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Cellular entry of white spot syndrome virus and antiviral immunity mediated by cellular receptors in crustaceans

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ABSTRACT

Enveloped virus usually utilizes the receptor-mediated multiple endocytic routes to enter permissive host cells for successful infection. Cellular receptors are cell surface molecules, either by helping viral attachment to cell surface followed by internalization or by triggering antiviral immunity, participate in the viral-host interaction. White spot syndrome virus (WSSV), the most lethally viral pathogen with envelope and double strand DNA genome in crustacean farming, including shrimp and crayfish, has been recently found to recruit various endocytic routes for cellular entry into host cells. Meanwhile, other than the typical pattern recognition receptors for recognition of WSSV, more and more putative cellular receptors have lately been characterized to facilitate or inhibit WSSV entry. In this review, recent findings on the endocytosis-dependent WSSV entry, viral entry mediated by putative cellular receptors, the molecular interplay between WSSV and cellular receptors, and the following *anti*-WSSV immunity are summarized and discussed, which may provide us a better understanding of the WSSV pathogenesis and further possible antiviral control of white spot disease in crustacean farming.

1. Introduction

White spot syndrome virus (WSSV) is the etiology of white spot disease (WSD) that has been resulting in huge economic losses to global shrimp cultural industries since its first outbreak in Asia in 1992 [1]. At present, WSSV is still considered as the most deadly viral pathogen in shrimp and many other crustaceans due to its high mortality rates of up 90–100% within 3–7 days after infection [2,3]. A wide range of hosts that almost cover all shrimp, crayfish, crabs and other crustaceans are sensitive to WSSV infection, including economically valuable shrimp species like *Litopenaeus vannamei*, *Penaeus chinensis*, *Macrobrachium rosenbergi*, *Marsupenaeus japonicas*, *Penaeus japonicus*, *Penaeus monodon* and *Fenneropenaeus chinensis*, crayfish species like *Procambarus clarkii* and *Cherax quadricarinatus*, as well as crabs species like *Scylla serrata*, *Charybdis feriatus, Portunus pelagicus*, *Portunus sanguinolentus* and so on [4]. Unfortunately, the effective control of WSSV disease remains unavailable at present.

WSSV is an enveloped virus with a large double strands DNA, which belongs to the genus *Whispovirus*, the only member of the family *Nimaviridae*. WSSV virions are ellipsoid to bacilliform in shape with 250–380 nm in length and 80–120 nm in diameter, along with an obvious thread-like extension at one end of the virion [5,6]. The complete genome sequence of several strains isolated from different geographical regions have been revealed since its first outbreak, including WSSV-TH strain, WSSV-TW strain, WSSV-KR strain, WSSV-EG3 strain, WSSV-CN strain, WSSV-CN01 strain, WSSV-CN02 strain and WSSV-CN03 strain, which share high nucleotide identity ranging from 293 to 307 kb that encodes 154 to 181 putative open reading frames [7,8]. Specifically, at least 20% sequences variation of 34 genes have been found among three WSSV strains, including WSSV-CN01, WSSV-CN02 and WSSV-CN03 [9]. This genetic variation of WSSV genome may lay the molecular basis contributing to the significant difference in virulence, in which these three isolates exhibit similar replication kinetics but with significantly different lethal times as well as the induced immune response in the animals.

2. WSSV proteome and infectome

Based on comprehensively proteomic analysis combined with proteins localization determination, WSSV has been proposed to contain at least 58 structural proteins, of which 33 envelope proteins and 9 nucleocapsid proteins were undeniably determined [10]. Particularly, VP28, VP26, VP24 and VP19 are the four most abundant envelope proteins, of which VP28 and VP26 account for approximately 60% of

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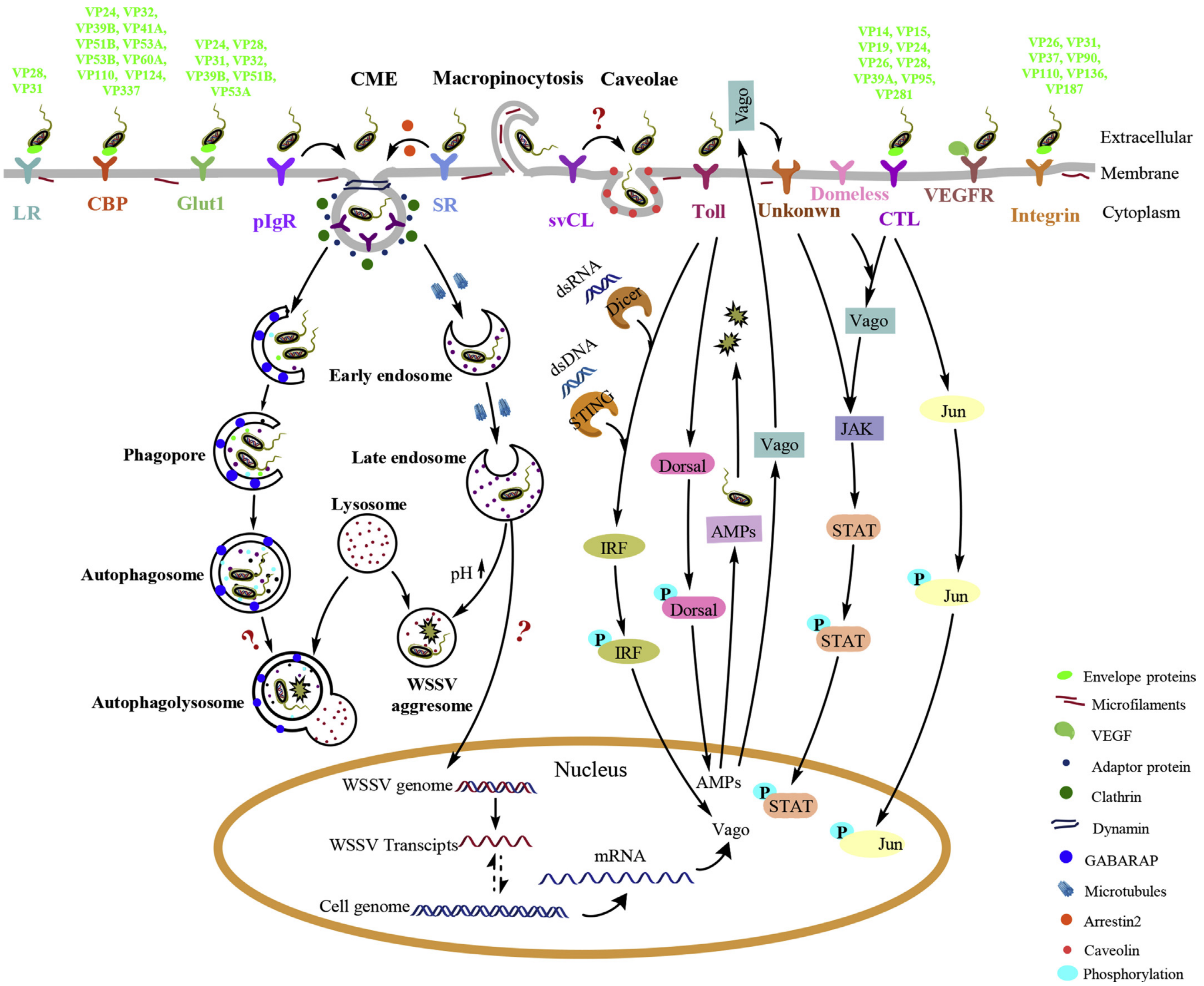

