culminate in an effective antiviral immune response. Although debates on whether invertebrates have antiviral immunity similar to the interferon system of vertebrates, growing evidences points to the existence of a parallel interferon-like defense system in some invertebrates, including shrimps.

As mentioned above, virus-derived dsRNAs can afford potent antiviral immunity in shrimps via the RNAi pathway in a sequence-specific manner. Interestingly, researchers have observed that various unrelated dsRNAs were able to induce antiviral protection against WSSV and TSV in a sequence-independent manner in shrimp [48]. This result demonstrated for the first time that an invertebrate (shrimp) immune system, like its vertebrate counterparts, can recognize dsRNA as a virus-associated molecular pattern, resulting in the activation of an innate antiviral response. Another group reported that shrimps injected with CpG ODNs developed resistance to WSSV; they observed that a higher survival proportion lowers WSSV copy numbers and increases the mRNA expression of Dicer and STAT in CpG ODNs pretreatment shrimps [49]. Improved survivals following a range of nucleic acids, including dsRNA mimics poly (I: C) and poly (C: G), ssRNA mimics CL097 and poly C, and DNA mimic CpG-DNA ODN2006 injection were found later after WSSV infection [50]. Notably, the injection of poly (I: C) was most effective in protecting shrimp from WSSV infection, which could be attributed to the viral dsRNA mimicking property of poly (I: C), because most viruses, including some dsDNA viruses, can produce significant amounts of dsRNA during their replication [51,52]. Later, the induction of the innate (non sequence-specific) and RNAi-related (sequence-specific) antivirus was found to engage through the same pathway in *L. vannamei* [28,53]. Indeed, some arthropods, including shrimps, *Drosophila*, and mosquito, use the core member of canonical RNAi pathway Dicer2 to activate Vago via an unidentified mechanism [30,54,55].

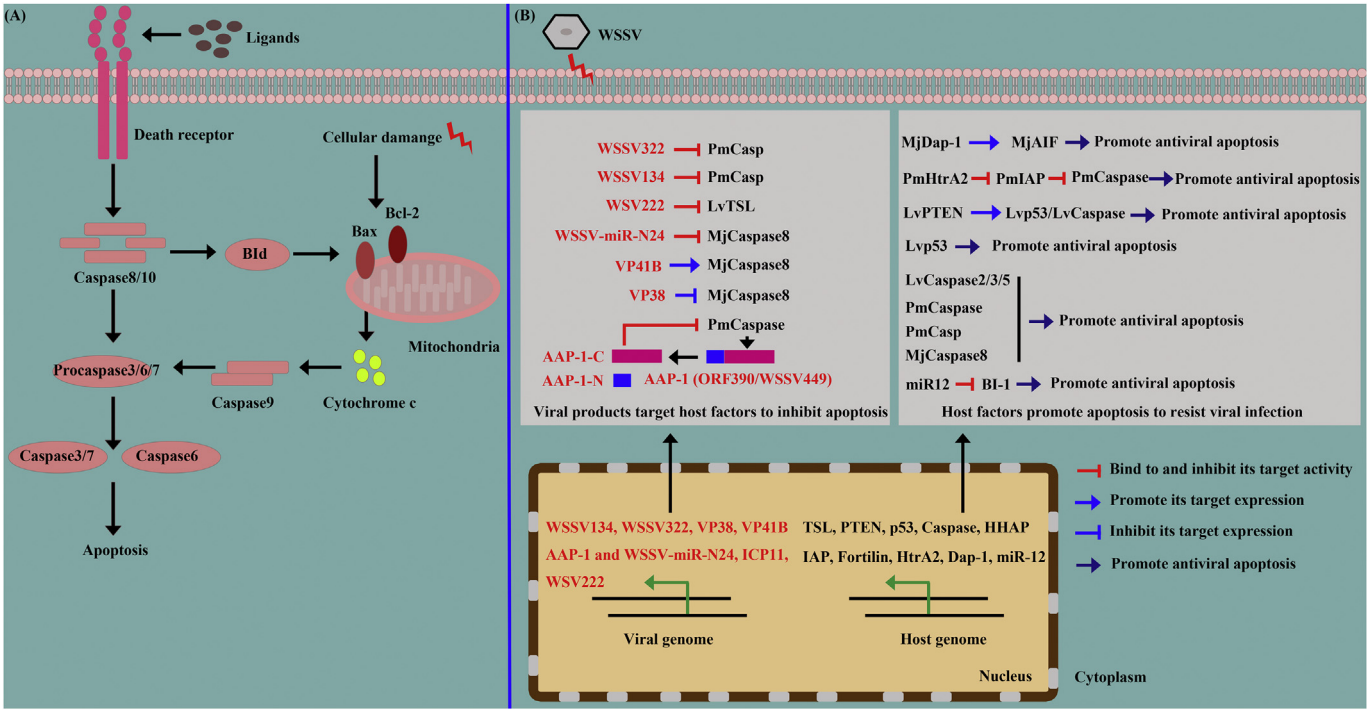

Fig. 2. The interplay between host apoptosis and WSSV. (A) Brief introduction of apoptotic signaling pathway in mammals. (B) A proposed model for the apoptotic interactions between shrimp cellular factors and WSSV (See the text for detail).

Vago is a secreted peptide that is able to activate the JAK/STAT pathway to trigger the expression of a set of genes that can inhibit viral replication and spreading [56]. Based on this description, Vago is thus considered as a functional homolog in arthropods and although structurally unrelated, as similar to vertebrate type I IFN cytokines [56].

Recently, some exciting progresses have been made for a deeper insight into the molecular and cellular mechanisms underlying the nucleic acid-induced shrimp's non-specific defense system. First, several key components homologous to the vertebrate type I interferon pathway have been found in shrimps (Fig. 3). These components include homologs of viral nucleic acids sensors, such as Dicer2 and many Toll receptors. Homologs of other interferon-signaling components include inhibitor of nuclear factor κB kinase ε (IKKε), interferon-regulatory factor (IRF), stimulator of interferon genes (STING), janus kinase (JAK), and signal transducer and activator of transcription (STAT). Besides, five Vago-related genes *Vago1-5*, the interferon-like molecules in arthropods, are also identified in shrimp *L. vannamei* [30]. Second, the patterns of signaling cascade in the parallel antiviral system in shrimp are similar to those of mammals. At the nucleic acid receptor level, *Drosophila* Dicer2 and shrimp Dicer2 also belong to the same DExD/H-box helicase family as do the RIG-I-like receptors in mammals. These genes are upregulated in immune responses against Poly (C-G) or WSSV challenge [30]. Moreover, shrimp *L. vannamei* LvToll1 and LvToll3 exhibited affinity to CpG ODN 2395 via direct interaction [57], which is similar to the sensing pattern of the mammalian TLR9 to detect dsDNA. Surprisingly, another group provided some evidences indicating that LvToll3 was also able to respond to dsRNA regardless of its sequence [58]. Our previous results showed that the *L. vannamei* IKKε was able to induce several interferon reporter genes in the background of HEK293T cells [50]. In a recent study, our findings provided important experimental evidence that shrimps have an IRF-like transcription factor, which directly interacts with the promoter of a secreted interferon-like cytokine Vago4 that activated a JAK/STAT signaling pathway [59]. Moreover, the shrimp IRF was also capable of recognizing mammalian ISG elements and inducing the expression of mammalian ISGs [59]. Notably, the IRF-Vago cascade appeared to be engaged by the receptors LvToll3 and Dicer2 in response to dsRNA [30,58]. In mammals, STING is the central regulator of the interferon-producing pathway in response to dsDNA species from DNA virus [60–62]. Our recent study demonstrates that *L. vannamei* STING (LvSTING) is able to induce the activation of the IRF-Vago cascade (unpublished data). Taken together, these results indicated that shrimps indeed have a Dicer/Toll-(STING)-IKKε-IRF-Vago/JAK-STAT signaling axis similar to the RIG-I/Toll-(STING)-IKKε-IRF-IFN/JAK-STAT signaling cascade in mammals. Finally, the functional analysis of these key components of shrimp during viral (mainly WSSV) infection has demonstrated their important roles in defense against viral infection. Specifically, the knockdown of Dicer2 [38], STING (unpublished data), IRF [59], Vago [59], or JAK [63] renders shrimps more susceptible to viral infection, as manifested by the higher mortality rates or/and viral loads observed in these key components-silenced shrimps. Nevertheless, in the next step, we need to determine whether this signaling cascade also confers shrimp general protection from other viral infections, such as YHV and TSV. Based on the three aspects, we can conclude that shrimp has an equivalent antiviral pathway to the vertebrate type I IFN pathway.