Fig. 1. Schematic representation of entry and sensing of WSSV by putative cellular receptors followed by induced antiviral immunity. The envelope proteins of WSSV bound to putative cellular receptors are indicated in green color and listed accordingly. All the molecules and signal pathways are selected and modified from previous publications, for more details please refer to the related references.Abbreviations: CME: clathrin-mediated endocytosis; pIgR: polymeric immunoglobulin receptor; VEGFR: vascular endothelial growth factor receptor; LR: laminin receptor; SR: scavenger receptor; CBP: chitin-binding protein; CTL: C-type lectins; svCL: stomach virus-associated C-type lectins; Glut1: glucose transporter 1; IRF: interferon regulatory factor; AMPs: antimicrobial peptides; JAK: the Janus kinase; STAT: signal transduction and activator of transcription. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the total amount of envelope proteins [11]. Both of VP28 and VP26 are located on the outer surface of the virion and expose β-barrel structure with an N-terminal regions protruding outside of the WSSV envelope, which therefore provides a potential structural basis for interaction with cellular receptors or to fuse with host cell membrane [12]. Meanwhile, a growing number of studies have found that VP28 could act as a key viral envelope protein to interact with a variety of cellular surface molecules on the host cell membrane, strongly highlighting the critical role of VP28 envelope protein in WSSV entry. More details about VP28 and its interplay with different cellular surface molecules will be discussed in the following section.

Unlike to VP28 and VP26, VP24 is another major envelope protein, which contains a nine stranded β-barrel fold with mostly antiparallel β-strands and the loops extending out the β-barrel at both ends of the polypeptide chain [13]. The crystal structure comparison between VP24 and VP28 or VP26 may reveal their differential roles in WSSV entry and infection. For example, VP24 was found to interact with chitin that present in the inner surface of the digestive tract for WSSV *per os* infection, exhibiting different function from other major envelope proteins [14].

Importantly, different structural proteins of virus can form a complex through interaction with each other, which is so-called "infectome", a presumable structural complex that consists of variously viral proteins to provide a structural basis for cellular tropism to targeting cells or cellular receptors. Several WSSV infectome complexes have been proposed until now. For instance, VP24 could be a presumably core envelope protein to form a complex, by which VP24 directly interacts with multiple envelope proteins, including VP26, VP28, VP38A, VP51A and WSV010, and also indirectly interacts with other envelope proteins, including VP19, VP37,VP51A, VP28 and VP51C, as well as a nucleocapsid protein VP664 [15]. Afterward, a new infectome complex of WSSV has also been proposed, which is dependent on their binding capacity with a putative cellular receptor, chitin-binding protein (CBP). This infectome is therefore categorized into two groups: one group includes VP19, VP26, VP28, VP38A, VP51A and VP51C, while the other group contains VP53A, VP28, VP31, VP32, VP39B and VP56. These two complexes are then connected via VP24 and further linked with VP56 by another linker VP31-VP32-VP39B [16]. In addition, VP11 and VP26 are likely to be assumed as a central "machinery" to form virion structural complex through their binding to other proteins or with each other, further suggesting a complicated mechanism in the infectome of WSSV [17].

Viral structural proteins complex not only provides a powerful structure for the protection of viral genome, but also acts as the attachment factor, by interacting with host cell surface receptors, for cellular entry, subsequent internalization and ultimate releasing of viral genome. It's no doubt that the envelope complex composed of more than one envelope proteins or "infectome" plays a crucial role in specific cell recognition and attachment. However, the molecular mechanisms, underlying key structural proteins of WSSV, mediate and determine the virus entry into targeted cells remain largely elusive which is definitely necessary for more investigations.

3. Cellular entry of WSSV into host cells

Virus entry into permissive cells is the first step for effective infection and propagation. In general, one or multiple entry pathways are employed for most of enveloped DNA viruses, including fusion with the plasma membrane, cell-cell fusion and endocytosis. Although a stably passaged cell line suitable for WSSV propagation remains unavailable, by virtue of the hematopoietic tissue (Hpt) cell of the red claw crayfish *Cherax quadricarinatus* and the secondary cell culture from the lymphoid organ of shrimp *Litopenaeus vannamei*, the cellular entry mechanism of WSSV into host cells have been recently elucidated [18,19]. Similar to the other enveloped DNA viruses, WSSV has also been found to employ multiple endocytic routes, including clathrin-mediated endocytosis (CME), macropinocytosis and caveolae-mediated endocytosis, for entry into crayfish Hpt cells on the basis of transmission electron microscopy combined with specific pharmacological inhibition assays and gene loss-of-function studies (Fig. 1) [18,20]. The clathrin-mediated endocytosis of WSSV was suppressed in the Hpt cells if pretreated with chlorpromazine, a specific pharmacological inhibitor used for disruption of the assembly of clathrin-coated pits on the cell membrane to block CME pathway. This was further supported by gene silencing of several key genes of CME pathway, such as the clathrin light chain (*Cq*CLC), medium subunit of clathrin-associated adaptor protein complexes (*Cq*AP50) and *Cq*dynamin, suggesting that CME pathway is indeed important for WSSV entry into crayfish Hpt cells. Recently, the endocytosis of WSSV through CME pathway was also found to be mediated by a shrimp polymeric immunoglobulin receptor-like protein (*Mj*pIgR), of whose extracellular domain could interact with envelope protein VP24 of WSSV and its intracellular domain could bind to calmodulin (*Mj*CaM), indicating that pIgR-CaM-Clathrin endocytosis pathway is an alternative receptor-mediated WSSV entry in a CME-dependent manner [21].

As we know that the efficiency of viral entry is strongly associated with a variety of cellular factors during the endocytosis process. For instance, a γ-aminobutyric acid receptor-associated protein (CqGABARAP), one of the traffic proteins and autophagy-related protein 8 (Atg8) homologs, has been well defined from crayfish to promote WSSV entry into Hpt cells in a CME-dependent manner. The recombinant CqGABARAP protein could enhance WSSV entry by protein overloading into Hpt cells, which was significantly suppressed when Hpt cells were pretreated with chlorpromazine, but not with other selected pharmacological inhibitors such as rottlerin and methyl-β-cyclodextrin (MβCD), specific inhibitors against macropinocytosis and caveolae-mediated endocytosis, respectively. Furthermore, the promoted WSSV entry by recombinant CqGABARAP protein was significantly increased after gene silencing of a small GTPase CqRac1 involved in CME pathway, suggesting a negative role of CqRac1 in regulating WSSV entry associated with the CME pathway [18]. However, the details about WSSV entry mediated by receptors and other cellular factors are still largely limited that is worth of more investigations.

Alternatively, the caveolae-mediated endocytosis is also necessary for WSSV entry into target cells, as proved by significant inhibition on virus entry into Hpt cells if pretreated with MβCD or nystatin, specific pharmacological inhibitors interfering with cholesterol involved in the structure maintaining of caveolae. This is also supported by an earlier study that the relative expression of the immediate early gene *ie1* of WSSV almost could not be detected in the primary hemocyte culture if pretreated by MβCD, implying that the caveolae was indeed recruited for WSSV entry. Previously, a soluble C-type lectin was shown to recognize WSSV in shrimp *M. Japonicas*, in which the subsequent virus entry facilitated by molecular chaperone calreticulin was likely in a cholesterol-dependent manner [22]. This is consistent with the speculation that WSSV entry is possibly through caveolae-mediated endocytosis. However, present evidences are not sufficient due to the lacking of gene functional studies of key molecules, like caveolae or related genes, involved in this process.

Meanwhile, the research on WSSV entry and infection has also been carried out in the other cell culture systems, for example, the secondary cell cultures from the lymphoid organ of *L. vannamei*. In this study, WSSV virion was shown to enter the lymphoid organ cells and further co-localized with the early endosomes at 30 min post viral infection. Subsequently, the uncoating of WSSV virion was occurred within 1 hpi in the cytoplasm. Additionally, the progeny virions were ultimately released at 12 hpi and culminated at 18 hpi in this cell culture [19]. However, the molecular events, underlying the interplay between WSSV virion and the host cells, have not been revealed in regarding to the viral life cycle, which severely limits the efficiently antiviral designing against both replication and assembly of progeny WSSV.

Generally, virus can use several alternative pathways to enter host cells, which are strongly dependent on the external topology of the virions and different target cell types, and more specifically on the viral attachment factors as well as cellular receptors. Whatever endocytic routes employed by WSSV, the diverse cellular receptors recognized by WSSV virion are indispensable, which are likely to determine the cell tropism of WSSV. In the following, we will summarize the putative cellular receptors that mediate WSSV entry. Meanwhile, cellular receptors, also known as pattern recognition molecule recognizing WSSV and inducing the antiviral immunity, are further discussed.

4. WSSV entry mediated by putative cellular receptors

Viral entry mediated by cellular receptors is an intricate process, with a variety of cell surface components and viral structural complexes or specific structural proteins, which generally starts with binding to specific cell-surface receptors followed by internalization via different endocytic routes. However, cell surface receptors may take different ways to function in viral entry. Some cellular receptors may be directly recruited by virus for endocytosis, while other cellular receptors may activate related signaling pathways or induce the surface structure conformation change of virus for helping viral entry. In addition, how many types of cellular molecular receptors would be recruited by virus is dependent on the specific types of viruses and their target cells. In the following, we will summarize the mainly putative cellular receptors that mediate WSSV entry and infection based on recent studies.