2.3. Apoptosis and WSSV

2.3.1. Brief introduction of apoptotic signaling pathway

Apoptosis plays an important role during development and homeostasis and represents an ancient form of innate host defense system, which is considered to be evolutionarily conserved across animal species [64]. The mechanism of apoptosis initiation and execution has been extensively studied in mammals, and the process is widely acknowledged to be required for the activation of caspase cascades (Fig. 2A). Caspases belong to a family of highly conserved cysteine-dependent aspartate-specific proteases and play a central and evolutionarily conserved role in mediating and executing apoptosis [65,66]. Mammalian caspases are grouped into three major categories based on their structural and functional considerations, namely apoptotic caspases, inflammatory caspases, and the keratinocyte differentiation-

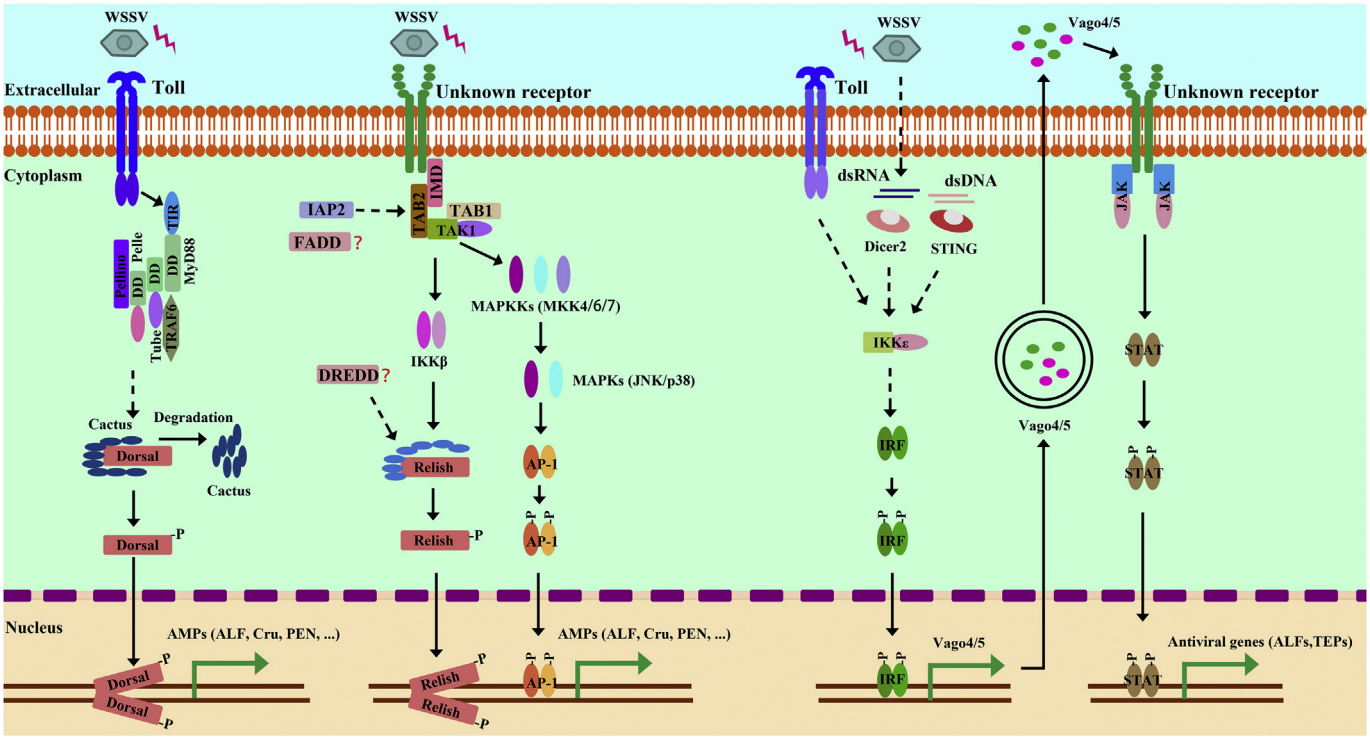

Fig. 3. Conceptual diagram of several evolutionarily conserved innate immune pathways of shrimp including Toll pathway, IMD pathway and nucleic acids induced nonspecific antiviral pathway within a JAK/STAT cascade. The activation of NF-κB related signaling pathways (Toll/IMD pathways) by WSSV infection via an unidentified sensing mechanism, leads to the stimulation of multiple transcription factors involving Dorsal, Relish and AP-1 that result in the expression of several sets of effectors such as AMPs some with antiviral ability. In the nucleic acids induced nonspecific antiviral pathway, Dicer2 and STING are proposed to sense the viral (WSSV) originated dsRNA and dsDNA species, respectively. Both proteins could engage the IRF-Vago cascade to induce the expression of Vago that can activate the JAK/STAT pathway to promote some antiviral genes expression to combat infection.

related caspase such ascaspase-14 [67]. The apoptotic caspases are further subclassified into initiator caspases and executioner or effector caspases. Two separate yet interlinked apoptotic signaling pathways in mammals are triggered by intracellular and extracellular stimuli, extrinsic pathway or death receptor pathway, and intrinsic pathway or mitochondria/cytochrome c pathway [68] (Fig. 2A). The extrinsic pathway is activated by the binding of death receptors to proapoptotic ligands, leading to receptor oligomerization, and the initiator caspases such as caspase8 and 10 are recruited to initiate death receptor signaling for apoptosis. The intrinsic pathway is activated by diverse cellular stress signals such as DNA damage, which leads to the permeabilization of the mitochondrial outer membrane and the release of proteins, such as cytochrome c [69]. Cytochrome c is able to induce the cleavage and activation of initiator caspase-9 with the involvement of other adaptor molecules. The extrinsic death receptor pathway can be linked to the intrinsic mitochondrial pathway via the cleavage of Bid [70]. The two pathways converge on the cleavage of the latent (zymogen) form of procaspase3/6/7 to form the activated effector caspase3, 6, and 7, which execute apoptosis by cleaving essential cellular proteins and ultimately lead to the cell's apoptotic demise [68,71]. Subsequently, the dying cells are removed and degraded via phagocytosis.

2.3.2. Shrimp caspases and apoptosis-related regulatory factors are involved in antiviral apoptosis against WSSV infection

Most of the recent studies on apoptosis in shrimp antiviral defense mechanisms are focused on the discovery of pathway proteins based on sequence homology with other organisms [10]. Until now, several caspases have been cloned and functionally characterized from multiple different shrimps (Fig. 2B). In *L. vannamei*, five caspases has been identified and named LvCaspase1 to LvCaspase5 based on the time order of discovery, among which the LvCaspase1 (also named as LvCap-3 in originally identified article [72]), LvCaspase2, and LvCaspase5 could be effector caspases, whereas LvCaspase3 and LvCaspase4 seem to be initiator caspases [72,73]. LvCaspase2/3/5 are proposed to possess antiviral roles against WSSV, as evidenced by higher expression levels of WSSV VP28 gene observed in LvCaspase2-, LvCaspase3-, or LvCaspase5-silenced shrimps after viral infection [73]. Interestingly, knocking down LvCaspase1(LvCap-3) via RNAi reduced mortality in *L. vannamei* challenged with a low dose of WSSV. This result suggests that LvCaspase1 may exacerbate rather than decrease mortality in WSSV-challenged shrimp [72]. Another caspase from *M. japonicus* (MjCaspase or designated as PjCaspase) shared sequence homologies to Caspase8 and 10 of other species and was thus deemed to be an initiator caspase [74] and also named as Mjcaspase8 in subsequent studies [75]. The study indicated that MjCaspase played an essential role in the WSSV-induced apoptosis progress, and MjCaspase-mediated apoptosis played a key role in the innate immune response to eliminate the virus-infected cells [74]. This conclusion was supported by the observation that virus-induced apoptosis was significantly inhibited and that the higher copies of WSSV virions were detected in MjCaspase-silenced shrimps [74]. Two different caspases from *P. monodon*, namely PmCaspase [76] and PmCasp [77] have been identified and shown to induce apoptosis. These two caspases have shown to play an important role in defense against WSSV and are thus frequently targeted by viruses to achieve immune evasion.

Some apoptosis-related regulatory factors such as Dap-1, PTEN, p53, Fortilin, HtrA2, IAP, TSL, and HHAP were also identified and have shown to play important roles in regulating antiviral apoptosis in shrimps (Fig. 2B). One study showed that Death associated protein 1 (Dap-1) from *M. japonicus* was helpful for amplifying WSSV in shrimp, which may be attributed to the promotion of hemocyte apoptosis in an AIF-dependent but not caspase-dependent manner [78]. As we mentioned that apoptosis functioned as an antiviral response against WSSV

in *M. japonicas* [74], this observation is surprising, and further study is needed to dissect the specific action of MjDap-1-mediated WSSV replication. Besides, a gene from *L. vannamei* named PTEN for Phosphatase and tensin homolog deleted on chromosome 10 has shown to participate in apoptosis by upregulating the downstream pro-apoptosis genes Lvp53 and LvCaspase1(LvCap-3) expression in response to infection [79]. Given that the knockdown of Lvp53 caused higher mortalities and virus loads under WSSV infection [80] and that it is able to induce apoptosis under some environmental stresses [81], we speculate that the shrimp PTEN-p53/caspase cascade-mediated apoptosis can play a critical role in defense against WSSV. One research showed that *Pm*-Fortilin, also known as translationally controlled tumor protein (TCTP), has similar anti-apoptotic properties to their human homologs, and its abundance can be greatly induced in *P. monodon* hemolymph during the early phase of WSSV infection; it has a key role in response to this virus [82]. Furthermore, HtrA2 is a member of the high-temperature requirement A (HtrA) family, and functions as an apoptosis-activating protein to enhance the apoptotic process by disrupting inhibitor of apoptosis protein (IAP)-caspase interaction, thus freeing caspase to trigger the apoptosis pathway [83]. A shrimp HtrA2 homolog (PmHtrA2) was identified from *P. monodon* [84]. PmHtrA2 was able to interact with PmIAP, and functioned as an apoptosis-inducing factor via recovering PmCaspase activity from the inhibition by PmIAP; the apoptosis was induced [85]. Interestingly, LvHtrA from *L. vannamei* was positively related to the transcription of LvCaspase1 (LvCap-3), pointing that LvHtrA seemed to positively regulate the apoptosis pathway. However, the knockdown of LvHtrA2 led to delay shrimp mortality during WSSV infection, suggesting that LvHtrA2 may be involved in apoptosis-mediated mortality rather than providing immune protection during WSSV infection [86]. Thus, HtrA2 could have different roles in response to WSSV infection in different shrimp species. A shrimp *L. vannamei* tumor suppressor-like protein (LvTSL) homologous to the human tumor suppressor OVCA1 (ovary cancer gene 1) has been shown to have pro-apoptosis activity in the mammalian BHK cells [87]. A *P. monodon* hemocyte homeostasis-associated protein (PmHHAP) has been shown to regulate hemocyte homeostasis by inhibiting apoptotic cell death through caspase activation. In a recent study, an apoptosis signal-regulating kinase 1 (ASK1) homolog from *L. vannamei* (LvASK1) was identified and characterized. LvASK1 knockdown via RNAi *in vivo* significantly reduced the apoptotic ratio of hemocytes collected from WSSV-infected *L. vannamei*, and decreased the cumulative mortality of *L. vannamei* after WSSV infection [88]. The result was similar to those in previous studies on pro-apoptosis gene voltage-dependent anion channel (VDAC), which was critical in inducing apoptosis through the promotion of mitochondrial membrane permeabilization (MMP) [89]. Therefore, the authors proposed that reduced cumulative mortality in the LvASK1 downregulated group could be due to the reduced hemocyte apoptosis level upon WSSV infection [88]. In summary, the extent of apoptosis is related to the severity of the WSSV infection, varies across different tissue/organs, and also depends on the species of the infected host [10].

Another type of regulatory factor is the host encoded miRNAs, which are directly or indirectly implicated with antiviral apoptosis in shrimps. It has been discovered that shrimp miR-100 was up-regulated at 24 h after WSSV infection, and that knockdown of miR-100 expression decreased the mortality of WSSV-infected shrimp from 24 h to 72 h post-infection [90]. Further, the authors found that knockdown of miR-100 induced the apoptosis of shrimp hemocytes, in other words, the up-regulated expressed miR-100 could play a role to inhibit apoptosis, which could be implicated with WSSV pathogenesis. However, the actual function of host miR-100 related to apoptosis is still unclear. As mentioned above, Lvp53 could be able to induce antiviral apoptosis in *L. vannamei*. Interestingly, a result was observed from Gong et al. that *M. japonicas* p53 could also regulate the apoptotic activity, but play a negative role in defense against WSSV as shown by that knockdown of Mjp53 resulted in significant decreases of WSSV copies in shrimp [91]. It is noted that Mjp53 could be targeted by host encoded miR-1000, and the miR-1000 silencing resulted in significant increases of apoptotic activity and virus infection, indicating that miR-1000 took great effects on apoptosis and virus infection by targeting Mjp53. In addition, another study provided evidence that *M. japonicas* encoded miR-12 could trigger the antiviral apoptosis by downregulating expression of the BI-1 gene (transmembrane BAX inhibitor motif containing 6), which encodes an inhibitor of apoptosis [92]. However, the precise mechanism of how these regulatory factors induce antiviral apoptosis in shrimps is still largely unknown and needs to be determined further.