4.1. Immunoglobulin-superfamily receptors

Immunoglobulin-superfamily (IgSF) receptors are those proteins contain immunoglobulin domains, which constitute one of the largest repertoires of cellular surface receptors in metazoan. Most of them are localized on the cellular membranes and function in recognizing and binding of exogenous pathogens, which is then followed by the subsequent activation of immune signaling. For instance, the polymeric immunoglobulin receptor (pIgR), a member of IgSF, is a highly conserved type I transmembrane glycoprotein that is abundantly expressed in epithelial cells. The extracellular domains of pIgR include five immunoglobulin-like domains and a highly variable domain containing a conserved 9 amino acids sequence (FAEEKAVAD). The reason that pIgR raises extensive concern of researchers is due to its crucial role associated with mucosal immunity. It's well-known that the mucosal barrier in the gastrointestinal tract is the first line of mucosal defense system to protect host from millions of enteric microbes. In regarding to that most of WSSV infection is via the digestive tract of crustaceans, the mucosal immunity around the digestive tract may play crucial roles in immune defense against WSSV infection. One of the most important immunological mechanisms at mucosal barrier is the secretion of polymeric immunoglobulins including polymeric IgA or IgM, of which secretory component depends on the binding to the pIgR [23]. Inversely, pIgR could also be hijacked by some pathogens to facilitate their own infection, such as Epstein-Barr virus (EBV), which can be successfully internalized into non-susceptible epithelial cells by recruiting pIgR for permissive infection [24].

Interestingly, a pIgR like protein was recently proved to act as a receptor for WSSV entry in shrimp *M. japonicus*. Similar to vertebrate pIgR, *MjpIgR* is comprised of an Ig domain, two Ig-like domains, a transmembrane region and an intracellular region, which was widely expressed in hemocyte, heart, hepatopancreas, intestine, gill and stomach in shrimp [21]. Besides, the expression of *MjpIgR* in both transcript and protein levels was found to be markedly up-regulated in hemocyte and intestine post WSSV infection. Meanwhile, both the transcripts and the copy number of WSSV were significantly reduced by gene silencing of *MjpIgR*, which was accompanied with the higher survival rate of shrimps compared to those of control animals. Consistent with above results, the transcripts of *vp28* gene was reduced in the shrimp by antibody blocking against *Mj*pIgR, which was further supported by the increased gene expression of *vp28* and the copy number of WSSV in shrimp intestine with overexpression of *Mj*pIgR. Furthermore, *Mj*pIgR was found to be co-localized with Dil-labeled WSSV virion in hemocyte, and the internalization rate of WSSV virion was reduced by gene silencing of *Mj*pIgR in hemocyte. Importantly, the “susceptibility” as indicated by the “entry” of WSSV into human HEK293T cells, known as a kind of non-permissive cell line for WSSV infection, was clearly increased by overexpression of *Mj*pIgR [21]. Taken together, these findings demonstrate that *Mj*pIgR can serve as a cellular receptor for WSSV entry and facilitate the subsequent internalization of WSSV into shrimp cells.

Besides, some cell surface molecules with immunoglobulin-like domains may play similar roles to IgSF receptors described above. For example, the vascular endothelial growth factor receptor (VEGFR) is a key member of the vascular endothelial growth factor (VEGF) signaling pathway, which can be triggered by binding of VEGFRs to their ligands and thus plays vital roles in activation of endothelial cells for angiogenesis [25]. In *L. vannamei*, both *Lv*VEGFR and *Lv*VEGFR2, with 6 immunoglobulin-like domains, have been shown to promote WSSV infection as proved by the decreased copy number of WSSV *in vivo* after gene silencing of *LvVEGFR* or *LvVEGFR2* [26,27]. It's well-known that the activation of VEGF signaling pathway is induced through the interaction of VEGFRs with their corresponding ligands. Consistently, *Lv*VEGFR was found to interact with its ligands, such as *Lv*VEGF3, *Lv*VEGF4 and *Lv*VEGF5, while *Lv*VEGFR2 could interact with *Lv*VEGF2 and *Lv*VEGF3 but not the other VEGFs. Thus, different VEGFRs may bind to distinct ligands for certain cellular processes. Due to its localization on cell membrane, it is likely that VEGFR can regulate WSSV entry through interaction with specific VEGF, which mechanism is worth of further studies for benefiting the *anti*-WSSV control.

4.2. 2 Integrin

The integrin families are well-known cell-surface receptors that play vital roles in modulating a variety of biological processes, including cell adhesion, cell migration, endocytic trafficking and signaling transduction. Totally, integrins are composed of 18 α-subunits and 8 β-subunits, which can generate 24 functionally various integrin heterodimeric receptor molecules. As one of the multifunctional adhesion molecules, the function of integrin is accomplished through cell-to-cell, cell-to-extracellular cellular matrix (ECM) or cell-to-pathogen interactions [28]. Therefore, integrins are usually served as the indispensable receptors or co-receptors for many viruses, such as Kaposi's sarcoma-associated herpesvirus and Herpes simplex virus-2, to enter host cells by internalization since its ubiquitous distribution on the cell membrane [29,30]. Importantly, interaction between integrin and virus leads to the clustering of integrin subunits, which then promotes the viral binding capacity and activation of cytoskeleton activity necessary for viral attachment and the subsequent internalization of virions. Meanwhile, structural conformation changes of virions required for viral entry are also induced after binding between integrin and the virion. Taken together, both of integrin and viral structure are altered after their binding to each other, which helps to expose the key domains of integrin and viral proteins for successful entry into host cells.