2.3.3. WSSV evasion of apoptotic signaling

Apoptosis has been shown to play a critical role in vertebrate and invertebrate defense against viral pathogens. Invertebrates, such as insects lack adaptive immunity, and apoptosis has been widely recognized as a powerful antiviral action to limit viral replication, infectivity, and spread [93,94]. In shrimps, coming to a final and clear conclusion that the relative contribution of apoptosis to viral pathogenicity and/or antiviral immune responses is difficult. The viral accommodation theory says that viral-triggered apoptosis may be a major cause of mortality, and reduced rates of cell death may allow for the attenuation of viral pathogenicity in shrimp [95,96]. Later, Wongprasert et al. reported that cells displaying nuclear condensation and fragmentation characteristic of apoptosis did not contain WSSV virions, whereas those containing WSSV virions were not apoptotic [93]. This latter observation suggests that WSSV could have evolved some strategies to counter host antiviral apoptosis (Fig. 2B), which is supported by increasing evidence as follows.

Caspases are critical protease mediators of apoptosis, and WSSV have been shown to encode several genes to target the host caspases and disturb their activities. The viral AAP-1, encoded by WSSV449 or ORF390, has been identified as an anti-apoptosis protein. The overexpression of AAP-1 in insect cell is able to replace the anti-apoptosis protein P35 to inhibit the apoptosis induced by a p35-deleted mutant AcMNPV (Autographa californica multiple nucleopolyhedrovirus), thereby rescuing the multiplication of this virus [97]. AAP-1 contained two putative caspase-9 cleavage sites, VETD233G and LEHD303G, as well as a caspase-3 cleavage site, DEVD272G [97]. Further study showed that two of these sites, LEHD303G and DEVD272G, were required by PmCaspase in targeting and cleaving AAP-1 [98]. The cleaved AAP-1 (C-terminal of AAP-1), but not the full-length AAP-1, can bind with PmCaspase, which lead to inhibit PmCaspase activity and its mediated apoptosis [77]. PmIAP functioned as an anti-apoptosis protein by binding with PmCaspase, but this interaction has been shown to have only a slight anti-apoptosis role. AAP-1 showed a stronger inhibited effect than PmIAP to PmCaspase, as AAP-1 can completely block the pro-apoptotic activity of PmCaspase in SF-9 cells, whereas PmIAP showed only negligible anti-*Pm* caspase activity. In view of the full-length of AAP-1 as a substrate for PmCaspase and the cleaved AAP-1 (AAP-1-C) with ability to bind with PmCaspase [77,98], AAP-1 could not only compete with others pro-apoptosis related substrates or host inhibitors of apoptosis such as IAP for caspase but also bind to caspase via its cleaved part to alter caspase activity, both of which lead to inhibition of apoptosis.

Another effector caspase the PmCasp from *P. monodon* has been demonstrated as target for two viral proteins to inhibit apoptosis [99]. WSSV proteins WSSV322 and WSSV134, also known as the WSSV structural protein VP36A, have been shown to bind with the p20 domain of PmCasp through yeast two-hybrid screening and further confirmed by co-immunoprecipitation. In the background of insect cell (Sf-9), either WSSV134 or WSSV322 was able to inhibit PmCasp mediated apoptosis. This research also showed that the activity of PmCasp cannot be blocked by the previously identified anti-apoptosis protein-1 (AAP-1) that targeted another caspase (PmCaspase) [98]; these results revealed diversity in effector caspases and their viral protein inhibitors in *P. monodon* [99]. One study further showed that WSSV134 or VP36A

contains three potential caspase binding sites, corresponding to D54, D104 and D259, but site-directed mutagenesis assay indicated that only the residue D104 was important for PmCasp inhibition [100]. Of note, WSSV134 is able to interact with a host anti-apoptosis factor hemocyte homeostasis-associated protein (PmHHAP), which can regulate hemocyte homeostasis by suppressing caspase mediated apoptotic cell death during WSSV infection [101,102]. Although the interaction between WSSV134 and PmHHAP was observed, they were able to inhibit caspase-induced activation of PmCasp in a non-competitive manner *in vitro* [102]. Besides, *in vivo* experiment showed that co-silencing PmHHAP and WSSV134 counteracted the effects on WSSV infection. Based on these finding, the authors suggested that PmHHAP might counteract WSSV134 and control apoptosis triggered by WSSV to protect shrimp from their demise rather than causing shrimp death [102]. Further research is required to reveal the exact mechanism underlying the WSSV134-mediated anti-apoptosis during viral infection.

The MjCaspase from shrimp *M. japonicus* (designated as PjCaspase [74,103] or Mjcaspase8 [75]) was also identified to be a target for several viral products including two viral structural proteins VP38 and VP41B and a microRNA WSSV-miR-N24. Huang et al. found that the WSSV encoded miR-N24 was able to target the host Mjcaspase8, which led to repressed antiviral apoptosis of shrimp hemocytes *in vivo*. This result was consistent in that the number of WSSV copies in shrimp *in vivo* was significantly increased compared with the control level (WSSV only) [75]. Surprisingly, WSSV encoded VP38 and VP41B are viral envelope proteins, but the streptavidin-bead pulldown assay demonstrated VP38 and VP41B were able to bind to MjCaspase promoter [103]. Luciferase reporter assay further showed that VP38 and VP41B could upregulate and downregulate the promoter activities of MjCaspase, respectively. These results showed the first report on WSSV envelope proteins with potential transcriptional activity, and provide some novel insights into the function of WSSV structural proteins in gene regulation. However, the exact function of VP38 and VP41B during viral infection is still unknown.

In addition to target the caspases, several viral genes have been shown to regulate apoptosis-related regulatory factors to manipulate host antiviral apoptosis. A study from He et al. showed that wsv222 encoded a viral E3 ligase, which mediated ubiquitination degradation of the host tumor suppressor-like protein (TSL) with pro-apoptosis activity [87]. This research indicated that WSSV encoded E3 ligase can indirectly inhibit apoptosis via reducing the abundant of pro-apoptosis related regulated factor. Besides, the ICP11, a highly expressed gene after WSSV infection [104], appeared to have apoptosis-inducing activity [105]. ICP11 was found to form a histone-binding DNA mimic that disrupted nucleosome assembly. The overexpression of ICP11 in HeLa cells was able to induce apoptosis, which was likely to attribute to the incidental consequence of its destabilization of nucleosomes [105]. The involvement of ICP11 in apoptosis *in vivo* needs to further investigation.

3. Humoral immunity and WSSV

3.1. Evolutionarily conserved NF-κB pathways and WSSV

Nuclear Factor-κB (NF-κB) related signaling pathways form a fundamental part of innate humoral immune system and are conserved throughout the animal kingdom [106,107]. Two NF-κB signaling pathways, namely, the Toll and the IMD pathways, have been well-identified and functional characterized in *Drosophila* [108,109]. In shrimps, the constituent proteins and function of the Toll and IMD pathways have been previously reviewed [110,111]. Many significant progresses have been made in the mechanism of activation and regulation of these pathways.