Previously, β-integrin was found to interact with the viral envelope protein VP187, and the WSSV infection was significantly inhibited by gene silencing of β-integrin in *M. japonicas*, suggesting a promoted WSSV infection by β-integrin [31]. Later, several homologs of integrins or its subunits were reported to be associated with WSSV attachment and recognition in different penaeid shrimp species. An integrin β subunit (*Fc*βInt) with classical structural feature of β integrin, identified from Chinese shrimp *F. chinensis*, was mainly expressed in granular and semigranular hemocyte along with active response to WSSV infection, while lower expression of *Fc*βInt was present in the hyaline cell with weak response to WSSV infection [32]. Further study found that *Fc*βInt was specifically bound to VP187 and other three WSSV envelope proteins VP31, VP37 and VP110 by Far-Western blotting assay, and the binding activity of WSSV to the hemocyte membrane could be partially blocked by using the mouse antibody specifically against recombinant protein of extracellular domain of *Fc*βIntin (*anti*-r*Fc*βInt-ER). Meanwhile, higher survival rate was shown with the shrimp infected with WSSV pre-incubated by *anti*-r*Fc*βInt-ER antibodies [33]. In addition, a homologue of β-integrin identified from *L. vannamei* was also involved in the recognition of various structural proteins from WSSV, including envelope proteins VP26, VP31, VP37, VP90 and nucleocapsid protein VP136 [34]. Several cell adhesion motifs, such as RGD (Arg-Gly-Asp), YGL (Tyr-Gly-Leu) and LDV (Leu-Asp-Val) motif, displayed on these viral proteins were shown to play important roles for binding to β-integrin. Particularly, RGD motif exhibits more strong binding capacity than the other two motifs [34]. Other viruses, more than DNA envelope viruses, have been found to have an RGD motif displayed on their envelope glycoproteins for binding to integrins [28]. In particular, all of the WSSV structural proteins mentioned above also contain the cell attachment RGD motif, which is known as one of the most universal motif for binding to a large number of cellular receptors during virus infection. However, other two motifs, including YGL and LDV motifs present in several WSSV envelope proteins, could also bind to integrin in shrimp, which indicates that RGD motif is not a prerequisite for virus-integrin interaction.

Intriguingly, the alternative role of β-integrin such as immunomodulation has also been shown in *L. vannamei*, which was proved by the decreased phagocytosis-related parameters, increased activity of both prophenoloxidase and antioxidant due to gene silencing of β-integrin [35]. Therefore, it is likely that integrins may function in both the recognition and attachment of WSSV, through directly targeting the structural proteins of WSSV, and immunomodulation of innate immune system in crustaceans, but the underlying molecular mechanism needs to be further revealed.

4.3. Laminin receptor

Laminin receptor was originally identified as a binding protein with laminin, an extracellular matrix glycoprotein, for cellular adhesion. It has been proved to participate in diverse cellular processes, not only in cell anchoring but also in development, differentiation, signaling transduction, cancers and microorganism infections [36]. Given its functional properties, viral entry into permissive cell mediated by laminin receptor has been described in some viruses such as Sindbis virus and Dengue virus. In shrimp, laminin receptor has been found as a receptor protein for both DNA virus and RNA virus [37]. In the case of WSSV, laminin receptor (*Pm*Lamr) identified from *P. monodon* was found to interact with an envelope protein VP31 of WSSV by yeast two-hybrid screening and protein pull-down assay. Moreover, the binding specificity and affinity between recombinant *Pm*Lamr protein and viral VP31 was further confirmed in a dose-dependent way by competitive enzyme-link immunosorbent assay. Meanwhile, the cumulative motility of shrimp was clearly delayed when infected by the WSSV if pre-incubated with *Pm*Lamr or VP31 recombinant protein *in vivo*, which highlighted the key role of *Pm*Lamr in WSSV infection [38]. Additionally, a laminin receptor-like molecule, with a conserved laminin-binding domain from red claw crayfish, was identified to interact with another major envelope protein VP28 of WSSV. Moreover, both the WSSV entry and viral replication were significantly inhibited by gene silencing of *laminin receptor-like* gene in Hpt cells from red claw crayfish Hpt cells, in which the presence of intracellular WSSV virion was significantly reduced at an early stage of WSSV infection, and the relative expression of both the immediate early gene *ie1* and the late gene *vp28* was clearly decreased post WSSV infection [39]. Based on its known localization on cell membrane of various cells, laminin receptor or laminin receptor-like molecule is likely to be a putative cellular attachment receptor or co-factor for WSSV entry through binding to certain viral structural proteins, of which molecular mechanism needs more in-depth study.

4.4. Chitin-binding protein and glucose transporter 1

Chitin-binding protein (*Pm*CBP) and glucose transporter 1 (Glut1) were first identified in *P. monodon* with yeast two-hybrid screening by using a WSSV envelope protein VP53A as a bait protein, both of which are alternatively putative cellular receptors involved in WSSV infection [40]. In the case of chitin-binding protein, *Pm*CBP was found to be co-localized with VP53A on the hemocyte membrane, suggesting that *Pm*CBP is a cell surface receptor. Meanwhile, the shrimp infected with WSSV pre-incubated with *Pm*CBP recombinant protein could decrease the mortality towards WSSV infection, indicating that *Pm*CBP participates in WSSV infection [41]. This finding was also supported by another study in which the mortality upon WSSV infection was reduced in *P. vannamei* after gene silencing of *CBP* [16]. Additionally, further study showed that *Pm*CBP could interact with other envelope proteins of WSSV, including VP24, VP110, VP53B, VP337, VP32, VP124, VP41A, VP51B, VP60A and VP39B, suggesting its key role in mediating WSSV infection [41]. In contrast to CBP, Glut1 was reported to be the receptor for cellular entry of several viruses, such as Human cytomegalovirus, Feline leukemia virus C and Human T-cell lymphotropic viruses. Glut1 protein is another putative cellular receptor that was identified with yeast two-hybrid assay by using the same envelope protein VP53A as a bait protein from WSSV. As a member of a large superfamily of transporters, the major facilitator superfamily, Glut1 was predicted with 12 transmembrane regions to be localized in the cell membrane of hemocyte from *L. vannamei*. Furthermore, the recombinant protein of extracellular portion of Glut1 could bind to WSSV and then delay WSSV infection by both *in vitro* and *in vivo* neutralization assay, while the mortality was decreased by gene silencing of *Glut1* in shrimp post WSSV infection [42]. All of these results indicate that Glut1 plays an essential role in WSSV infection. More importantly, protein interaction assay further showed that Glut1 could interact with seven envelope proteins of WSSV, including VP24, VP28, VP31, VP32, VP39B, VP51B and VP53A, in *L. vannamei* [43]. In consideration to that Glut1 could bind to *Pm*CBP, *Pm*CBP and Glut1 are likely to act as alternatively putative cellular receptors or co-receptors for benefiting WSSV entry due to their localization on the cell membrane and interplay with multiple envelope proteins of WSSV.