3.1.1. Toll pathway

In contrast to that fungi and most Gram positive bacteria mainly activate the Toll pathway and Gram-negative bacteria activate the IMD pathway in *Drosophila*, the activation of the Toll and IMD pathways in shrimps appears to be induced by both Gram-positive and Gram-negative bacteria, as evidenced by the remarkable expression changes of core components of both pathways were observed after several bacterial infections including *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *Staphylococcus aureus* [110,111]. Likewise, WSSV has been shown to activate Toll and IMD pathways in shrimps [110–112]. Signal transduction in both pathways displays some striking similarities, as well as some notable differences, compared to these *Drosophila* Toll and IMD pathways (Fig. 3). Indeed, studies showed that shrimp Tolls function as pattern recognition receptors are similar to TLRs in mammals and are able to sense microbial derived products, such as LPS, PGN and CpG ODN via direct interaction with each other [57,113]. In our very recent research, we cloned a total of nine Tolls from *L. vannamei*, among which the new identified Toll4 could be a pattern recognition receptor for WSSV [112]. *Drosophila* Toll1 is shown to bind to a cytokine Spaetzle in response to microbial challenge indirectly [108]. Nevertheless, we cannot rule out the possibility that shrimp Tolls have the ability to sense and interact with Spaetzle, as several Spaetzles have also been found in different shrimps and showed to activate the promoters of Toll pathway controlled AMPs in insect cells [114,115] and the synthesis of several AMPs *in vivo* [116,117]. In the intracellular signaling aspect, the shrimp Toll pathway has revealed striking similarities to that of *Drosophila* (Fig. 3). Upon Toll receptor activation, MyD88 is recruited through a homotypic TIR interaction and forms a heterotrimeric complex with other two death domain (DD) containing proteins Tube and Pelle [118]. Two more proteins Pellino and TRAF6 are shown to be involved in the Toll pathway signaling via their interaction with Pelle and Tube, respectively [119,120]. Shrimp Pelle has a kinase domain that is similar to *Drosophila* Pelle, which is proposed to phosphorylate the IκB protein Cactus, triggering its polyubiquitination and degradation by the proteasome. Whether shrimp Pelle is able to phosphorylate the shrimp IκB protein Cactus needs to be determined further [121]. Regardless of this mechanism in shrimps, the activation of Toll pathway directs the signaling to the NF-κB transcription factor Dorsal, which then translocates into the nucleus and activates the transcription of collective sets of AMPs [121].

3.1.2. IMD pathway

In *Drosophila*, the activation of the IMD pathway by bacterial DAP-type PGN from most Gram-negative bacteria and some Gram-positive bacteria requires the sensing receptors of peptidoglycan recognition proteins (PGRPs) [109,122]. Still now, no PGRP homolog is reported and found in shrimps, although we have tried our best to search for homologous sequences in the available transcriptome data from ours [123] and others submitted in NCBI (data not shown). Other sequence unrelated proteins might function as receptors for IMD pathway to sense PGN and initiate signaling in shrimps. Some core components of IMD pathway from shrimps have been identified, including IMD [124], TAB2 [125], TAB1 [126], TAK1 [127], IKKβ [128], IAP2 [129], and the transcription factor Relish [130,131]. However, the two core proteins FADD and DREDD, which are critical for the initiation of downstream signaling events, including their mediated cleavage, ubiquitination of IMD, and cleavage of relish in *Drosophila* [109,122], are not identified from shrimps. Intracellular signaling in the IMD pathway of shrimps is also likely to involve a receptor-proximal multi-protein complex, which is similar to that in *Drosophila* [122]. Some interplay between these core components have been observed in shrimps. For example, shrimp *L. vannamei* LvTAK1 has been shown to interact with LvTAB2 [127], and both LvTAK1 and LvTAB2 can regulate the expression of several AMPs in insect cells and *in vivo* [125,127]. In addition, *L. vannamei* LvTAB1 combines with LvTAK1 and Lvp38, indicating that LvTAB1 can function as a regulator for the activities of TAK1 and p38 [126]. However, the mechanism of activation and regulation of shrimp IMD pathway is still largely unclear. In general, after bacterial infection, the IMD pathway

directs signaling to Relish, whose activation requires phosphorylation and cleavage to remove the C-terminal IκB-like domain. The N-terminal Rel homology domain (RHD) then translocates into the nucleus and triggers the expression of several sets of AMPs. Recombinant intact LvRelish in S2 cells can be cleaved into two fragments, namely, the N- and C-terminal cleavage products, and the molecular weight of the former is identical to the predicted RHD domain of LvRelish [130]. EMSA analysis and dual-luciferase reporter assays support that the RHD domain of LvRelish can bind with the κB motif that are frequently observed in the promoter region of shrimps and insect AMPs [130]. In a separate arm of the IMD pathway, shrimp TAK1 transmits signaling to MAPK kinases, which predominantly targets activation of AP-1 transcription factors. Our results indicate that LvTAK1 can bind to and phosphorylate LvMKK4, LvMKK7, and LvMKK6 (unpublished data), which activates downstream MAPK pathways, including LvMKK7-LvJNK [132] and LvMKK6-Lvp38 cascades [133]. The activation of MAPK pathways culminates in triggering the phosphorylation and nuclear translocation of the AP-1 transcription factors, such as c-Jun, which leads to the expression of several AMPs [134].

3.1.3. *WSSV subversion of NF-κB pathway*

In response to WSSV infection, the activation of Toll and IMD signaling pathways results in the up-regulated activities of the transcription factors Dorsal, Relish, and AP-1 (Fig. 4). Several AMPs, such as ALFs, Crustins, LYZs and C-type lectin regulated by the shrimp Toll and/or IMD signaling pathways, can inhibit WSSV [112,135–139]. Shrimp Tolls from *L. vannamei*, *Cherax quadricarinatus*, *Procambarus clarkii,* and *Macrobrachium rosenbergii* can restrict WSSV infection through inducing the expression of AMPs [112,140–142]. However, the successful infection of WSSV requires the activation of host NF-κB-related signaling pathways. WSSV has developed several strategies to subvert or hijack host NF-κB-related signaling pathways for its genome replication and gene expression (Fig. 4). In particular, WSSV encodes a non-structural protein WSSV449 (wsv390) with 15.7%–19.4% sequence identity to Tube homologs from insects [120]. WSSV449 plays a similar function to host Tube in activating the NF-κB pathway, as illustrated by its ability to activate promoters of Toll-signaling pathway-controlled AMPs and viral self-genes, including wsv069 (ie1), wsv303, and wsv371, with κB sites in their promoter regions as a result of WSSV449 over-expression in insect cells [120]. Several core members of shrimp Toll signaling pathway, including Pellino and MyD88, induce the promoter activities of wsv069 (ie1), wsv303, and wsv371 *in vitro* [119,143]. Dorsal is the transcription factor of Toll-signaling pathway, and its activity may represent the outcome of the activation of this pathway. Therefore, these results suggest that the activation of Dorsal can facilitate WSSV pathogenesis. Similarly, decreased viral loads and cumulative mortalities are observed in the Dorsal- or Relish-silenced shrimp *L. vannamei* [144]. The host microRNA miR-1959, which mediates a positive feedback loop between Dorsal and Cactus that can continuously maintain the activation of the NF-κB factor Dorsal [145], is induced and hijacked by WSSV to favor its infection in *L. vannamei* [146]. However, the two WSSV encoded miRNAs WSSV-miR-N13 and WSSV-miR-N23 are targeted to host Dorsal to escape Spz-Toll-Dorsal-ALFs signaling pathway-mediated antiviral action in shrimp *M. japonicas* [147]. These observations suggest that the interplay between host Toll-Dorsal signaling pathway and WSSV can be complex and dynamic during this viral infection. Similarly, the IMD pathway, including the Relish branch and the AP-1 branch, is utilized by WSSV to favor its infection. For instance, Relish binds to the promoter of wsv069 in a κB site-dependent manner and increases its promoter activity [148]. Notably, wsv069 can also induce itself promoter activities [148], indicating that a potential positive feedback loop mediated by wsv069 can continuously annex the shrimp Relish system to enhance the expression of viral genes. LvIKKβ, a core regulator of the IMD-Relish signaling pathway, induces the promoter activities of up to ten viral genes, including wsv051, wsv059, wsv069, wsv083, wsv090, wsv107,

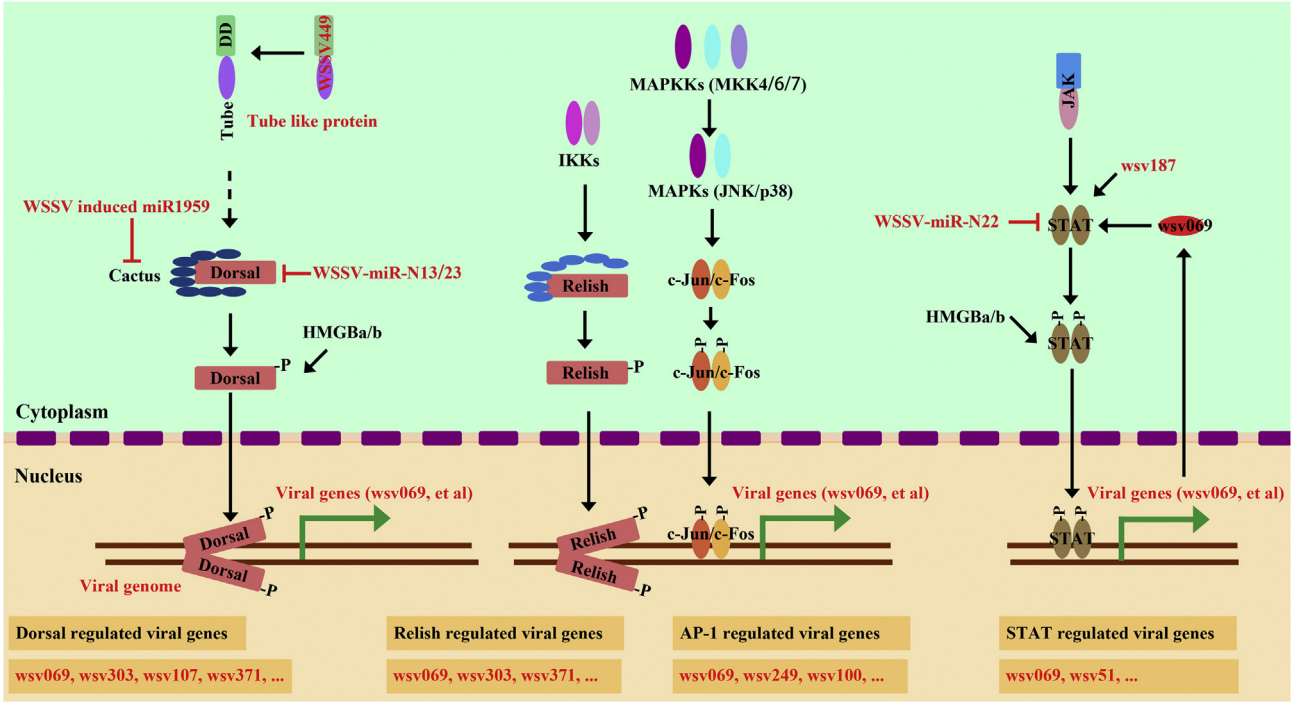

Fig. 4. WSSV modulation of innate immune signaling pathways including Toll pathway, IMD pathway and JAK/STAT pathway. WSSV encoded WSSV449 (wsv390) mimics a host factor the Tube protein, a core member of the Toll pathway, to activate NF-κB factor Dorsal that can translocate into the nucleus where it regulates the expression of viral genes such as wsv069. The Relish and AP-1, the transcription factors from two branches of the IMD pathway, is shown to be hijacked by WSSV to induce the expression of viral genes such as wsv069. The WSSV encoded WSSV-miR-N22 targets the host STAT to evade the JAK/STAT mediated antiviral action. In addition, several other viral or host factors such as host miR1959 and wsv187 are involved in regulating the activity of host signaling pathways and viral genes expression (See the text for detail).

wsv244, wsv303, wsv371, and wsv445, in *Drosophila* S2 cells [128]. Some core members of IMD-AP-1 signaling pathway, including MKK6, JNK, c-Jun, and c-Fos, have also been shown to regulate the expression of a wide range of viral genes, such as wsv069, wsv100, and wsv249 *in vivo* or *in vitro* [133,134,149,150].

3.2. Interference with the JAK/STAT pathway by WSSV

The JAK/STAT pathway, a separate immune response pathway that does not involve NF-κB factors, is critical to control viral infections in insects and mammals. As mentioned above, the JAK/STAT pathway is a fundamental part of nucleic acids inducing nonspecific antiviral signaling pathway in shrimps. The knockdown of LvJAK from *L. vannamei* causes increased mortality rate and viral load after WSSV infection [63]. In addition, knockdown of LvSOCS2, the inhibitor of LvJAK, decreases shrimp viral load and susceptibility to WSSV, further demonstrating that the JAK/STAT pathway can exhibit anti-viral immunity in shrimp [151]. WSSV encoding a WSSV-miR-22 can suppress shrimp *M. japonicas* STAT, supporting that STAT is a key restrict factor against WSSV infection [152]. Interestingly, ectopic expression of wsv187 activates JAK/STAT pathway in *Drosophila* and confers resistance against DCV infection [153]. However, the effect of wsv187 on the JAK/STAT pathway of natural hosts, such as *L. vannamei* and *M. japonicas,* during WSSV infection is still unclear. On the other hand, WSSV has evolved several strategies to subvert host JAK/STAT pathway to facilitate viral gene expression. Host STAT is the originally identified factor that is hijacked by WSSV to promote viral gene expression (wsv069) [154,155]. Notably, wsv069, which is regulated by STAT, can interact with STAT again and promote its phosphorylation [156]. This process creates a positive feedback loop mediated by STAT/wsv069, which can continuously maintain the activity of STAT. The expression of wsv069, the first identified immediate early gene from WSSV, is dependent on several host factors, such as YY1 [157] and HMGBa/b [158], in addition to the previously mentioned NF-κB factors Dorsal and Relish and AP-1 factors c-Jun and c-Fos. Based on these observations, how WSSV orchestrates the interplay between host and virus is very fascinating but poorly understood. However, the successful of infection can be proposed to require WSSV to subvert the host JAK/STAT pathway to promote the expression of viral genes, such as wsv069, in the early stage of infection. Consistently, wsv069 is the immediate early gene that is expressed in very early stage of infection. The established WSSV infection then utilizes other viral genes, such as WSSV-miR-22, to escape the JAK/STAT pathway-mediated antiviral action.