5. Sensing of WSSV and triggering of innate immune response by cellular receptors

Over the few decades, germline-encoded pattern recognition receptors (PRRs) in the innate immune system have been well-characterized across animals and plants. PRRs can sense extraneous pathogens by directly binding to pathogen-associated molecular patterns, such as peptidoglycan lipotechoic acid, lipopolysaccharide, glucans, CpG DNA, and single-strand and double-strand DNA or RNA. After recognition on PAMPs, PRRs can trigger intracellular immune signaling pathways and induce both the expression and release of immune effectors for clearance of invading pathogens [44]. Canonical PRRs involved in viral infection, including Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors, nucleotide-binding oligomerization domain-like receptors, scavenger receptors (SRs) and C-type lectins (CTLs), have been well-investigated in vertebrate and some invertebrate animals. Whereas, only some PRRs such as Toll-like receptor, C-type lectins and scavenger receptors, have been intensely described in crustaceans like both shrimp and crayfish. In this section, we will summarize several known pattern recognition receptors that associated with sensing of WSSV and the subsequent initiating of antiviral immunity in crustaceans (Fig. 1).

5.1. Toll-like receptors

Toll-like receptors are considered as one cannonical type of PRRs for sensing of pathogens, including bacteria, fungi and viruses, in almost all metazoans. Since their first discovery involved in immune defense against fungal infection in *Drosophila* in 1988, ten and at least thirteen Toll-like receptors homolog have been described in human and mouse, respectively [45]. All Toll-like receptors share highly conserved structural characteristics, which consist of leucine-rich repeat in the amino-terminal, a transmembrane domain and Toll-interleukin-1 receptor homology domain in cytoplasmic carboxy-terminal. According to their localization and functional recognition, two groups of TLRs are classified: the TLRs localized on cell membrane for recognition of microbial membrane components such as LPS, flagellin, peptidoglycans and viral structural proteins, while the other TLRs localized on endosomes and lysosomes for recognition of intracellular nucleic acids. Among these identified TLRs in mammalian, several TLRs have been found to be associated with antiviral immunity [46]. For examples, TLR3, TLR7/8 and TLR9 have been greatly well-characterized by directly sensing of dsRNA, ssRNA and CpG DNA for initiating intracellular innate immune signaling pathways, and the subsequent activation and release of interferon cytokines as well as proinflammatory cytokines, both of which strongly contribute to the clearance of invasive pathogens. In addition to sensing of nucleic acids released from virus into intracellular cytoplasm, several TRLs localized on the cell surface have also been proved to mediate inherent immune response via direct recognition of viral proteins. Specifically, TLR2 was found to directly interact with the envelope glycoproteins B and H of human cytomegalovirus, and then led to stimulation of inflammatory cytokine. The envelope glycoproteins gH/gL and gB of Herpes simplex virus were also recognized by TLR2 followed by the subsequent initiating of NF-κB signaling pathway. In contrast to mammalians Toll-like receptors, the role of Toll-like receptors involved in defense against virus is relatively limited in

Table 1
Putative cellular receptors for entry and sensing of WSSV in crustaceans based on previous studies.

Putative cellular receptors	Isoforms	Domains	Viral ligands	Effect	Species	Reference
pIgR	–	Ig-like domains	Envelope protein VP24	Recognize and promote WSSV entry	*M. japonicus*	[21]
VEGFR	VEGFR, VEGFR2	Ig-like domain, Ig subtype PK domain	–	Recognize and indirectly facilitate WSSV infection	*L. vannamei*	[26,27]
Integrin	*Fc*βInt	βA domain, 4 EGF-like domains	Envelope proteins VP31, VP37, VP110, VP187	Recognize and facilitate WSSV infection	*F. chinensis*	[32,33]
	Integrin	βA domain	–		*M. japonicas*, *P. clarkii*	[34]
	Integrin β	–	Envelope protein VP187		*L. vannamei*	[35]
	*Lv*Int	–	Envelope proteins VP26, VP31, VP37, VP90; Nucleocapsid protein VP136			[31]
	Lv-B	–	–	Activate immunomodulation		
Laminin receptor	LR		Envelope protein VP31	Recognize and facilitate WSSV entry and replication	*P. monodon*	[38]
	LR-like	Laminin-binding domain	Envelope protein VP28		*C. quadricarinatus*	[39]
Chitin-binding protein	*Pm*CBP, CBP	Chitin-binding Type 2 domain	Envelope proteins VP24, VP32, VP39B, VP41A, VP51B, VP53A, VP53B, VP60A, VP124, VP337, VP110	Recognize and facilitate WSSV infection	*P. monodon*, *L. vannamei*	[16,40,41]
Glucose transporter	Glut1		Envelope proteins VP24, VP28, VP31, VP32, VP39B, VP51B, VP53A	Recognize and facilitate WSSV infection	*P. monodon*, *L. vannamei*	[42,43]
Toll	Toll	LRR, TIR	–	Recognize WSSV and activate the antiviral immune response	*P. monodon* *S. serrata* *M. rosenbergii* *C. quadricarinatus*	[47–49]
	*Pc*Toll4				*P. clarkii*	
	*Lv*Toll1,4,5,6				*L. vannamei*	
C-type lectin	*Fm*LC1- *Fm*LC5	LDLR, CRD		Recognize WSSV and activate the antiviral immune response	*F. merguiensis*	[22,52–55,57]
	*Fm*LC6		Envelop protein VP28; Tegument protein VP39A; Capsid protein VP15			
	*Fm*LDLR					
	*Lv*LT		Tegument protein VP95; Envelope proteins VP28, VP26, VP24, VP19, VP14		*L. vannamei*	
	*Lv*CTL1					
	*Lv*Lectin-1, *Lv*Lectin-2					
	*Lv*CTL3					
	*Lv*LdlCTL					
	*Mj*LecA, Lec*B*, Lec*C*		Envelop proteins VP26, VP28, VP281		*M. japonicas*	
	*Mj*svCL		–			
Scavenger receptor	SRC	MAM, CCP	Envelope protein VP19	Recognize WSSV and activate the antiviral immune response	*M. japonicas*	[23,59,60]
	SCRBQ	CD36 domain	–	–		
	SRB				*S. paramamosian*	