3.3. ProPO system induced melanization and WSSV

3.3.1. ProPO system induced melanization in shrimps

The melanization is considered as an important facet of host defense in a wide range of invertebrates, which is controlled by the prophenoloxidase activating system (proPO system) in response to pathogenic infection. In shrimps, the melanization reaction mediated by proPO system has been well reviewed by Anchalee Tassanakajon et al. [159]. Melanization pathway involves three major processes: pathogen recognition, proteolytic cascade, and activation of proPO enzymes (Fig. 6 and [159]). Currently, several constituent proteins of melanization/proPO system have been identified in shrimps, including prophenoloxidase 1 (proPO1), proPO2, proPO-activating enzyme 1 (proPPAE1), and proPPAE2, from *L. vannamei* and *P. monodon* [160–164]. The proPO cascade is an efficient non self-recognition system [165]. Upon pathogenic infection, host utilizes pattern recognition proteins to detect and bind to pathogen-associated molecular patterns, for example, potential PGN binding protein (PGRP) senses PGN of Gram-positive bacteria, and LGBP senses LPS and β-Glucan from Gram-negative bacteria and fungi, respectively. This recognition leads to the activation of a not yet well defined serine proteinase cascade, which subsequently activates PPAE by cleaving an inactive zymogen proPPAE [165]. The activated PPAE renders the enzymatically inactive proPO cleave into active phenoloxidase (PO), which is a type of tyrosinase that is a copper containing enzyme. The activation of PO catalyzes oxidation of mono- or *o*-diphenols to the corresponding o-quinone. The quinines crosslink together to form melanin, which is deposited onto the surface of the target and form a capsule like structure to prevent the growth, reproduction, and survival of the pathogen. The progress of melanization arises with the synthesis of reactive oxygen and nitrogen intermediates that work together to kill the invading pathogen. In shrimps, melanization plays a vital role in defense against multiple type of pathogens, including bacterial (*V. harveyi*), fungal (*Fusarium solani*), and viral (WSSV) infection [165]. Silencing of proPO(s) from *P. monodon* by RNAi elevates shrimp lethality rate after WSSV infection, whereas incubation of WSSV with an *in vitro* melanization reaction prior to injection into shrimp significantly increases the shrimp survival rate, which strongly indicates a key role of the melanization in defense against WSSV [166].

3.3.2. WSSV evasion of ProPO system induced melanization

WSSV have evolved strategies to evade immune control mechanisms. The isolated granular cells (GCs, one type of hemocytes) from sham-injected crayfish were melanized but not in the GCs from WSSV infected crayfish, which was the first report of WSSV with the ability to inhibit melanization [167]. In this report, the inhibition of melanization in cells was affected by an upstream of PO or alternatively by depletion of the native substrate for PO. The PO activities in hemocyte lysate supernatant of both sham-injected and WSSV-injected crayfish were observed to be the same as proPO expression. However, other research in *P. monodon* showed that the hemolymph PO activity of WSSV-infected shrimp was severely reduced at days 2 and 3 post-injection [166]. These results suggested that WSSV probably inhibited multiple proteinases activity in the proPO cascade in different shrimps. The authors also demonstrated that WSSV encoded WSSV453, a non-structural viral protein, to be able to interact with PmproPPAE2, and this interaction reduced PmPPAE2 activity toward PmPO1 or PmPO2 [168]. Interestingly, WSSV453 exhibited no effect on activated PmPPAE2, which was supported by the observation that addition of active PmPPAE2 to WSSV-infected shrimp plasma can rescue PO activity [168]. Furthermore, another viral protein WSSV164 inhibited melanization by interacting with host proPO system components PmproPO1 and PmproPO2, which can attribute to that binding of WSSV164 to PmproPO1 and 2 that failed to produce functional PO [169]. Taken together, WSSV have evolved several strategies to overcome the host antiviral melanization, as mentioned previously, WSSV encoded proteins, such as WSSV453 and WSSV164, can target multiple levels of host proPO cascade (Fig. 5).

4. WSSV manipulation of metabolic reprogramming

Virus infections trigger metabolic changes in host cells that support the bioenergetic and biosynthetic demands of viral replication. Similar to some mammalian virus, WSSV infection induced a metabolic shift in shrimp that resembles a Warburg effect, which was characterized by enhanced glucose uptake, glycolysis, and lactate fermentation [89]. Studies have shown that this effect was achieved via the activation of the PI3K-Akt-mTOR pathway and was usually accompanied by the activation of other metabolic pathways, such as the pentose phosphate pathway (PPP), nucleotide biosynthesis, glutaminolysis, and amino acid biosynthesis [170]. WSSV is also able to use this metabolic shift (Warburg effect) to counteract the high levels of reactive oxygen species produced by host cells in response to viral infection, which represents an immune evasion strategy [171]. Previous research works demonstrate that WSSV genome replication takes approximately 22 h–24 h, and the 12 and 24 h post infection (hpi) represent the beginning of the genome replication and late stages, respectively [89,172]. At the initial stage of WSSV infection (0.52 hpi), host cells transiently produced high

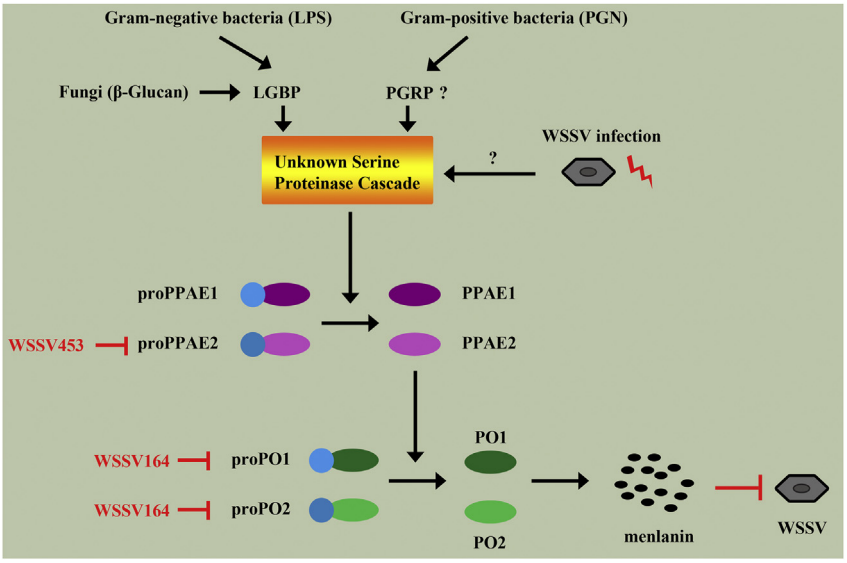

Fig. 5. WSSV evasion of ProPO system induced antiviral melanization. Several viral proteins involving WSSV453 and WSSV164 are shown to target the host members of ProPO system to evade host antiviral immune response (See the text for detail).

levels of ROS against the invading WSSV (Fig. 6). Subsequently, after 6 hpi, the ROS concentrations returned to normal, which can attribute to the elevated levels of nicotinamide adenine dinucleotide phosphate and glutathione that is the outcome of the Warburg effect triggered by this virus. Therefore, the authors concluded that the PI3K-Akt-mTOR-regulated Warburg effect neutralized ROS production and reduced the oxidative stress defenses of the host, thereby restoring the cell to a state of redox balance starting at 6 hpi and allowing the WSSV to successfully replicate [171].

In addition, WSSV induced metabolic reprogramming of host cells is crucial to achieve the viral life cycle, including viral gene expression, viral genome replication, and virion assembly. A series of studies on this aspect at two stages of WSSV infection (6–12 hpi and 24 hpi) are present. Thus, a proposed model for WSSV induced host metabolic shift to facilitate viral pathogenesis was proposed (Fig. 6). More specifically, in the early stage of WSSV infection (6–12 hpi), the PI3K-Akt-mTOR pathway was activated by an unidentified mechanism, which led to the change of at least four identified metabolic routes in shrimps. Firstly, the activation of PI3K-Akt-mTOR pathway by WSSV can induce 4E-BP1 phosphorylation, which up-regulates amino acid metabolites that are useful for protein synthesis and especially those that are involved in glutaminolysis and the synthesis of nucleic acids. This activation presumably benefits viral genome replication, which is supported by that shrimps pretreated with Rapamycin or Torin1, which are inhibitors of the upstream mTORC1 of 4E-BP1, showed the lower virus loads after WSSV infection at 12 hpi [170]. Secondly, the PI3K-Akt-mTOR pathway was activated after WSSV infection and metabolic changes resembling the Warburg effect (aerobic glycolysis) associated with an increase of energy production (lower ADP/ATP ratio) were observed in infected cells at 12 hpi [89]. Moreover, the activity of Glucose-6-phosphate dehydrogenase, which is a key enzyme in the PPP, was up regulated at 12 hpi, which can be conducive to the synthesis of the nucleotides and nucleic acids necessary for viral genome replication [89]. Thirdly, at WSSV genome replication stage (12 hpi), glutamate can be taken up by