Abbreviations: pIgR: polymeric immunoglobulin receptor; VEGFR: vascular endothelial growth factor receptor; EGF: epidermal growth factor; LR: laminin receptor; LRR: leucine-rich repeat; TIR: Toll/interleukin-1 receptor; LDLR: low-density lipoprotein receptor class A domain; CRD: carbohydrate recognition domain; SRC: class C scavenger receptor; SRB: class B scavenger receptor; SCRBQ: class B scavenger receptor Croquemort; MAM: domain in meprin, A5, receptor protein tyrosine phosphatase mu; CCP: complement control protein domains.

crustaceans.

Given its crucial role in immune response, some Toll-like receptor homologs or members have also been found in crustaceans, including shrimp, crayfish and crab (Table 1), which generally exhibits its antiviral function via induction of antimicrobial peptides (AMPs) that directly targeting virus during WSSV infection. Actually, Toll-like receptors are also as one of the well-characteristic PRRs associated with virus infection in crustaceans. For example, Toll4 was recently identified as a critical pattern recognition receptor to recognize WSSV from a total of nine Tolls in *L. vannamei* by RNAi screening. Upon sensing of WSSV, Toll4 leads to nuclear translocation and phosphorylation of the NF-κB transcription factor Dorsal and then induces the production of two types of antiviral molecules, i.e. *anti*-LPS factor (ALF) and lysozyme, to inhibit viral infection through interacting with several envelope proteins from WSSV, including VP19, VP24, VP26, VP28, wsv134 and wsv321. Indeed, Toll4-Dorsal-AMPs cascade is important for the *anti*-WSSV response underlying the Toll-mediated signaling pathway in shrimp [47]. In addition, the Toll3 has also been shown to participate in antiviral immune response through activating the expression of interferon regulatory factor (IRF) and downstream factor Vago4/5 in shrimp *L. vannamei*, of which is independent on the canonical Toll signaling pathway [48]. Moreover, the gene expression of a Toll-like receptor *CqToll* was also shown to be responsive to WSSV infection in red claw crayfish *C. quadricarinatus*. And the transcripts of both *ie1* and *vp28* of WSSV were markedly enhanced by gene loss-of-function of *CqToll* gene in Hpt cells, which was accompanied with the reduced relative expression of *Cq*ALF, an antiviral antimicrobial peptide, but not with another AMP of *Cq*Crustin [49]. In regarding to that CqALF inhibits WSSV by disruption of the viral envelope [50], this finding implies that *Cq*Toll may function in battling with WSSV infection through triggering the production of *Cq*ALF in *C. quadricarinatus.* Additionally, similar findings were also found in other species of shrimp and crayfish, such as *M. rosenbergii* and *P. clarkii*, which strongly indicate that the induction of antiviral antimicrobial peptide mediated by Toll or TLRs is a highly conserved pathway for defensing against WSSV infection in crustaceans. However, the molecular mechanism underlying the Tolls-like receptors-mediated regulation of antiviral immunity in crustaceans needs further more investigations due to abundant Toll-like receptors present in a variety of crustacean species.

5.2. 2 C-type lectins

As a group of pattern recognition receptors, C-type lectins (CTLs) have been shown to play important roles in immune defense in metazoan, which could bind to carbohydrates via functionally characteristic carbohydrate-recognition domains (CRDs), also known as C-type lectin-like domains, in a calcium-dependent manner. By owning 110–130 residues in length, CRD has a characteristic double-loop stabilized by two or three conserved disulfide bonds, and contains four Ca^{2+}-binding sites involved in carbohydrates binding and structure maintenance. The CTLs superfamily has been categorized into 17 distinct classes on the basis of structural features. This high diversity of different CTLs members exhibits various cellular functions, including sensing of pathogen, signaling transduction, cell adhesion and so on [51]. Particularly, C-type lectins can act as PRRs capable of sensing pathogen-derived ligands, such as viral envelope glycoprotein, to trigger antiviral immune response. On the other hand, C-type lectins could be recruited by some viruses for cellular entry into host cells. In the following, the dual roles of C-type lectins involved in WSSV recognition and the subsequent antiviral effect or subversion for benefiting infection by virus will be discussed.