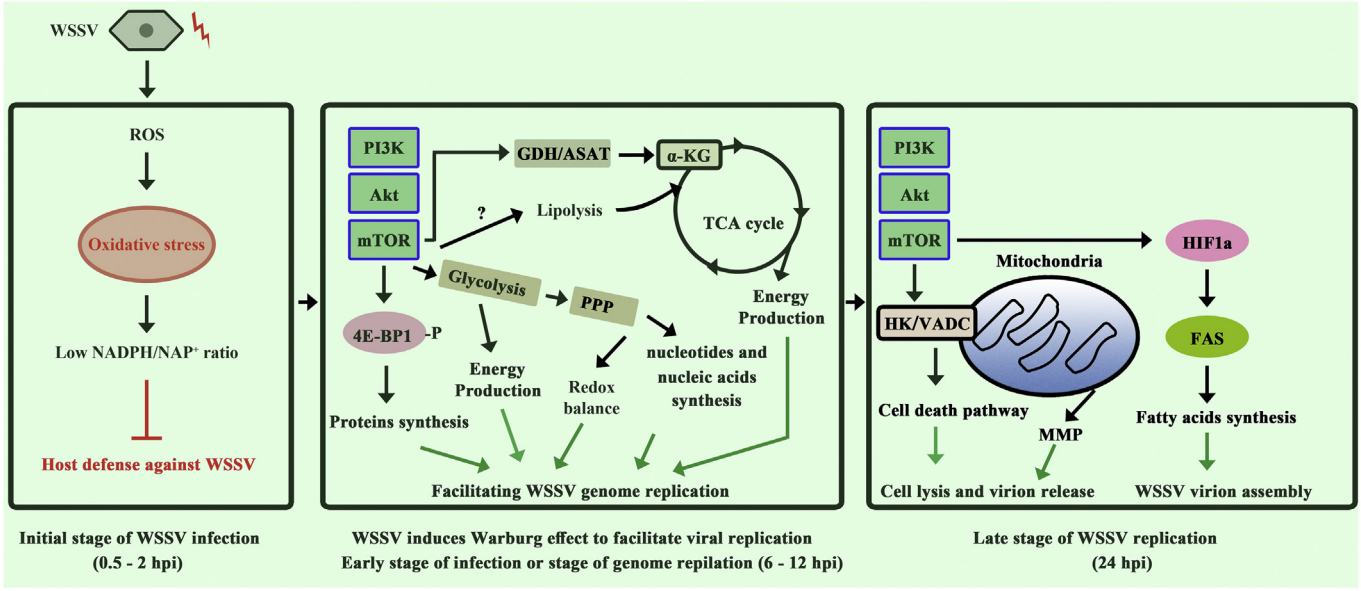

Fig. 6. Schematic representation of the WSSV-induced metabolic changes at the viral genome replication stage (6–12 hpi) and the late stage (24 hpi) of the first WSSV replication cycle according to the previous studies [89,170,171,173,174]. See the text for detail.

the hemocytes of WSSV-infected shrimp and converted to α-ketoglutarate by the two enzymes glutamate dehydrogenase (GDH) and aspartate aminotransferase (ASAT), which provides anaplerotic replenishment of the tricarboxylic acid (TCA) cycle [173]. This process is also required for viral replication, as indicated by that silencing of the two enzymes GDH and ASAT rendered shrimp with reduced VP28 expression levels and viral copy numbers after WSSV infection compared to those of control group [173]. In addition, at 12 hpi, the WSSV infected shrimp cells used the triacylglycerols (TAG) to participate in lipolysis, which may support the anaplerotic effect by increasing the availability of the building blocks and energy through TCA cycle [174]. All these metabolic changes induced by WSSV have been demonstrated to be beneficial to viral genome replication.

After the completion of genome proliferation, WSSV subverted host metabolic processes to facilitate viral genome assembly and virion release at the late stage of viral replication (24 hpi). The PI3K-Akt-mTOR pathway is important in the involvement of these metabolic shifts especially on two aspects (Fig. 6). In particular, the Warburg effect had ceased at 24 hpi, WSSV induced the activation of PI3K-Akt-mTOR pathway that increased in most of the long chain fatty acids (LCFAs), which exhibited a direction correlation with the favor of WSSV virions formation [174]. Two factors HIF1α, a transcription factor of hypoxia-inducible factor 1α, and fatty acid synthase (FAS), an enzyme with the ability to catalyze the conversion of acetyl-CoA into LCFAs, appeared to play a vital role in the progress of lipogenesis [174]. This proposal is further supported by that the WSSV virion formation was impaired in the presence of the FAS inhibitor C75 and the HIF1α inhibitor 2-ME [174]. Therefore, WSSV seems to use the PI3K-Akt-mTOR-HIF1α-FAS pathway to induce lipid biosynthesis at 24 hpi to support viral morphogenesis by providing the lipid materials necessary to meet the requirements of WSSV virion assembly. Additionally, at 24 hpi, WSSV-infected hemocytes showed several metabolic changes associated with cell death, including the generation of MMP, the accumulation of ROS, decreased glucose consumption, and disrupted energy metabolism [89]. The VDAC protein played a pivotal role in promoting this progression. WSSV infection led to an up-regulated level of VDAC and a loss of mitochondrial membrane potential at 24 hpi, as well as silencing of the VDAC reduced WSSV-induced mortality and virion copy number. Increased expression of the VDAC caused a loss of mitochondrial membrane potential, which in turn led to MMP and cell death. In other words, VDAC is probably engaged by the WSSV to regulate host mitochondrial functions, which led to cell death and lysis, and in turn allowed the new virions to be released.

However, the strategy that WSSV used to induce different metabolic shifts at the viral genome replication stage (12 hpi) and the late stage of viral infection (24 hpi) remains unknown. Furthermore, an interesting subject is to identify the putative viral factors that are involved in the activation of the PI3K-Akt-mTOR pathway and the metabolic shifts at two life stage of viral cycle.

5. Concluding remarks

Accumulating research evidence has provided a wealth of information regarding host-pathogen interactions during WSSV infection. In this review, we have described how advances have increased our understanding of how host factors hijacked by viruses to promote infection, as well as antiviral factors responsible for subverting viral infection. WSSV have adopted many different strategies to inhibit or subvert diverse intracellular signaling events: NF-κB related signaling pathways can be actively subverted to manipulate the homeostasis environment and benefit virus, a metabolic shift that resembles a Warburg effect is induced by WSSV to support its genome replication, constituent proteins of apoptosis, and melanization can be disabled to escape their mediated antiviral responses. However, most of these studies have shown only the phonotype, which are insufficient to give the nature of how viral genes regulate shrimp immune response. Further works are required to explore the exact mechanism by which WSSV used to evade or annex host immune system. In addition, information about the detailed molecular mechanism of shrimp innate immunity is still limited, and deserves more attention in the future. Increasing research works are focused on the interaction between host and WSSV, which will provide additional information for understanding WSSV pathogensis and searching new potential anti-WSSV strategy. In the near future, we can expect a more complete description of this host-pathogen interaction, and development of effective strategies for WSSV control will be important.

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