A numbers of CTLs have been found in many shrimp species, including *F. merguiensis*, *F. chinensis*, *L. vannamei*, *P. monodon*, *F. indicus*, and *M. rosenbergii* (Table 1). In *L. vannamei*, several divers isoforms of C-type lectins have been characterized, including C-type lectin (*Lv*LT), *Lv*CTL1, *Lv*Lectin-1, *Lv*Lectin-2, *Lv*CTL3 and *Lv*LdlrCTL, most of which exhibit similar function in immune response toward WSSV infection based on the conserved characteristic domain. Importantly, *Lv*CTL1 has a strong binding activity to WSSV and interacts with several envelope proteins of WSSV, including VP95, VP28, VP26, VP24, VP19 and VP14, to exert direct antiviral effect against WSSV infection. The *anti*-WSSV activity was further supported by the higher survival rate of shrimp infected with WSSV that pretreated with *Lv*CTL1 recombinant proteins [52]. Additionally, three lectins, termed as *Mj*LecA, *Mj*LecB, and *Mj*LecC, were identified by screening a phage display library of *M. japonicas* by using WSSV envelope proteins VP26, VP28 and VP281, of which *Mj*LecA and *Mj*LecB also exhibited directly antiviral capacity against WSSV infection [53]. In *F. merguiensis*, several C-type lectins, including *Fm*LC1 to *Fm*LC6 and *Fm*LDLR, were shown to be associated with WSSV infection. Among these *Fm*LC, *Fm*LC6 with dual carbohydrate recognition domains was found to bind directly to structural proteins of VP15, VP39A and VP28 from WSSV, and thus to function in the antiviral immune defense [54]. Another C-type lectin *Fm*LDLR, with a unique low-density lipoprotein receptor (LDLR) domain, was also shown to have similar binding activity with structural proteins of WSSV as mentioned above [55].

In contrast to the lectins above, some C-type lectins do not possess antiviral activity in WSSV infection. For instance, a soluble C-type lectin of *M. japonicas* induced by WSSV infection in the stomach has been proved to promote WSSV infection likely through *Mj*svCL-calreticulin pathway in a cholesterol-dependent manner [56]. In addition, a recent study also showed that *Lv*LdlrCTL negatively regulates WSSV infection likely through direct or indirect modulation of the NF-κB pathway and the JAK-STAT pathway, which was proved by the down-regulated expression of c-JNK, Vago4, Dorsal and STAT *in vivo* after gene silencing of *Lv*LdlrCTL. Therefore, *Lv*LdlrCTL may function in WSSV infection through a complicated immune regulatory network in crustaceans [57]. Although highly diverse members of CTLs have been extensively studied in human and mammalian, the corresponding homolog of each class among these CTLs family members are poorly understood. More types of C-type lectins from a wide range of shrimp or crayfish species and the molecular mechanism involved in *anti*-WSSV effect or subversion of antiviral response by WSSV are needed to be further elucidated, which will contribute to our better understanding of C-type lectins in innate antiviral immunity in crustaceans.

5.3. 3 Scavenger receptors

Scavenger receptors were first identified to recognize and clear danger-associated molecular patterns, such as modified low-density lipoprotein that are related to pathogenesis of atherosclerosis. In mammalian, scavenger receptors are structurally heterogeneous and thus subdivided into eight classes on the basis of the domain structure [58]. In addition to its key role in the maintenance of homeostasis and the pathogenesis of various diseases, scavenger receptors have been emerged as crucial pattern recognition receptors to recognize microorganisms and therefore involved in the innate immunity. Unsurprisingly, scavenger receptors are also expanded in other species in invertebrate, such as *Drosophila melanogaster* and *Aedes aegypti*, which mediate phagocytosis of bacteria and the recognition of dengue virus, respectively.

Until now, two classes of scavenger receptors, both the class B and class C scavenger receptor, have been found to be associated with WSSV infection in crustaceans (Table 1). For example, a class B scavenger receptor Croquemort (*Mj*SCRBQ) was shown to be responsive to WSSV infection in kuruma shrimp *M. japonicus* [59]. Particularly, a class C scavenger receptor (*Mj*SRC) identified from *M. japonicus* could be markedly up-regulated upon WSSV challenge at both the transcript and protein levels in hemocyte. Moreover, the quantity of WSSV was increased after gene silencing of *MjSRC* in shrimp, suggesting the antiviral role of *Mj*SRC. Meanwhile, the phagocytotic rate of WSSV was reduced in hemocyte after gene silencing of *MjSRC*, which was further proved by the promoted phagocytosis of WSSV with overexpression of *Mj*SRC in hemocyte. And the antiviral effect of *Mj*SRC was exerted through the direct interaction of its extracellular domain with WSSV envelope protein VP19 and then oligomerized to a trimer for internalization into hemocyte. Following internalization, an adaptor protein *Mj*β-arrestin2 was then recruited to bind to the intracellular domain of *Mj*SRC for promoting the internalization, which eventually proceeded in the clearance of WSSV via clathrin-mediated endocytosis and lysosomes promoted degradation [60] (Fig. 1).

6. Conclusion and future perspective

Many viruses recruit single cell surface molecule as their specific receptor for entry into host cells. Meanwhile, diverse types of cellular molecules could also be utilized by some viruses in a complicated manner. Various cellular molecules that contributed to WSSV entry and effective infection exhibit a dependency of intricate receptors or cofactors (Table 1). Alternatively, different members of identical cellular molecules play dual roles during WSSV infection, either by helping viral entry and internalization or by inducing innate immunity to remove WSSV via recognition molecules (Fig. 1). However, as one of the largest envelope DNA virus, many key molecular events involved in WSSV entry and internalization have not been completely elucidated, which raise us many questions valuable for further investigations. For examples, which cellular receptor (s) is the determinant factor for WSSV recognition and entry, if there is, among these identified putative cellular receptors as mentioned above? Whether other novel types of cellular surface molecule recognizing WSSV are present in crustaceans? Which endocytosis pathway is predominant in facilitating the WSSV entry? Particularly, the potential molecular mechanism underlying the antiviral immunity triggered by cellular surface molecules, are largely remained unknown. Addressing of these questions will further benefit the complete elucidation of WSSV pathogenesis, which will undeniably provide putatively effective antiviral designing against white spot disease.

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